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TITOLO TESI:

Identification and characterization of novel tumor-associated proteins as potential tumor markers for diagnosis and therapy

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ABSTRACT

This study deals with the discovery and characterization of EXN6 and EXN11 as novel tumor-associated proteins and promising therapeutic targets for cancer at high morbidity and mortality. These proteins were discovered in the context of an immuno-histochemistry (IHC) screening of a collection of approximately 1600 murine polyclonal antibodies towards membrane/secreted human proteins on tissue microarrays (TMA) containing cancerous and normal sample specimens of breast, colon, lung, ovary and prostate (5 patients per cancer). Both proteins were over-expressed in more cancer types, with concomitant negligible expression in corresponding normal samples.

An expanded IHC analysis on 50 samples per cancer confirmed that EXN6 is mainly present at abnormal levels in breast and ovary cancers (40 and 35%) while it is less frequently found in colon and lung cancer (less than 10%). Concerning EXN11, it is mainly detected in colon cancer (40%). Most importantly, EXN11 is expressed in hepatic metastasis from colon cancer, suggesting that this protein could be important for tumor progression and dissemination.

A molecular and biological characterization of the two proteins was undertaken to understand whether these proteins could be exploited as molecular targets for therapeutic interventions. Concerning EXN6, results showed that it is endogenously expressed and surface exposed in different breast and ovary cancers cell lines, as confirmed by gene silencing. Knock down of EXN6 expression significantly affects relevant cancer processes *in vitro,* such as cell invasiveness and proliferation, thus providing the first evidence that this protein could be a potential therapeutic target. Five highly specific monoclonal antibodies (mAbs) towards EXN6 were generated, able to bind the surface of EXN6 positive cells, as judged by FACS and confocal microscopy. Interestingly these antibodies are efficiently internalized by cancer cells, a property that makes them amenable for the generation of antibody-drug-conjugates (ADC). In agreement with this, both antibodies are capable to drive a toxin-conjugated secondary antibody into cancer cells and induce cell lysis. Moreover, one of them also shows ADCC activity. Thanks to these encouraging results, efficacy studies are ongoing to test the ability of the anti-EXN6 mAbs to prevent tumor formation or progression in mouse cancer models, either as naked antibodies or as ADCs.

Concerning EXN11, an expression profile analysis in human cancer epithelial, stromal and endothelial cells showed that the protein is normally endogenously expressed at very low level

while it is specifically up-regulated by hypoxia. Interestingly, this protein is secreted and partially associated with the exosomial fraction, suggesting that it could be released in the systemic circulation of oncologic patients and it may act in an endocrine way. Moreover, we showed that EXN11 exerts pro-angiogenetic activities on human differentiated endothelial cells by stimulating their motility, invasiveness and capability to form capillary-like networks, whereas it does not stimulate endothelial progenitor cells. Finally, EXN11 promotes vascularization *in vivo* in the mouse matrigel sponge assay. Experiments are ongoing to understand the role of EXN11 in cancer angiogenesis.

Overall, this study highlights the relevance of EXN6 and EXN11 as potential cancer markers and molecular targets for novel therapies. The identification of the molecular ligands and the cellular pathways in which they are involved, currently under investigation, would significantly facilitate the design of specific drugs. MAbs offer interesting therapeutic opportunities for both proteins. Concerning EXN6, this study already contributed to this aspect by providing novel anti-EXN6 mAbs with therapeutic potential.

1. INTRODUCTION

1.1 Cancer, the disease

Cancer represents the second and third cause of death in the developed countries and worldwide, respectively. Cardiovascular and infectious diseases are the other two major killer diseases. However, in the near future, cancer is expected to become the leading cause of death. The first reason is that deaths from cardiovascular and infectious diseases are declining, thanks to the scientific achievements in the understanding of the pathogenic mechanisms and risk factors associated with these diseases, and to the overall improvement of sanitary conditions. The second reason is due to the genetic mutations that occur every day, because of metabolic reactions and of environmentally induced cellular damages, which accumulate because of the extension of human life span. The National Cancer Institute of the United States of America (NCI) recently estimated that a 2013 newborn has a 40% possibility to get a cancer during his/her life. In the last decade, malignancies affecting breast, colon, ovary, prostate and lung cancers had the highest incidence and mortality, and are therefore considered as the "five killers". Prostate, breast and lung cancers have been the tumors at the highest incidence, followed by colorectal and ovary cancers. Table 1 reports the epidemiological data of the five tumors in 18 SEER geographic areas of the United States for the first six months of 2013 (http://www.cancer.gov/statistics/).

Table I – Expected Incidence and mortality rates of major tumors in the USA in 2013							
Cancer	Estimated new cases	% of all new cases	Estimated death	% of all cancer deaths	Incidence per 100,000	Mortality per 100,000	Prevalence
Breast	232,340	14.1	39,620	6.9%	123.6	22.6	2,829,041
Colon and rectal	142,820	8.6	50,830	8.8	45.0	16.4	1,154,481
Lung and bronchus	228,190	13.7	159,480	27.5	61.4	49.5	399,431
Prostate	238,590	14.4	29,720	5.1	152	23	2,617,682
Ovary	22,240	1.3	14,030	2.4	12.5	8.1	186,138

Despite cancer is still as a devastating disease, cancer-associated deaths are constantly declining since 1995 (Figure 1), thanks to the significant progress in the understanding of tumor biology, improved and early diagnosis, and the identification of appropriate therapies.
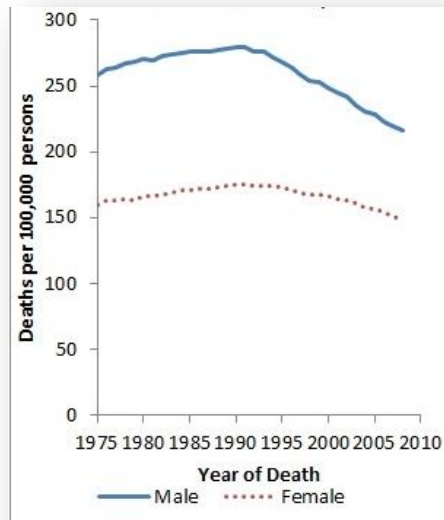
Figure 1 – Cancer death rates in the United States from 1975 to 2010 (NCI:http://seer.cancer.gov/csr/1975_2010/)

This favorable trend will probably continue and improve in the next years. In an optimistic view of the future of cancer prevention and treatment, cancer will be a sort of "chronic disease" that will allow oncologic patients to live with a good quality of life with for several years, similarly to what now occurs with diabetes, hypertension, and other once deadly pathologies.

The fight against cancer is based on the combined action of: i) prevention, ii) early diagnosis, iii) accurate surgery, and iv) optimized and personalized therapy (1)

- *Prevention* – A number of environmental factors and personal behaviors are risk factors for cancer, with higher relevance for specific pathologies such as smoking and lung cancer, ultraviolet light exposure and melanoma, excessive meat assumption and colon-rectal cancer. Moreover, different microorganisms are responsible for aggressive forms of cancer, such as HBV and HCV for hepatocarcinomas, HPV for cervical cancers, and *Helocobacter pylori* whose chronic infection can lead to stomach cancer. Thus, a proper surveillance of healthcare and correction of wrong behaviors aimed at reducing the exposure and prolonged contact with these tumorigenic agents should have a profound effect on cancer control.

- *Early and accurate diagnosis* – Most cancer deaths are the consequence of the cancer progression and disseminated metastases deriving from primary tumors that are generally refractory to the current therapies. Therefore, the success of a cancer treatment strongly

depends upon the ability to diagnose and treat cancer before the metastasis process has started. Moreover, an important aspect is the characterization of the cancer aggressiveness and metastatic potential that has an impact in the clinicians' decision on the most appropriate treatments. In fact, it is now well established that tumors fall into three classes (Http://cancer.gov/cancertopics): i) low invasive and metastatic cancers which remain in such a state during the life time of the patients; ii) highly aggressive cancers with a high metastatic potential that, in most cases have already disseminated by the time of diagnosis; iii) cancers of intermediate grade that have the potential to disseminate. Presently, a common way of thinking is that only the last group of cancers is really worthy of treatment. Indeed, death rates associated to indolent low invasive tumors is low. In addition, it has been shown that surgery may even provoke dormant cancers to become clinically apparent, or even aggressive. Concerning the highly aggressive cancers, they are generally refractory to the available therapies, whose toxicity sometimes overcome their palliative curative effects. Finally, the treatment of tumors with intermediate aggressiveness have a high probability to lead to a long-term, even relapse-free response. However, at present, the ability of differentiating tumors is still rudimental and all classes are treated similarly, with limited success and high socio-sanitary economical costs.

Cancer markers are tumor-associated molecules that enable oncologists to distinguish different type of markers, characterize them for their severity, and monitor their progression. Moreover, markers could allow to discriminate cancers that are likely to respond to specific therapy, thus allowing a more tailored treatment of oncologic patients. Despite the incredible effort of academic and industrial research centers in the cancer markers field, due to the heterogeneity and complexity of cancer, the number of clinically relevant markers is still limited.

Surgery – Surgery is still the first medical intervention to fight cancer and will remain a key therapeutic solution in the years to come. Surgical practice of primary tumors is considered effective in reducing the risk of metastatic relapse. For instance, the five year overall survival of colorectal cancer patients that receive a surgery at early stages of the diseases is 95%. An effective surgery is accompanied to an early and accurate diagnosis and avails of high-resolution imaging and endoscopy systems. Moreover, surgery is becoming less invasive and more selective, to the great benefit of patients in terms of time of recovery and quality of life.

1. *Optimized and targeted therapies* - Radiotherapy and chemotherapy based on alkylating agents, platinum-based drugs and anti-metabolites (such as analogs of DNA bases) have been the first therapeutic strategies still widely used in clinical oncology in association to surgery. These strategies are active against rapidly dividing cells, a characteristics share by all tumors. However, they can be very toxic, poorly tolerated by patients and often leading to secondary tumors (both radiation and anti-cancer drugs are carcinogens themselves). Moreover, they can be ineffective, since often cancer can become resistant to most conventional therapies. For these reasons, cancer therapy is seeking for more selective and personalized approaches, targeting components, which are specifically or abnormally present in cancer cells of oncologic patients. In the last decades, the molecular characterization of cancer has led to the discovery of components that can be selectively targeted by cancer drugs, such as (i) oncogenic proteins responsible for uncontrolled signaling of cell cycle and proliferation, (ii) mutated tumor suppression proteins, and (iii) cancer-specific surface-associated proteins. A plethora of novel targeted therapies are under clinical validations, whose individual components can be categorized in three major types: 1) small molecules, specifically selected to block aberrant signaling proteins or biological pathways activated in cancer due to the absence of properly functioning tumor suppression genes, 2) passive immunotherapy with mAbs able to bind cancer-specific surface proteins and capable of killing cancer cells, and 3) active immunotherapy in which tumor markers are exploited as antigens and delivered to patients with proper adjuvant formulation able to break the immune-tolerance and elicit cytotoxic immune responses.

Overall, targeted cancer therapies give doctors a better way to tailor cancer treatment, especially when a target is present in some but not all tumors of a particular type, as is the case for HER-2-positive breast cancers. Ideally, treatments could be personalized based on the unique set of molecular targets produced by the patient's tumor. Targeted cancer therapies also hold the promise of being more selective for cancer cells than normal cells, thus harming fewer normal cells, reducing side effects, and improving quality of life.

Nevertheless, targeted therapies have some limitations. Chief among these is the potential for cells to develop resistance to them. In some patients who have developed resistance to imatinib, for example, a mutation in the *BCR-ABL* gene has arisen that changes the shape of the protein so that it no longer binds this drug as well. In most cases, another targeted therapy that could overcome this resistance is not available. It is for this reason that

targeted therapies may work best in combination, either with other targeted therapies or with more traditional therapies.

Overall, the discovery highly selective tumor-specific markers will enable an early and accurate diagnosis and offer new perspective for the development of novel targeted drugs, among which mAbs are emerging therapeutic opportunities.

1.2 Tumor Markers

Ideal tumor markers are molecules able to accurately distinguish a cancerous state, discriminate different cancer types and become targets for selective therapy capable of specifically recognizing and destroying tumor cells without damaging the surrounding normal tissues. Tumor marker can be categorized in four major classes based on their clinical use.

1- Diagnostic markers are used for the early detection of cancer. Moreover, their aberrant expression level can be used to monitor cancer progression in patients subjected to a given therapeutic regimen. For instance, a marker decrease or return to a normal level could be associated to a relapse-free state or indicate that cancer is responding to therapy. Of particular relevance are markers released in biological fluids of cancer patients and can be detected with non-invasive immunological assays.

2- Prognostics markers are indicative of the severity and likely outcome of the disease at time of diagnosis. The prediction of poor or favorable prognosis helps the clinician in the decision making process and improve the patients' management.

3- Predictive markers are helpful to predict the patients' response to drug therapies and allow clinicians to select the most appropriate therapeutic regimen while avoiding ineffective treatments.

4- Therapeutic markers are molecules, frequently receptors, oncogenes or key components of molecular pathways that can be used to develop tumor-specific ligands able to block their action, such as small molecules and mAbs.

Table 2 reports the protein markers currently used in clinic and their application (Http://cancer.gov/cancertopics/factsheet/detection/tumor-markers)

Table 2. Clinical cancer markers currently in use in clinic

TUMOR MARKER	CANCER TYPES	TISSUE	APPLICATION
ALK gene	Lung cancer	Tumor	To help determine treatment and prognosis
AFP(alpha-fetoprotein)	Liver cancer and germ cell tumors	Blood	Diagnostic and prognostic
B2M (Beta-2-microglobulin)	Multiple myeloma, chronic lymphocytic leukemia, and some lymphomas	Blood and urine	Diagnostic and prognostic
Beta-Hcg (Beta-human chorionic gonadotropin)	Choriocarcinoma and testicular cancer	Blood and urine	Diagnostic, prognostic and predictive
BCR-ABL	Chronic myeloid leukemia	Blood and/or bone marrow	Diagnostic and prognostic
BRAF mutation V600E	Melanoma and colon cancer	Tumor	Therapeutic
CA15-3/CA27.29	Breast	Blood	Predictive
CA19-9	Pancreatic cancer, gallbladder cancer, bile duct cancer, and gastric cancer	Blood	Predictive
CA-125	Ovary tumor	Blood	Diagnostic, predictive and therapeutic
Calcitonin	Medullary thyroid cancer	Blood	Diagnostic, prognostic and predictive
CEA	Colon and breast cancer	Blood	Diagnostic, prognostic and predictive
Chromogranin A (CgA)	Neuroendocrine tumors	Blood	Diagnostic, prognostic and predictive
Chromosome 3, 7, 17and 9p21	Bladder cancer	Urine	Prognostic
Cytokeratin fragments 21-1	Lung cancer	Blood	Prognostic
EGFR mutation analysis	Non small cells lung cancer	Tumor	Therapeutic and prognostic
Estrogen receptor (ER)/progesterone receptor (PR) (ER/PR)	Breast cancer	Tumor	Therapeutic
HE4	Ovary tumor	Blood	Predictive
HER2/neu	Breast cancer, gastric cancer, and esophageal cancer	Tumor	Therapeutic
Immunoglobulins	Multiple myeloma and Waldenström macroglobulinemia	Blood and urine	Diagnostic, prognostic and predictive
KRAS mutations	Colorectal cancer and non-small cell lung cancer	Tumor	Therapeutic and prognostic
Nuclear Matrix protein 22	Bladder cancer	Blood	Prognostic
Prostate specific antigen (PSA)	Prostate cancer	Blood	Diagnostic, prognostic and predictive
Thyroglobulin	Thyroid cancer	Tumor	Prognostic
21 Gene signature	Breast cancer	Tumor	Prognostic
70 Gene signature	Breast cancer	Tumor	Prognostic

Currently, despite the importance of the cancer markers that account for a considerable area of oncology research, only a few are recognized as valid and used in the clinic, as they do not have

sufficient specificity and sensitivity to distinguish cancer cells and allow an accurate and timely diagnosis. For example, the "Carcinoembryonic Antigen" (CEA) and the "Prostate Specific Antigen" (PSA) , are proteins detectable in the sera of patients and routinely used in the clinic to predict the presence of the tumor or to monitor the patient's response to treatments for colon or prostate cancers, respectively. Concerning CEA, this marker has the advantage of being dosed accurately and in a reproducible way, with minimal costs, and for these characteristics it has been used in the past for the screening of colorectal tumors (2). Subsequent studies have shown that CEA is inadequate for this purpose, being expressed in a variety of extra-intestinal tumors , such as cancers of the lung , ovary and bladder. Similarly other markers used for the diagnosis of tumors of the colon, such as the "Tumor - Associated Glycoprotein -72" (TAG -72) , a high molecular weight glycoprotein expressed in a variety of tumors , and the " Carbohydrate Antigen " CA19 -9 were inadequate diagnostic tools (2,3). Regarding the PSA, this protein is organ specific rather than tumor-specific. Although this marker has been of great help to facilitate the detection of prostate cancer, it also presents limited specificity as its serological levels may be altered in inflammatory conditions, benign prostatic hyperplasia, and trauma. Recent studies have identified new molecular forms of PSA , such as free PSA (fPSA) and PSA derivatives, which could be more appropriate to discriminate prostate cancer from benign neoplastic states (4). Another marker sometimes used for screening of women with high risk of ovarian cancer is the "Cancer Antigen 125" (CA- 125). Even in this case, changes in the levels of serological CA- 125 are not sufficiently specific and sensitive for population screening. CA- 125 is mainly used to monitor response to cancer treatment and check for recurrence of ovarian cancer (3). In addition to the markers mentioned, in the last 30 years, other proteins, hormones and enzymes have been used, but their level increases even in benign conditions and during pregnancy.

Despite thousands of researchers around the world are engaged in the study of cancer, today many areas of biology and physiology of tumors are unexplored and there is a pressing need to identify new markers suitable for clinical use.

1.3 MAbs and their use in cancer therapy

Immunotherapy represents the most attractive opportunity for the treatment of cancer and it is pushing the clinical research from more than a century. In particular, mAb therapy is emerging as a powerful solution for the treatment of cancer. The popularity of mAbs stemmed from the advent of hybridoma technology in 1975 and progressed through the development of chimeric, humanized, and human antibodies (5). In fact, before mAbs can be used in humans, they are

"humanized" by genetic engineering by replacing as much as possible of the animal portion of the molecule with human portions to prevent the human immune system from recognizing the mAb as "foreign" and destroying it before it has a chance to interact with and inactivate its target. Being directed against tumor-specific or tumor-associated antigens, mAbs can selectively eliminate tumor cells while maintaining an acceptable toxicity profile. Moreover, an emerging immune-therapeutic strategy exploits mAbs that target immune cells with the goal of breaking local tolerance and stimulating the patient's anti-tumor immune response.

mAbs can be used as naked molecules or conjugated with cell payloads (radioisotopes, drugs or toxins) to direct kill tumor cells or to activate pro-drugs specifically within the tumors. These antibody-drug conjugates (ADC) can deliver a toxic load selectively to the tumor site while normal tissues are generally spared. ADC are of particular interest in that their therapeutic efficacy is stronger than that of naked antibodies. The concept that mAbs could be used as vehicles for the selective delivery of cytotoxic agents to tumors has been around almost since the beginning. However, until very recently, the idea has eluded successful implementation, probably for three reasons: (i) the use of antibodies against targets that were not sufficiently restricted to tumor cells, (ii) the use of drugs with insufficient potency or (in the case of bacterial or plant toxins) that were immunogenic, and (iii) the linker chemistry used to attach drugs to antibodies was not optimized. The most important property of antibodies to be used for the generation of ADC is their specificity for cancer cells, and the ability to be efficiently internalized by them so as to deliver the toxic compound in the intracellular compartment. In order to minimize toxicity, conjugates are usually engineered based on molecules with a short serum half-life (e.g. the use of IgG3 or IgG4 isotypes). Concerning the linker chemistry issue, this aspect has so far required an enormous empirical effort (6,7). For instance, a linker that is too labile allows a too rapid dissociation of the drug from the antibody causing high drug level in the blood and its exposure of normal tissues. Conversely, stable linkers require complete proteolytic digestion of the ADC to release the cytotoxic drug as the active metabolite (8). Similarly, the choice of drug required a great deal of trial and effort. So far, the most successful drugs are microtubule antagonists.

Currently, thirteen antibodies are approved for use in oncology with different therapeutic indications (9,10), described below and grouped on the basis of their major mechanism of action (http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted).

- MAbs blocking specific enzymes and growth factor receptors involved in cancer cell proliferation.

1.   Trastuzumab (Herceptin®) is approved to treat certain types of breast cancer as well as some types of gastric or gastroesophageal junction adenocarcinoma. The therapy is a mAb that binds to the human epidermal growth factor receptor 2 (HER-2). HER-2, a receptor with tyrosine kinase activity, is expressed at high levels in approximately 15-20% of breast cancers and also some other types of cancer. The mechanism by which trastuzumab acts is not completely understood, but one likely possibility is that it prevents HER-2 from sending growth-promoting signals (Figure 2). Trastuzumab may have other effects as well, such as inducing the immune system to attack cells that express high levels of HER-2.

2.   Pertuzumab (Perjeta™) is approved to be used in combination with trastuzumab and docetaxel to treat metastatic breast cancer that expresses HER-2 and has not been treated with chemotherapy or a HER-2-directed therapy. Pertuzumab is a mAb that binds to HER-2 at a region distinct from trastuzumab. This region allows HER-2 to interact with other receptors, such as Her3, to send growth-promoting signals. The drug likely prevents HER-2 from sending growth signals and induces the immune system to attack HER-2-expressing cells (Figure 2).

3.   Cetuximab (Erbitux®) is a mAb that is approved to treat some patients with squamous cell carcinoma of the head and neck or colorectal cancer. The drug binds to the external portion of EGFR, thereby preventing the receptor from being activated by growth signals, which may inhibit signal transduction and lead to antiproliferative effects.

4.   Panitumumab (Vectibix®) is approved to treat some patients with metastatic colon cancer. This mAb attaches to EGFR and prevents it from sending growth signals.

Other targeted therapies block the growth of blood vessels to tumors (angiogenesis). To grow beyond a certain size, tumors must obtain a blood supply to get the oxygen and nutrients needed for continued growth. Treatments that interfere with angiogenesis may block tumor growth.

5.   Bevacizumab (Avastin®) is a mAb that is approved for the treatment of glioblastoma. The therapy is also approved to treat some patients with non-small cell lung cancer, metastatic colorectal cancer, and metastatic kidney cancer. Bevacizumab binds to VEGF and prevents it from interacting with receptors on endothelial cells, blocking a step that is necessary for the initiation of new blood vessel growth.

- MAbs working by helping the immune system to destroy cancer cells.

6.   Rituximab (Rituxan®) is a mAb that is approved to treat certain types of B-cell non-Hodgkin lymphoma and, when combined with other drugs, to treat chronic lymphocytic leukemia (CLL). The therapy recognizes a molecule called CD20 that is found on B cells. When rituximab binds to these cells, it triggers an immune response that results in their destruction. Rituximab may also induce apoptosis.

7.   Alemtuzumab (Campath®) is approved to treat patients with B-cell CLL. The therapy is a mAb directed against CD52, a protein found on the surface of normal and malignant B and T cells and many other cells of the immune system. Binding of alemtuzumab to CD52 triggers an immune response that destroys the cells.

8.   Ofatumumab (Arzerra®) is approved for the treatment of some patients with CLL that does not respond to treatment with fludarabine and alemtuzumab. This mAb is directed against the B-cell CD20 cell surface antigen.

9.   Ipilimumab (Yervoy™) is approved to treat patients with unresectable or metastatic melanoma. This mAb is directed against cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4), which is expressed on the surface of activated T cells as part of a "checkpoint" to prevent a runaway immune response. By inhibiting CTLA-4, ipilimumab stimulates the immune system to attack melanoma cells.

-       *MAbs formulated as ADCs able to deliver the toxic drug into the cancer* cells

10.   Tositumomab and 131I-tositumomab (Bexxar®) is approved to treat certain types of B-cell non-Hodgkin lymphoma. The therapy is a mixture of mAbs that recognize the CD20 molecule. Some of the antibodies in the mixture are linked to a radioactive substance called iodine-131. The 131I-tositumomab component delivers radioactive energy to CD20-expressing B cells specifically, reducing collateral damage to normal cells. In addition, the binding of tositumomab to the CD20-expressing B cells triggers the immune system to destroy these cells.

11.   Ibritumomab tiuxetan (Zevalin®) is approved to treat some patients with B-cell non-Hodgkin lymphoma. The therapy is a mAb directed against CD20 that is linked to a molecule that can bind radioisotopes such as indium-111 or yttrium-90. The radiolabeled forms of Zevalin deliver a high dose of radioactivity to cells that express CD20.

12.   Brentuximab vedotin (Adcetris®) is approved for the treatment of systemic anaplastic large cell lymphoma and Hodgkin lymphoma that has not responded to prior chemotherapy or autologous stem cell transplantation. This agent consists of a mAb directed against a molecule called CD30, which is found on some lymphoma cells, linked to a drug called monomethyl auristatin E (MMAE). The antibody part of the agent binds to and is internalized by CD30-expressing tumor cells. Once inside the cell, the MMAE is released, where it induces cell cycle arrest and apoptosis.

13. Ado-trastuzumab emtansine (Kadcyla®), is approved for the treatment of Her2-positive metastatic cancer who have received prior treatment with Herceptin and Taxane chemotherapy. It consists in Trastuzumab, directed against HER-2, conjugated to the a maytansine derivative DM1 (Figure 2)
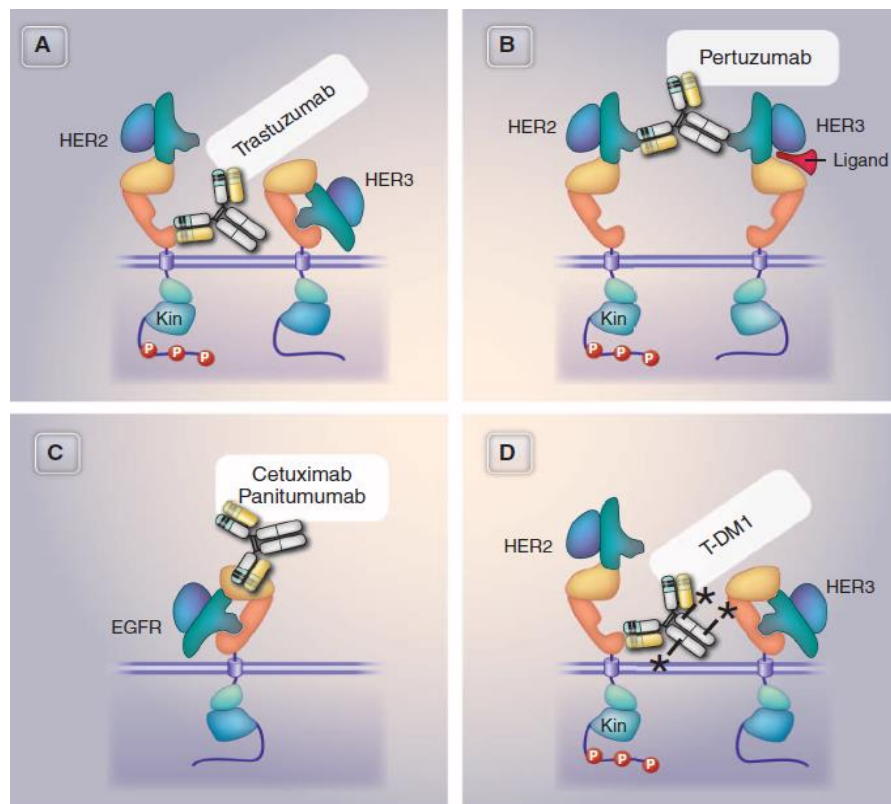
Figure 2. Specificity of and mechanism of action of therapeutic targeting HER2 and EGFR. The figure represents the epitope recognition of Trastuzumab (A), Pertuxumab (B), Cetuximab or Panitumumab (C), and the ADC ado-trastuzumab conjugated to DM1 (D). In the Her2 signaling, Her2 phosphorylates the Her3 cytoplasmic domain and this activates the phosphatidylinositol-3-phosphate kinase cascade. Conversely, Her3 activation by its binding ligans (e.g. can allosterically activate Her2 kinase). The anti-Her2 antibodies target the physical and functional interaction of Her2 with Her3. Pertuzumab block the heterodimerization of Her2 and Her3. In the case of Ado-trastuzumab conjugated to DM1, the cytoxic effect is potentiated by the DM1 drug (modified from Mark X, *Science* **341**, 1192, 2013)

Despite the encouraging clinical data, available mAbs are still insufficient to cope with the complexity of tumor biology and ultimately to provide satisfactory therapeutic interventions. Indeed, several mechanisms of resistance are emerging that limit the antibody therapy efficacy, including activation of alternative signaling pathways, genetic mutations and expression of immune inhibitory molecules. Altogether, these evidences prompt the search and development of new molecular targets and mAbs to improve future therapies and tumor patient health care. Moreover, the clinical success of antibody therapy is dependent on a thorough comprehension of their effects of on tumor cells and their mechanism of action. In this scenario, highly sensitive technologies are needed to understand the local physiological conditions present at a cellular level, the tumor microenvironment and the regulatory networks that might influence the behavior of molecular targets and their response to mAb therapy. Moreover, as with other treatment modalities, mAb-based immunotherapy is far from perfect and requires additional study to optimize clinical response and overcome therapeutic resistance.

1.3.1 Mechanisms of action of therapeutic mAbs

The mechanism by which naked therapeutic mAbs exert their anti-tumor effect can be grouped the following categories: 1) perturbation of tumor cell signaling, 2) activation of complement dependent cytotoxicity (CDC), 3) antibody dependent cellular cytotoxicity (ADCC), 4) induction of adaptive immunity (10).

Perturbation of tumor cell signaling. Antibodies of this class target soluble mediators (e.g cytokines) or membrane bound receptors to inhibit their ability to bind their cognate ligands and regulate signaling, and can act as agonists or antagonists. For examples, antagonistic antibodies target the epidermal growth factor receptor (EGFR), or other growth factors over-expressed in cancer, and inhibit their ability to mediate mitogenic signaling (11,12) . Other type of antibodies can inhibit immune suppressing receptors, e.g. CTLA-4, or enhance antigen presentation on APCs through the activation of receptors such as CD40 (13,14). Antibodies designed for signal perturbation frequently belong to the IgG2 and IgG4 subclasses, as they do not activate CDC or ADC and have fewer immune related adverse events due to non specific immune activation (15).

Complement Dependent Cytotoxicity (CDC). The human complement is a complex proteolytic cascade comprised of over thirty proteins involved in different innate immunity responses, such as acting to lysis of foreign cells through the assembly of the membrane attack complex (MAC),

stimulate inflammatory processes through anaphylatoxins, and remove opsonized targets (16,17). When two or more antibodies bind to a cell, the classical complement pathway is activated through the binding of the C1 complex, a serine protease consisting of C1q, C1r and C1s, to the antibody's Fc domains. This activates a proteolytic cascade that leads to the formation of the MAC and the release of potent anaphylatoxins and opsonins resulting in cell lysis and phagocytosis (16,18). The ability of human IgG to activate CDC varies depending on the isotype. IgG_3 followed by IgG_1 are the most effective isotypes for stimulating the classic complement cascade: both isotypes bind to C1q leading to formation of C3b on the surface of antibody-coated tumor cells near the site of complement activation. IgG_2 antibodies are less efficient in activating the complement cascade, whereas IgG_4 is unable to do so (19).

CDC is an important contributor to the anti-tumor activity of many therapeutic antibodies, such as Rituximab and Ofatumumab. As already said, Rituximab is a type I chimeric antibody targeting CD20 approved for treatment of many B cell malignancies and is a potent activator of CDC. It inhibits internalization and shedding of CD20 and shifts CD20 onto lipid rafts, increasing the likelihood of complement activation through the assembly of rituximab bound receptors (20). The connection of its therapeutic efficacy with CDC activity has been demonstrated in several studies. In one of them, using in vivo lymphoma model it was found that loss of C1q abrogated the protective effects of rituximab therapy (21). Data from clinical studies showed that polymorphisms in the C1qA gene in patients with follicular lymphoma are associated with response to rituximab therapy (22). However, some undesired effect of rituximab seems to due to its CDC activity. One small clinical study found that, following rituximab therapy, patients had high circulating levels of circulating complement components with associated severe toxicity (23,24).

Ofatumumab is another type-I anti-CD20 antibody that binds to a distinct epitope of CD20 and induces greater CDC com-pared to rituximab(25). The higher efficacy of Ofatumumab could be ascribed to the fact that it binds C1q with greater avidity than rituximab and efficiently kills rituximab-resistant large B-cell lymphoma cell lines. Moreover it is able to lyse cell lines expressing low levels of CD20, which are not efficiently killed by rituximab (26). Clinical trials data showed that ofatumumab has high response rates in patients with refractory chronic lymphocytic leukemia (CLL) (27).

Antibody Dependent Cell-Mediated Cytotoxicity (ADCC). ADCC is activated by the interaction of the Fc domain with FcγRs on effector immune cells,. The stimulation of immune-receptor

tyrosine-based activation motifs and immune-receptor tyrosine-based inhibitory motifs results in activating or inhibitory signals through FcγRs, respectively. There are three activating FcγRs: FcγRI (CD64), which binds to monomeric IgG and tends to be occupied by plasma IgG, the low-affinity FcγRIIA (CD32A), and FcγRIIIA (CD16A) which bind IgG aggregates or immunocomplexes, and one inhibitory receptor, FcγRIIB (CD32B) (28,29).

Natural killer (NK) cells, which predominantly express FcγRIIIA, are the main effector cells of ADCC, although macrophages and granulocytes cells have been shown to mediate ADCC to a lesser extent (28). These effector cells, through the involvement of FcγRs, recognize antibodies bound to cancer cell and cause their direct lysis through release of granzymes and perforin which culminates in antibody-dependent cell-mediated cytotoxicity (ADCC). Upon crosslinking of FcγRIIIa by the immune complex, the immunoreceptor tyrosine-based activation domain is phosphorylated by the SRC family tyrosine kinase LYN, thereby inducing the formation of a signaling complex (29). This signaling complex results in the activation of phospholipase C, which hydrolyzes phosphatidylinositol-3,4-bisphosphate [PtdIns(4,5)P2] into diacylglycerol (DAG) and inosititol-1,4,5-triphosphate [Ins(1,4,5)P3], thereby inducing a number of signaling events including calcium influx from the endoplasmic reticulum and the opening of calcium-release-activated calcium channels (29,30). The increased intracellular calcium induces the serine/threonine phosphatase calcineurin to dephosphorylate the nuclear factor of activated T cells (NFAT). Dephosphorylation of cytoplasmic NFAT exposes a nuclear localization sequence that causes translocation of this transcription factor to the nucleus where it induces the expression of a number of genes involved in the ADCC pathway (29,31). These effects are dependent on a number of factors including the density of the antigen on the cell surface and the isotype of the antibody. Of the drugable human IgG isotypes, IgG1 is reported to be the best at inducing effector function and is selected as the isotype for the antibody in indications such as the treatment of cancer where cell killing may be part of the mechanism of action. In cases where cell killing is not wanted, IgG2 or IgG4 are the isotypes of choice since they have limited ability to activate effector function,

Among others, two common mAbs with ADCC activity are trastuzumab and rituximab, which require functional activating FcγRs to exert their activity (32). Recent studies highlighted two determinants that could influence the clinical efficacy of therapeutic antibodies with ADCC activity. The first determinant is the balance between expression of activating and inhibitory FcγRs. In support of this notion, it has been shown that animals lacking expression of FcγRIIB

displayed a greater anti-tumor response when treated with therapeutic antibodies (32). The second determinant is the level of macrophages which can act as an effector for ADCC. High levels of macrophages are normally considered as prognostic factor for poor survival. However, two independent clinical studies have shown that follicular lymphoma patients with high levels of tumor associated macrophages have an improved response to rituximab (33), which might be ascribed to an increase in ADCC.

Induction of Adaptive Immunity. Numerous pre-clinical studies support the notion that tumor targeted antibodies can elicit adaptive immune responses, and a growing body of clinical evidence suggests that this mechanism may contribute to the clinical efficacy of antibodies. The mechanism by which this can happen is multiple and involves CDC, ADCC, or antibody dependent cell-mediated phagocytosis (ADCP). Tumor cell fragments and tumor antigens released during these processes can be taken up by professional APCs, such as DCs, to initiate tumor directed adaptive immunity. In addition, antibodies can trigger adaptive immunity by acting as an opsonin and triggering Fc dependent phagocytosis of tumor cells by APCs. Tumor antigens are processed by DCs through the endocytic pathway and presented on MHC II to prime CD4+ T cells. In addition, DCs are capable of presenting engulfed tumor antigens on MHC I molecules and elicit tumor-specific CD8+ cytotoxic T-cells (CTLs). Upon activation, CTLs can directly kill tumor cells that present the cognate peptide on MHC I, or further differentiate into tumor specific memory T cells (34).

Preclinical studies showed that DCs loaded with ovarian and melanoma cells coated with antibodies were able to elicit tumor specific CTLs able to kill primary ovarian and melanoma cells (35). A recent study showed that colon cancer cell lines coated with cetuximab, were able to induce tumor specific CTLs from autologous human DCs (36). Overall, the elicitation of adaptive immunity could potentially sustain anti-tumor immune responses in patients.

Immunotherapy approaches to activate the immune systems.

A number of molecular engineering approaches are undertaken to enhance the antibody capacity to activate immune effector mechanisms and/or to overcome the suppressive tumor microenvironment. One approach is based on the generation of non-fucosylated antibodies, In fact, it has been shown that highly fucosylated Fc significantly impair immune activation. Advanced clinical studies with non-fucosylated antibodies are currently ongoing with promising results (37). Another approach is that of bispecific tri-functional antibodies (so called triomabs) which have

two distinct Fab regions capable of binding two distinct epitomes, which can simultaneously bind tumor cells and immune cells while maintaining the capacity to mediate Fc dependent effector functions. To this class belongs Catumaxomab, a tri-omab that targets the tumor antigen EpCAM and the T-cell stimulatory receptor CD3, which allows for direct stimulation of CTLs in the tumor microenvironment (38). However, the complexity and the very high cost associated with this technology are preventing its widespread development.

Finally, a promising cancer immunotherapy strategy is to target the effector cells that largely contribute to the immune suppressive tumor microenvironment. As already said, Ipilimumab inhibits the potent immune suppressor molecule CTLA- 4 that is expressed by Tregs and consequently, the capacity of Tregs to inhibit the anti-tumor immune response is diminished, resulting in increased levels of CTLs, CD4+ T cells, and APCs (14,39). Following the success of ipilimumab, other mAbs that are emerging from clinical studies are those that antagonize the interaction of programmed death–1 (PD-1), another negative regulator of T cells with its ligands PD-L1 and PD-L2 (40). The PD-1/PD-L1 axis serves as another front-line mechanism of immune suppression in tumor, whose role is to prevent the unrestrained activation of T cells that have been previously activated and probably to contribute to maintain peripheral tolerance. During antigen presentation by dendritic cells, PD1 can act as a checkpoint inhibitor which can sent a negative signal by its binding to either PD-L1 or the closely related (and dendritic cell–specific) negative regulatory ligand PD-L2.. The addition of mAbs to either PD-1 or PD-L1 blocks their interaction, thereby rescuing T cell cytotoxic activity. Often, this results in rapid and substantial tumor shrinkage coupled with long-term responses (40). Two antibodies against PD-1 (nivolumab and lambrolizumab) and one to PD-L1 (MPDL3280A) are the most advanced in the clinic studies, and show less serious adverse effect than Ipilimumab.

Antibodies that block immune checkpoints and immune suppression in the tumor bed have so far produced long-term, durable patient responses rarely seen with other therapeutics and, as such, their development is expected in increase in the coming years.

Overall, in the last decade mAb therapy has revolutionized the treatment of cancer and it will continue to dominate in the years to come, in conjunction with other treatment chemotherapy. Presently, the best hope to maximize the efficacy of antibody therapy is represented by rational combinations of mAbs targeting tumor-specific antigens, either as naked or as ADCs, with antibodies targeting the immunosuppressive tumor microenvironment. The use of these antibody

combinations is expected to significantly lower the amount of cytotoxic chemotherapy that is still the main pillar of most systemic oncology treatments.

1.4 Externautics approach for tumor marker discovery

This PhD project has been conducted at Externautics, a Company located in Siena at the incubator Toscana Life Sciences (TLS). Externautics is a research company whose objectives consist in the discovery and pre-clinical validation of novel cancer markers and therapeutic targets, and the generation of mAbs for the diagnosis and treatment of prevalent human cancers. Briefly, the Externautics' approach for marker discovery and development of mAbs exploits the availability of an in-house generated collection of about 2600 murine polyclonal antibodies (pAbs) directed against secreted and membrane-associated human recombinant proteins only marginally characterized in the scientific literature (41). The library of pAbs is used screen Tissue Microarrays (TMAs) carrying clinical samples from pedigreed patients affected by breast, colon, lung, prostate and ovary tumors, generated at the European Oncology Institute (Milan, Italy). The concomitant presence on the TMAs of both cancerous and normal tissues from the same patients allows to discriminate the pAbs able to selectively cancer samples, thus providing a first-hand indication that their target proteins are over-expressed in tumor. After their initial discovery, the novel potential cancer markers are extensively validated using molecular and cellular biology experiments aimed at identifying whether they could be exploited as diagnostic markers or therapeutic targets, including analysis of antibody specificity, gene expression profiling in tumor cell lines, marker cell localization and biological characterization. Finally, murine mAbs (mAbs) are generated against the markers by immunizing animals with the recombinant protein and/or selected domain of the protein. Afterwards, the mAbs are further characterized so as to select the most promising candidates to be brought to the pre-industrial development phase, ready to enter the clinical development phase.

The strategy workflow is expected to generate tumor markers and mAbs with potential applications in the cancer diagnostics, small molecules and antibody therapeutics, and vaccine fields.
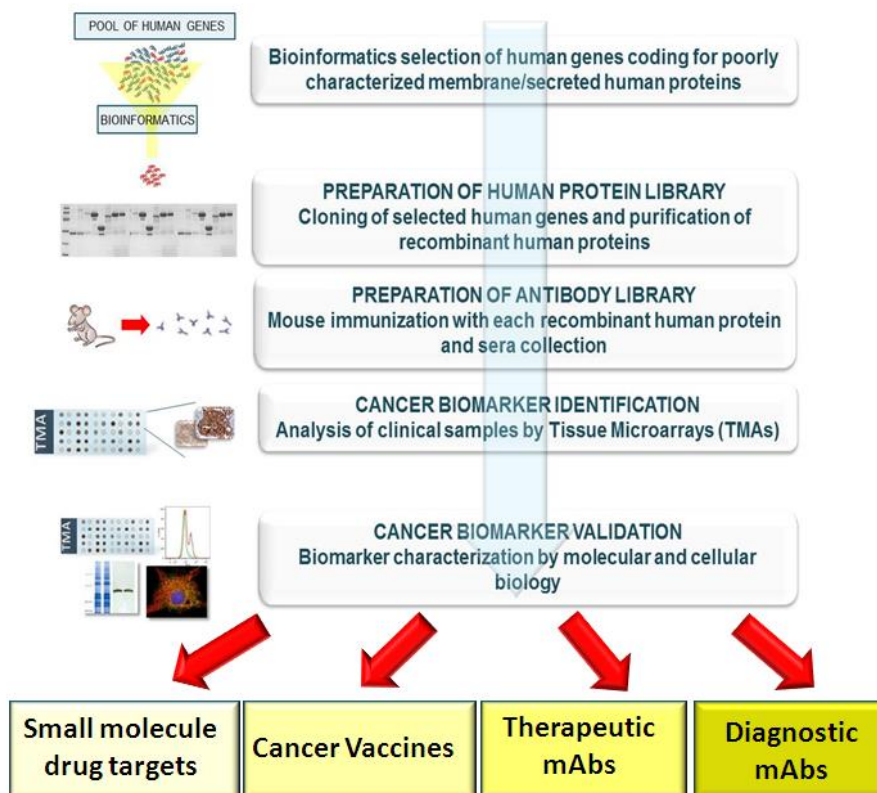
Figure 3 – Experimental approach used by Externautics for tumor marker discovery and characterization.

The screening of the pAb collection on TMAs carrying clinical specimens from 5 patients per each tumor has currently allowed the identification of 89 novel tumor-associated proteins differentially expressed in one or more tumors. These proteins are distributed in all the five tested tumors with approximately one fifth selectively over-expressed in only one tumor, one third in two tumors and the remaining part in three or four tumor types (Figure 4A). Most of these proteins show several annotated variants, resulting from alternative splicing events, predicted to be found in different cellular compartments. Bioinformatic analysis predicts that approximately 30% of the 89 proteins have at least a variant associated to the plasma membrane, 50% have a secreted isoform and the remaining proteins are associated to the internal cell membranes, such as those belonging to the Golgi apparatus and mitochondrion (Figure 4B).
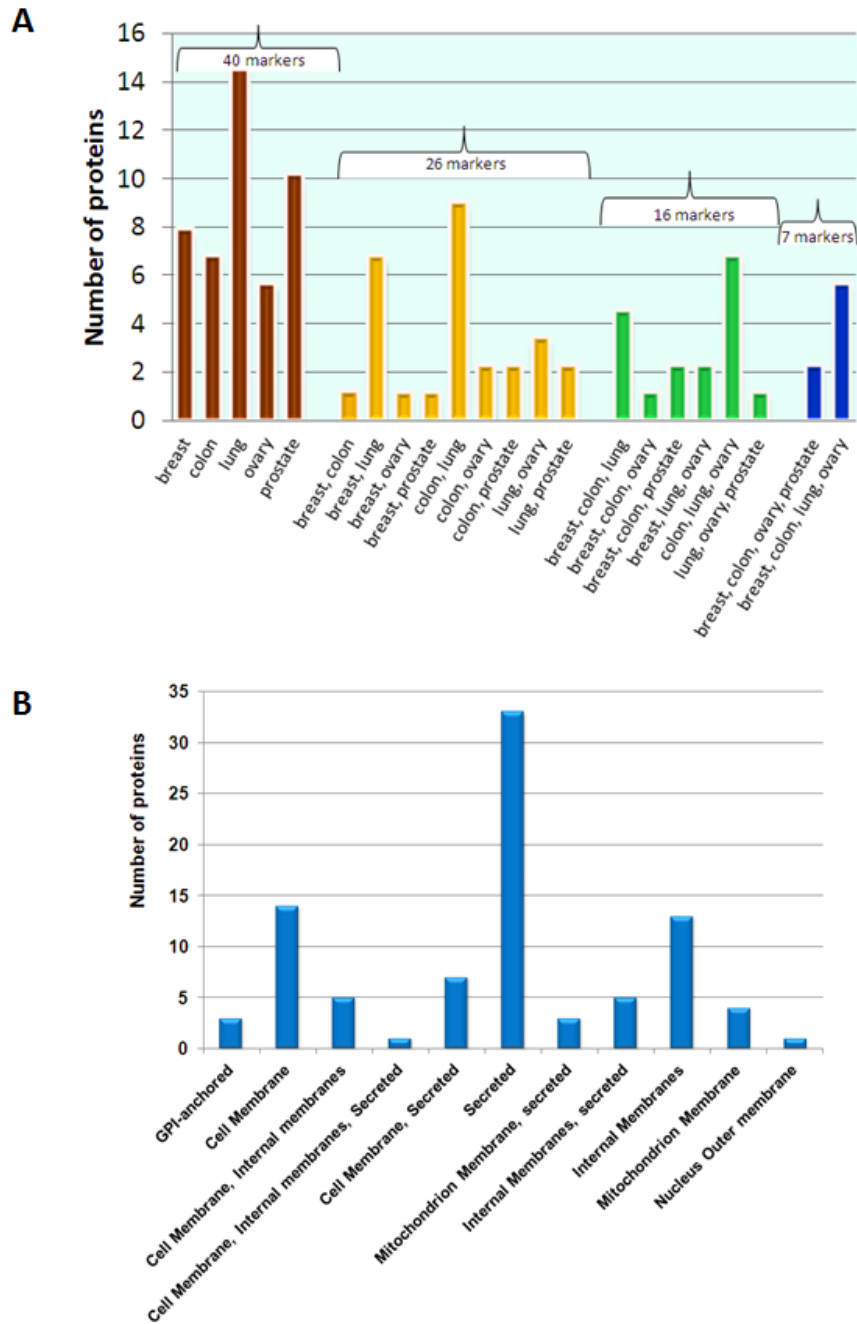
Figure 4 – Representation of the 89 novel markers discovered by Externautics. A) Distribution of IHC positivity of the 89 markers in the five tumor types. B) Predicted cell localization of the markers.

The 89 proteins are currently under characterization. An expanded IHC analysis so far conducted for 27 proteins on 50 patients per tumor showed that 19 of them are over-expressed in one or more cancers with frequencies ranging from 20% to 96% (9 pending patents: PCT/EP2010/000503, PCT/EP2010/000502; PCT/EP2010/066147, PCT/EP2010/066144, PCT/EP2010/066146, PCT/EP2010/066154, PCT/EP2010/066134, EP10161559.9).

1.5 EXN11 and EXN6, two novel tumor-associated proteins

The present study focuses on EXN11 and EXN6, two novel markers identified in the primary TMA screening. EXN6 is a single-pass type II membrane protein with a carbohydrate-binding domain. Apart from sequence annotation, EXN6 is almost unknown and no data are available on its expression, biological function and association to cancer. As far EXN11 is concerned, this protein shows homology the angiopoietin-like (ANGPTL) proteins, a family of secreted proteins with structural similarity to members of the angiopoietin (ANG) family, with which they share a coiled-coil domain at the N-terminus and a fibrinogen-like domain at the C-terminus. Most members of this family are emerging as central players of different cell processes and human diseases. Several ANGPTL proteins potently modulate angiogenesis (42-45), though for some of them the effect remains controversial. Similarly, their involvement in tumor and tumor-angiogenesis has been only partially elucidated. ANGPTLs are also involved in other processes independently from angiogenesis, such as augmentation of energy expenditure, induction of inflammation, and metabolism (44-47), as well as in associated diseases, such as metabolic and cardiovascular diseases (48). Concerning the EXN11, its role in angiogenesis and expression in human cancer has not been reported yet.

2. AIM OF THE PROJECT

Scope of the experimental work of this PhD thesis was twofold. The first objective of the project was the confirmation and characterization of EXN6 and EXN11 as novel tumor-associated proteins that could be exploited as diagnostic, prognostic, predictive or therapeutic biomarkers. The second objective was to develop specific mAbs able to specifically detect the markers and to interfere with tumor-related processes in vitro and/or in vivo.

1) *Marker confirmation and characterization.* The activity consisted in confirming the positivity of the cancer-reactive antibodies towards EXN11 and EXN6 in an expanded number of patients, using TMA representing tissues from 50 patients per each tumor. This confirmatory analysis allows to obtain a preliminary statistical analysis on the frequency with which these proteins are over-expressed in the tumors in which they were originally discovered. Afterwards, a marker characterization study was undertaken, aimed at defining their most likely use of EXN6 and EXN11 as diagnostic and/or therapeutic tools. It consisted in different molecular and cell biology analyses that allow to assess: i) the marker expression in human cell lines, either endogenous or in response to specific environmental stimuli; ii) the marker localization on commercially available

tumor cell lines; iii) the marker biological role, by investigating the perturbation of major cell processes relevant for tumor development caused by the alteration of marker expression.

3) *Generation and validation of mAbs*. The activity intended to develop marker-specific mAbs, with priority for the surface-associated protein EXN6. It consisted in generating panel of EXN6-specific mAbs and confirming the mAb ability to detect the target protein in cancer cells using different immunoassays, such as Western blot (WB), flow cytometry, confocal microscopy and immuno-histochemistry. The expected achievement was the availability of mAbs able to detect EXN6 on the surface of cancer cells and, possibly, to interfere with cellular processes relevant for cancer growth.

3. RESULTS

The Results section separately describes the major scientific achievements related to EXN6 and EXN11.

3.1 EXN6
3.1.1 Confirmation of EXN6 detection in human cancers

The screening of the antibody collection on TMA carrying tumor and matched normal samples of 5 patients per each tumor provided the first experimental evidence that EXN6 is over-expressed in breast, lung, colon and ovary tumors. We then confirm the altered expression of EXN6 in larger set of samples. To this regard, tumor-specific TMA were generated at IEO carrying clinical specimens of breast, colon, ovary and lung cancer (representing 50 patients/tumor) and analyzed with the anti-EXN6 pAb. EXN6 was mainly detected in breast (40% positivity) and ovary cancer samples (35%). In colon and lung cancer specimen samples it was detected with lower frequency (less than 10%, respectively). In these cancers the immuno-histochemical staining mainly accumulated at the plasma membrane, leading us to hypothesize that the protein is surface exposed (Figure 5).

We then investigated the presence of EXN6 in fresh bioptic specimens of breast cancer by WB. Since surgical biopsies are very inhomogeneous and contain epithelium, stroma, and connective tissues in variable amount, this analysis does not allow a marker quantitative measurement, but it was meant to assess the presence of EXN6 species in cancer, compared to normal samples. Total protein extracts were prepared from four biopsies of breast cancer and matched adjacent normal

samples, loaded on PAGE-SDS (25 µg/lane) and subjected to immunoblot. As shown in Figure 5, EXN6 bands (ranging from 55 to 65 kDa) were detected in 3 of the 4 samples, which were absent or only marginally visible in normal samples.
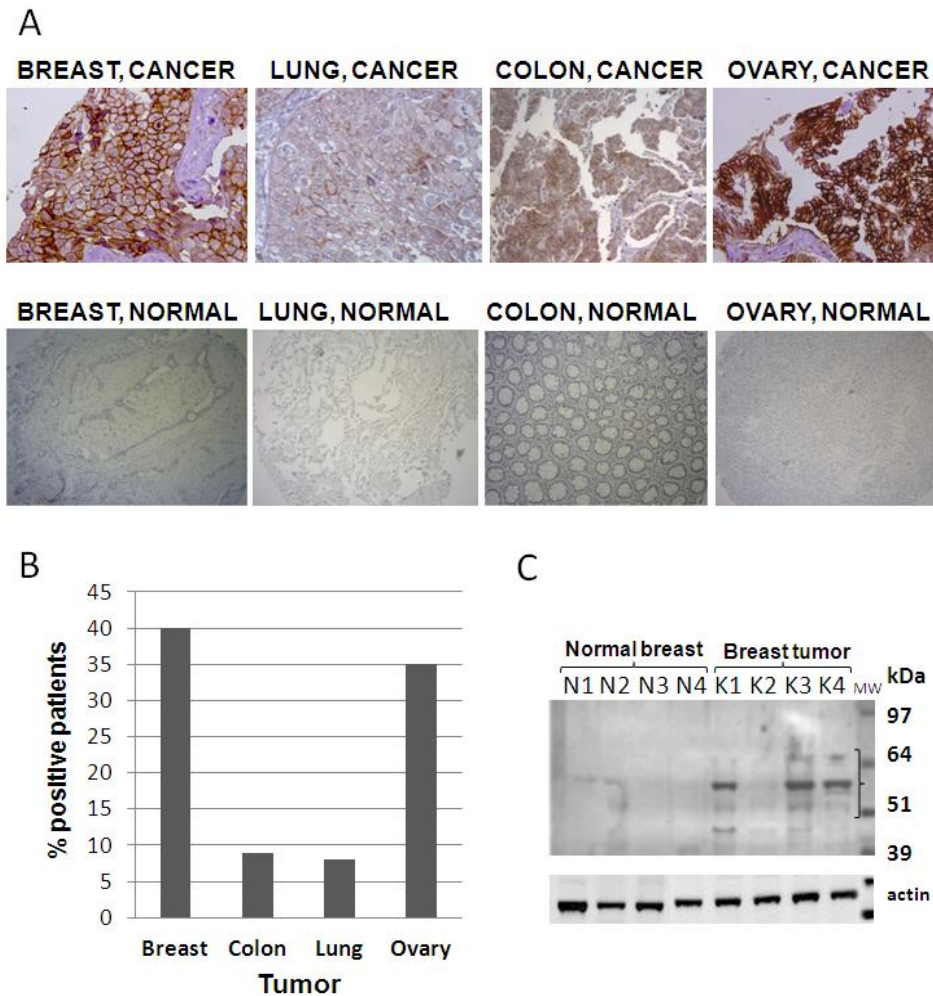
Figure 5. EXN6 detection in clinical sample specimen by IHC. A) IHC images of positive cancer specimens and corresponding normal samples stained with the anti-EXN6 pAb. B) Graph reporting the EXN6 detection frequency in clinical specimens from 50 patients/cancer. C)WB analysis of cancer (K) and normal (N) breast specimens stained with the anti-EXN6 pAb. Actin was used as sample normalization control.

3.1.2. The IHC-reactive anti-EXN6 polyclonal antibody specifically recognizes its target protein

The first experimental step necessary to start EXN6 validation and characterization was to assure the specificity of the anti-EXN6 pAb showing reactivity on cancer tissues. This was done by analyzing its ability to recognize EXN6 expressed in mammalian cells by WB and confocal

microscopy. The EX66 coding sequence was cloned in vector pcDNA3.1D and the resulting plasmid was used to transiently transfect HeLa and/or Hek293T human cell lines. After 24 hours, total cell extracts were prepared with and without DTT, separated by SDS-PAGE (25 micrograms/lane, corresponding to approximately $1x10^5$ cells) and subjected to immunoblot with the anti-EXN6 pAb. As controls, total extracts of EXN6-transfected cells were run in parallel. Under reducing condition the anti-EXN6 pAb was able to detect a EXN6 band (approximately 50 kDa) over-expressed by HeLa cells upon transfection. When WB was conducted under not reducing condition, other more intense high molecular weight bands ranging from 90 to 200 kDa were detected, likely corresponding to EXN6 dimers and multimers (Figure 6A).

For confocal microscopy analysis, EXN6 transfected cells were fixed with para-formaldehyde and incubated with the anti-EXN6 pAb, before or after treatment with BRJ96, a detergent used to permeabilize the plasma membrane. The anti-EXN6 pAb was able to bind EXN6 transfected cells without cell permeabilization (Figure 6B) indicating that, in agreement with its prediction, EXN6 is surface exposed.
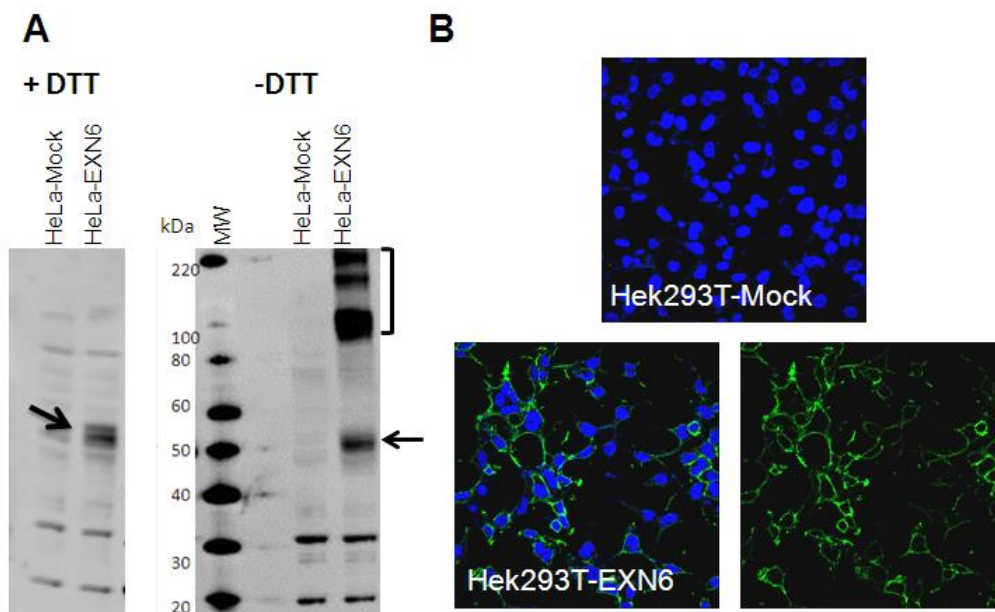
Figure 6 –Specificity of the IHC reactive anti-EXN6 polyclonal antibody. Cells were transfected with a EXN6-coding plasmid and the empty vector (mock) and subjected to WB (A) and confocal microscopy (B). For WB, total extracts from EXN6 and Mock transfected HeLa cells were prepared with or without DTT and used for immunoblot with the anti-EXN6 pAb. The EXN6 monomer and multimer bands are marked by arrow and bracket, respectively. For confocal microscopy, trasnfected HEK293T cells were fixed, incubated with the anti-EXN6 pAb, followed by incubation with an Alexa 488-conjugated secondary antibody. DAPI was used to visualize nuclei.

3.1.3 EXN6 is endogenously expressed and surface exposed in breast and ovary cancer cell lines

Confocal microscopy analysis of EXN6 transfected cells confirmed that EXN6 is surface exposed. We then investigated EXN6 endogenous expression and localization in cell lines derived from breast, ovary and colon cancer cell lines by flow cytometry, using a surface staining setting. Among 15 cell lines globally analyzed, the breast cell lines SKBr3, MCF7 and T47D and the ovary cell line Ovcar 3 showed FACS positivity (Figure 7A). WB analysis under not reducing condition confirmed the presence of EXN6 in these cells (Figure 7B). Moreover, the specificity of the EXN6 detected band was confirmed by gene silencing. SKBr3 cells transfected with EXN6 specific siRNAs o with an irrelevant siRNA (scrambled siRNA) and at 72 hours later EXN6 loss of expression was assesses by WB. As shown in Figure 7B, treatment with EXN6 siRNA resulted in the reduction of a band of approximately 90 kDa band, likely corresponding to EXN6 dimer. EXN6 transcription was significantly reduced at 48h after siRNA treatment, as judged by q-RT-PCR (not shown).

Overall, the results confirmed that EXN6 is endogenously expressed in a subset of ovary and breast cancer cells, in which it is surface exposed. This evidence suggests that EXN6 is a potential drug target in breast and ovary cancers.
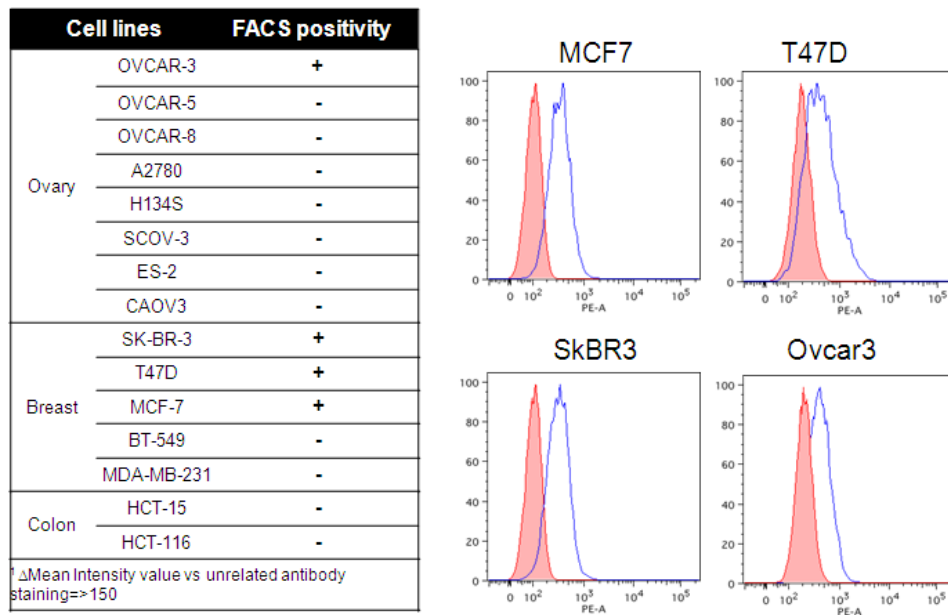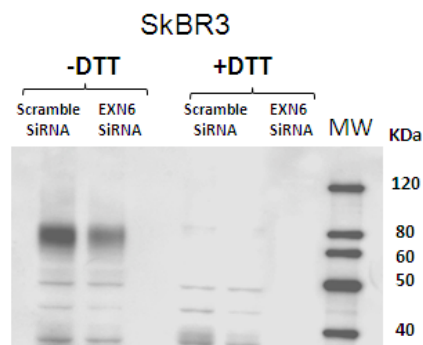
Figure 7. EXN6 expression and localization in cancer cell lines. A) FACS analysis. The table reports the panel of cell lines tested by surface staining using an anti-EXN6 pAb. The four FACS positive cell lines are represented in the graphs (Bleu, EXN6-pAb; red,unrelated negative antibody control). B) WB analysis of SKBR3. Cells were treated with EXN6-specific or scrambled siRNAs and 72 hours later total extracts were prepared with/without DTT for immunoblot, probed with the anti-EXN6 pAb. A major band of approximately 90 kDa was detected in cell total extracts, only under not reducing conditions. This band showed a weaker intensity in EXN6 silenced cells.

3.1.4. EXN6 is involved in cell proliferation and invasiveness

Ideal therapeutic targets are key player in signaling pathways frequently activated or altered in cancer. This type of proteins enables the generation of drugs able to inhibit their expression and function, ultimately leading to the arrest of proliferation, the induction of apoptosis, or alteration of cell motility properties. To obtain a preliminary indication of EXN6 relevance in tumor development we investigated the phenotypic effect of EXN6 knock-down in the viability,

proliferation and migration of breast cell lines. In these experiments, EXN6 silencing was verified by q-RT-PCR (not shown). MCF7 cells transfected with EXN6 siRNA or scramble siRNA were incubate for 72 either without FBS, for viability analysis, or in the presence of 2,5% FBS, with and without 10 ng/ml of EGF, to estimate proliferation. Cell viability and proliferation was assessed by the MTT assay, a colorimetric assay that allows to monitor the enzymatic conversion of a tetrazolium salt into a purple-colored formazan product in viable cells, by spectrophotometric measurement of the adsorbance at 570 nm. As shown in Figure 8A, EXN6 silencing caused a 2-fold decrease in cell viability and cell proliferation.

Cell migration / invasiveness was assessed in the Boyden-Matrigel-Assay. This assay employs a trans-well chamber in which the upper and lower compartments are separated by a microporous membrane. Cells are seeded on the upper compartment and are stimulated to migrate the lower compartment under a chemo-attractant stimulus, crossing the porous membrane. When used to measure invasiveness, cells must degrade a 3D extracellular matrix (Matrigel) before crossing the membrane. After 16h, the number of cells that have migrated to the lower side of the membrane is counted. In this assay, MCF7 and SKBR3 cells treated with EXN6-specific siRNAs for 72 hour showed a significant decrease of the invasive phenotype, as compared to control cells (Figure 8B). The effect was more pronounced in MCF7 (5 fold) than in SKBR3 cells (2 fold).
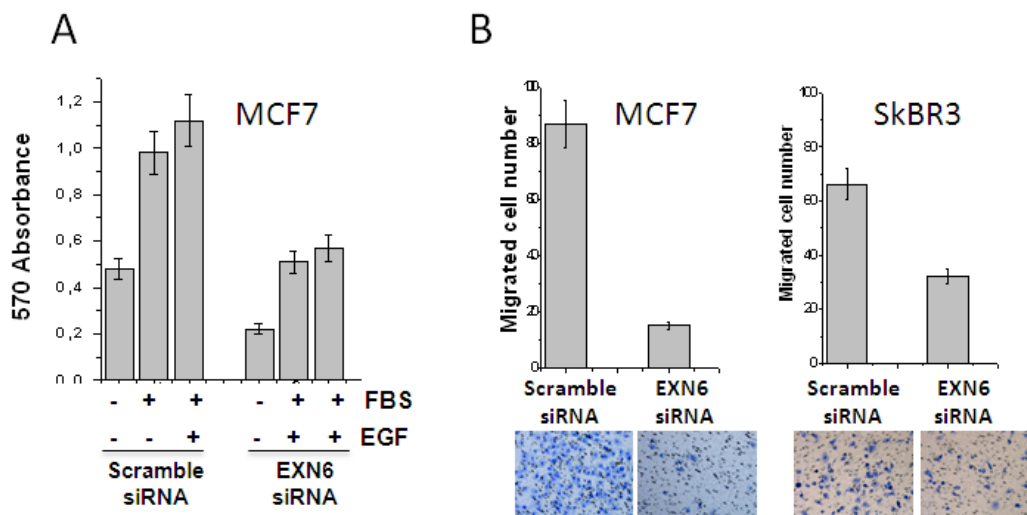
Figure 8– EXN6 is involved in cell proliferation and invasiveness. MCF7 and/or SK-BR-3 cells were transfected with EXN6-specific or scrambled siRNA. After 72 hours cells cell viability/proliferation and invasiveness was assessed. A) MTT assay. MCF7 cells were exposed to 2.5% FBS, 2.5% FBS+10 ng/ml EGF or serum-starved and cell viability/proliferation was measured by the MTT and optical absorbance reading. B) Boyden assay. MCF7 and SK-BR-3 were seeded on the upper chamber of a Matrigel-coated 96-well plates and the number of migrated cells was evaluated after Diff-Quick staining by counting cells in six randomly chosen fields. Images below the graphs show the visual counting.

3.1.5. Anti-EXN6 mAbs specifically recognize the protein on the surface of breast and ovary cancer cells.

To achieve the proof of concept that EXN6 could be a promising target for mAb therapy, mAbs were generated towards recombinant EXN6 using the standard hybridoma technology based on the fusion of splenocytes from immunized BALB/c mice with Ag8 myeloma cells (mAb production carried out at The Fourth Military University, X'ian, China). After screening by ELISA on the recombinant proteins, approximately 20 EXN6-specific mAbs were obtained. Afterwards, these mAbs were further screened by WB and FACS on EXN6 transfected HeLa/Hek293T cells. In total, five anti-EXN6 mAbs (labelled from 1 to 5) specifically recognized EXN6 transfected cells both by WB and FACS (Figure 9A). The five EXN6 mAbs were also tested for their ability to detect EXN6 on the surface of breast and ovary cancer cell lines (see Figure 7). As shown in Figure 9B, the five mAbs were able to bind the surface of MCF7, T47D, SKBr3 and Ovcar3 cell line (Figure 9B). The mAb specificity was confirmed in the EXN6-positive cells by treating them with different concentration of EXN6 siRNA (ranging from 10 to 50nM) and monitoring the disappearance of surface binding. EXN6-siRNA treated cells showed a significant loss of surface staining, as opposed to cells treated with a scrambled siRNA (see exemplary data for anti-EXN6 mAb1 and mAb2 in SKBR3, Figure 9C). WB analysis on EXN6 silenced cells further confirmed the mAb specificity (data not shown).
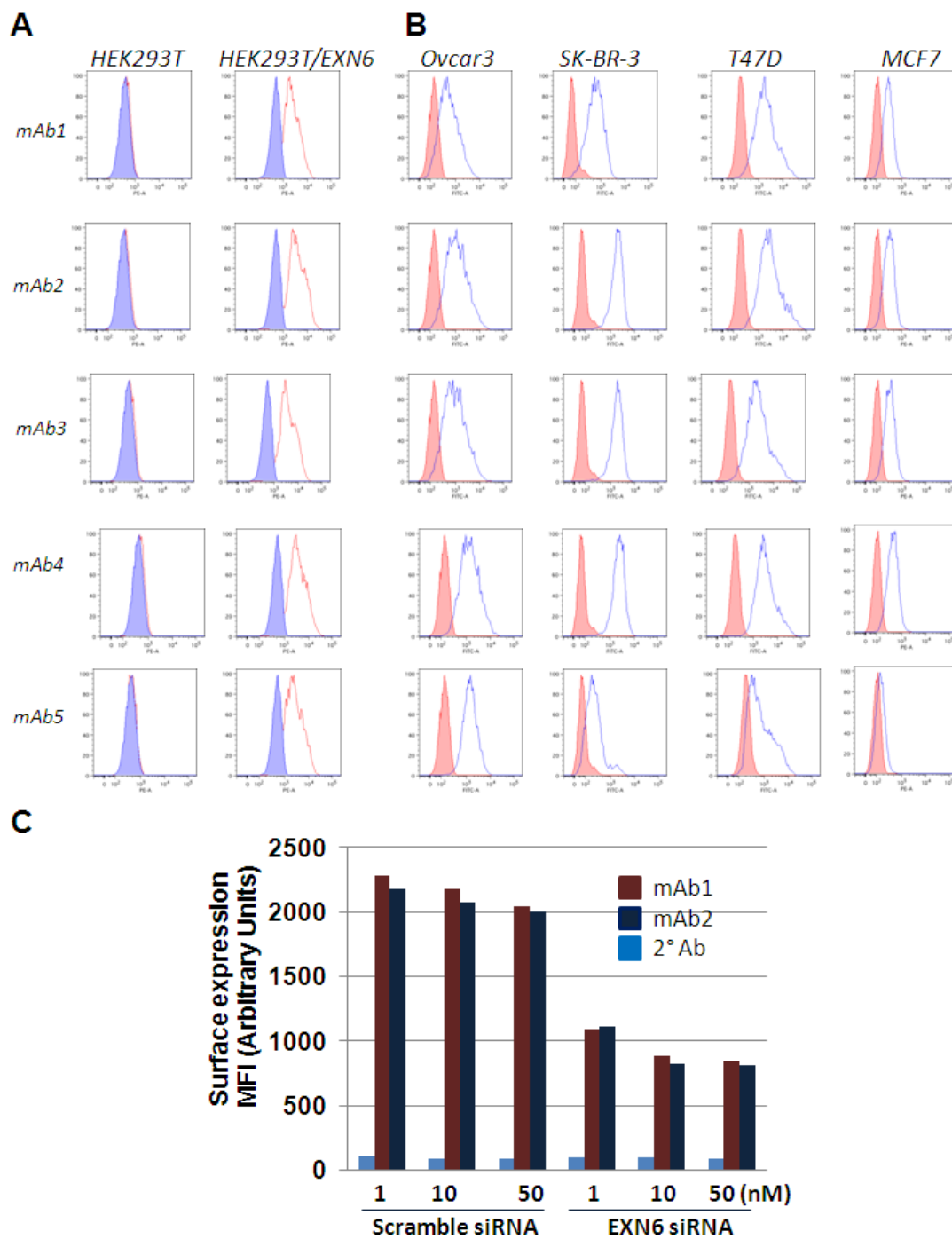
Figure 9 – The anti- EXN6 mAbs bind the surface of breast and ovary cell lines. A) FACS analysis of EXN6 transfected HEK293T cells with the five anti-EXN6 mAbs (red peak, EXN6 mAbs, Blue peak, irrelevant isotype control); B) FACS analysis on the EXN6-positive breast and ovary cell lines (blue peak, EXN6 mAbs; red peak irrelevant isotype control). C) FACS analysis in EXN6 knocked down SKBR3 cells. Cells were transiently transfected with different

concentrations of EXN6-specific sirRNA and 72 hours later the residual mAb surface binding was measured by flow cytometry as compared to cells treated with an irrelevant siRNA.

3.1.6 Two anti-EXN6 mAbs are efficiently internalized by breast and ovary cancer cells

Antibody-drug conjugates (ADC) are powerful tools to increase the anti-tumor activity of naked antibodies. As already said, the most important property of antibodies suitable for ADC development is the ability to be internalized by cancer cells shortly after their surface binding on cancer cells, so as to efficiently drive their drug-conjugates into the cells. To assess whether the anti-EXN6 mAbs have this important property, internalization experiments have been carried out by FACS and confocal microscopy, in breast and ovary EXN6-positive cell lines. Cells were incubated for 30'on ice with the five anti-EXN6 mAbs (10μg/ml) to allow surface binding, subsequently washed to remove the excess of unbound mAbs and then shifted to 37°C for up to 2 hours to permit the internalization process to occur. As negative control, cells were also incubated with a mAb toward CD81, a multipass plasma membrane protein that is not internalized. As controls, samples were kept on ice and analyzed in parallel. For FACS analysis, cells were then incubated with a fluorescently-labeled secondary antibody, and the disappearance of the anti-EXN6 mAbs from the cell surface was monitored as function of time. Two of the 5 anti-EXN6 mAbs (mAb1 and 3) quickly disappeared from the cell surface upon temperature shift (after 30' approximately 50% of surface bound antibodies disappeared from the cell surface) (Figure 10A). Conversely, the other 3 mAbs remained associated on the cell surface under the observation period (mAb2 example, in Figure 10A). To confirm that the loss of mAb surface binding visible upon temperature shift was effectively due the mAb internalization, confocal microscopy was also carried out to monitor the formation of intracellular immuno-complexes. In this experiment, after 1h incubation at 37°C, cells were methanol-permeabilized and incubated with a fluorescently labeled secondary antibody. As shown in Figure 10B, upon temperature shift the surface bound anti-EXN6 mAb 1 and mAb3 were not detectable on the plasma membrane and accumulated in the intracellular milieu, visible as fluorescent spots, confirming that they were efficiently internalized (mAb3 example, Figure 10B).
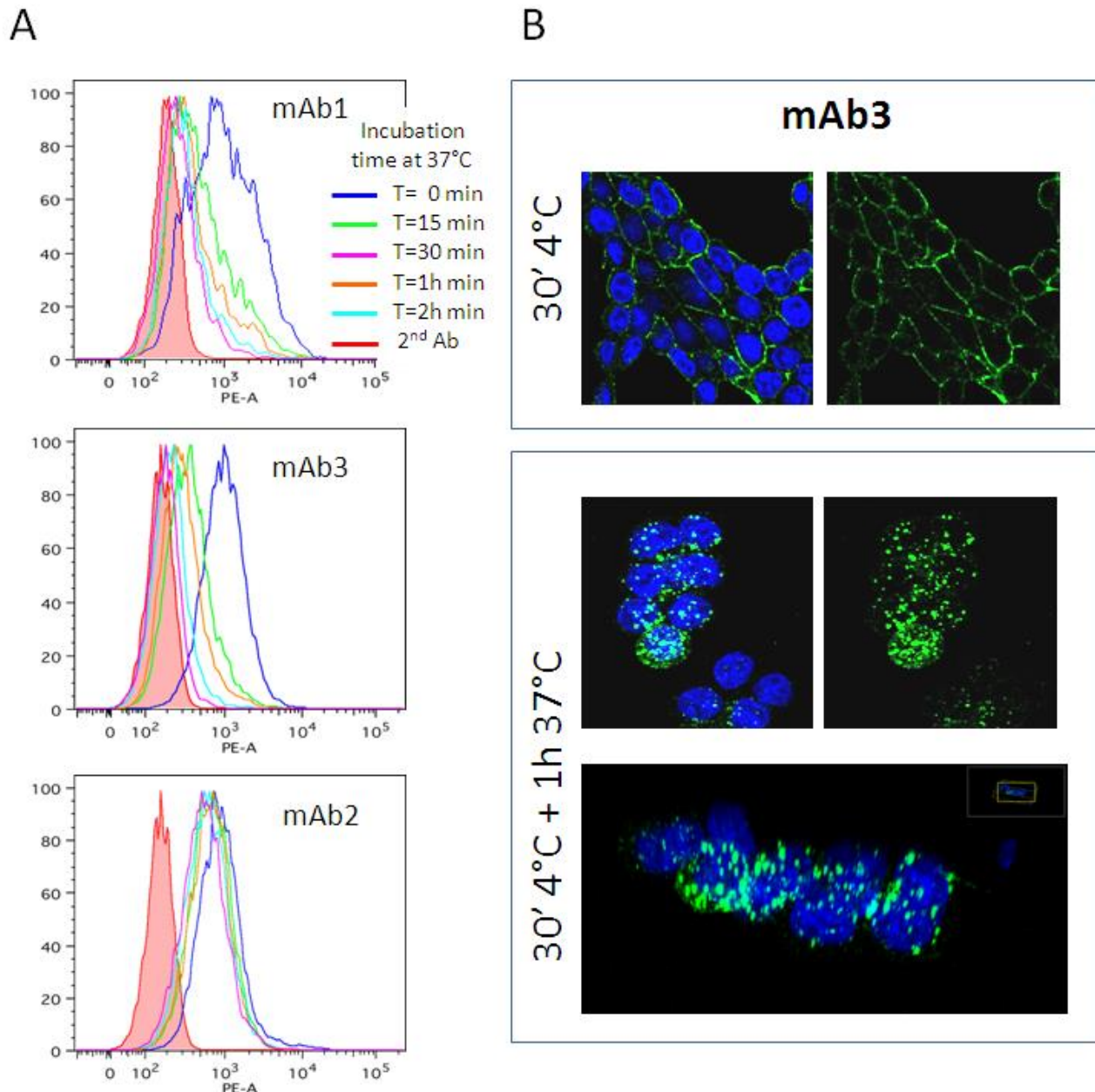
Figure 10 – *Two anti-EXN6 mAbs are efficiently internalized by tumor cells*. Cells were incubated for 30' on ice with the EXN6 mAbs, Afterwards, cells were washed and shifted to 37°C for up to 2h. For FACS analysis (A), cells were incubated at different time point with an Alexa488-conjugated secondary antibody and the residual mAb surface binding was assessed. For confocal microscopy (B), cells were fixed and permeabilized before (upper panels) and after incubation at 37°C (lower panels) and the formation of intracellular immune-complexes was detected by incubating cells with the secondary antibody. DAPI was used to visualize nuclei.

3.1.7 The two anti-EXN6 mAbs promote cell killing in an indirect ADC assay.

To further confirm that the anti-EXN6 mAbs 1 and 3 could be efficient as ADC, an indirect killing assay was used which allows to assess the ability of mAbs to bring a toxin-conjugated secondary antibody in the endosomic pathway and release the cytotoxic toxin within the cells.

T47D cells were incubated for 30' on ice with different concentration of mAbs 1 and 3 (from 1 to 40 μg/ml) to allow surface binding and subsequently incubated for 72h at 37°C in the presence of a secondary antibody linked to saporin (Fab-ZAP system), a ribosome inactivating toxin, and cell killing was measured with the MTT assay and adsorbance reading, as compared to treated with an irrelevant mAb. As shown in Figure 11, both anti-EXN6 mAbs were able to promote cell killing (up to 40%).

Overall, the results indicate that these two anti-EXN6 mAbs hold promise as candidates for ADC development.
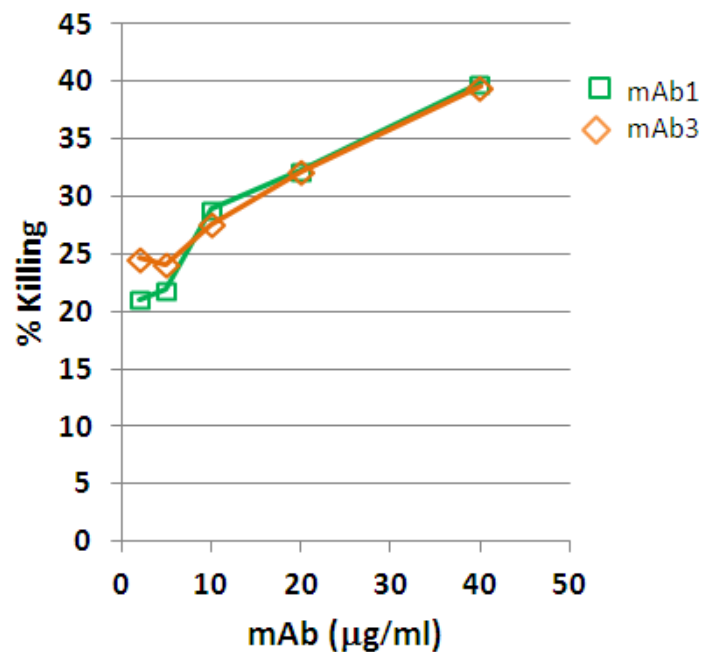
Figure 11. The anti-EXN6 mAbs promote FabZAP-based killing of cancer cells. T47D cells were incubated 30' on ice with different concentration of mAbs 1 and 3 (from 1 to 40 μg/ml) or with an irrelevant mAb, then shifted at 37°C and added with a secondary antibody linked to saporin (Fab-ZAP system). Cell viability was estimated by the MTT assay. The percentage of cell killing was determined by detracting the Adsorbance value of mAb-treated cells from that of control samples, expressed as percentage.

3.1.8 The anti-EXN6 mAb1 shows ADCC activity

As described in the Introduction, ADCC mechanism of action is triggered by antibodies that bind their target antigens on the cell surface and, with their Fc effector portion, also bind to FcγRIIIa receptors on the cell surface of effector cells, predominantly NK, generating multiple cross-linking of the two cell types and ultimately leading to the killing of target cells (49).

We assessed the ADCC activity of the anti-EXN6 mAbs using an ADCC-reporter assay. This method detects the Fc effector function of mAbs by measuring the activation of the nuclear factor of activated T-cells (NFAT) signaling pathway in Jurkat cells that have been engineered to stably express the FcγRIIIa receptor (V158 high affinity variant) and a NFAT response element driving expression of firefly luciferase. ADCC activity is quantified with luminescence readout through the luciferase produced as a result of NFAT pathway activation. This assay has important advantages over conventional ADCC systems, in that it is a non radioactive method, it does not employ human peripheral blood mononuclear cells (PBMCs) from healthy donors as source of NK, while offering accuracy, sensibility and reproducibility of the measurement at the same time. In our study, three anti-EXN6 mAbs were incubated at concentrations (ranging from 0 to 20 µg/ml) with T47D target cells (T) and the engineered effector Jurkat cells (E) (E:T ratio: 3). After 6 hours, the amount of luciferase was determined. Jurkat cells incubated with mAb1 showed an increase in the amount of luciferase (about 3 fold) whereas the other antibodies did not stimulated its production (Figure 12).
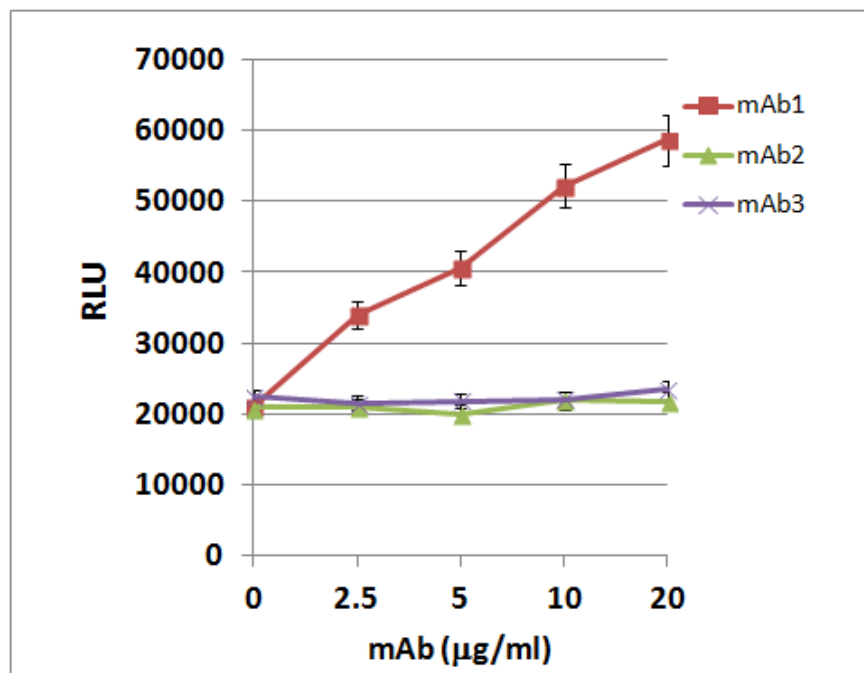
Figure 12 –An *anti-EXN6 mAb with ADCC activity.* mAb1, mAb2 and mAb3 were incubated at different concentrations with T47D and engineered effector Jurkat cells at an E:T ratio of 3:1 (75,000 and 25,000 cells/well, respectively). After 6 hours the amount of luciferase produced by NFAT activation was quantified by luminescence reading, as compared to untreated cells. RLU: Relative Luminescence Unit.

3.1.9. The anti-EXN6 mAbs show marginal reactivity on normal human tissues.

An important aspect in the development of mAbs with therapeutic potential is to assess the reactivity with normal human tissues. Indeed, very frequently cancer markers are expressed at endogenously level at different site. Alternatively, the mAb coud cross-react with other human proteins. To address this aspect we analyzed the immune-reactivity of the anti-EXN6 mAbs on the 33 normal human tissues requested by the Food and Drug administration to assess the mAb specificity.

The anti EXN6 mAb 1 and mAb3 were tested on commercially available tissue microarray carrying the 33 FDA normal human tissues (MNO961, Pantomics) by IHC, using the same staining protocol and mAb concentration used to stain cancer tissue TMA. As shown in Table 2, the two antibodies only gave weak cytoplasmic staining pancreatic Langherans cells and they were almost negative in all the other tissues. This indicates that these antibodies to not have relevant surface associated off-targets in normal tissues and have in general limited reactivity.

Table 2. IHC reactivity of anti-EXN6 mAbs in normal human tissues

Anatomic site	mAb1-mAb3 staining	Anatomic site	mAb1-mAb-3 staining
Adrenal gland	negative	Ovary	negative
Bladder, urinary	negative	Pancreas	cytoplasmic staining in Langherans cells
Bone, bone marrow	negative	Parathyroid	negative
Eye	ND	Pituitary gland	negative
Breast	negative	Placenta	negative
Brain, cerebellum	negative	Prostate	negative
Brain, cerebral cortex	negative	Skin	NV
Fallopian tube	negative	Spinal cord	negative
Esophagus	negative	Spleen	negative
Stomach	negative	Skeletal muscle	negative
Intestine, small intestine	negative	Testis	negative
Intestine, colon	negative	Thymus	negative
Intestine, rectum	negative	Thyroid	negative
Heart	negative	Tonsil	negative
Kidney	negative	Ureter	negative
Liver	negative	Uterus, cervix	negative
Lung	negative	Uterus, endometrium	negative

3.2. EXN11

3.2.1 Confirmation of EXN11 detection in human cancers

The primary IHC screening showed that the EXN11 is expressed in colon cancer (3/5 patients), where it showed a significant expression, and it was also detected breast (1/5) and ovary (1/5) cancers with lower expression level. We then confirmed EXN11 expression in 30 primary colon cancer specimens and 20 samples of liver metastasis from colon cancer. Concerning primary colon cancer, EXN11 was detected in 40% of the samples, with concomitant negligible staining in the corresponding normal tissues (Figure 13A). In agreement with the fact that EXN11 is a secreted protein the immune-histochemical staining was generally moderate and in some cases extended to the cell secretion products, as usually observed with antibodies targeting released products. Concerning the colon metastasis specimens, it was detected in over-expressed in 50% of the cases as compared to normal hepatocytes surrounding the cancerous lesions. In some cases, the IHC staining accumulated in the intra-hepatic biliary ducts (Fig 13C).

To confirm the presence of EXN11 in cancer samples, total protein extracts prepared from cryo-preserved biopsies of colon cancer (4 patients) and ovary cancer (3 patients) were analyzed by WB. Surgical resections from normal controls were run in parallel, including 4 colon samples from the same patients and 2 ovary samples from different donors. As shown in Figure 13B, a band of the expected molecular weight (approximately 45 kDa) was clearly detected in the 4 colon and in one ovary cancers samples. In normal samples, the same band was either absent or had a weaker intensity.
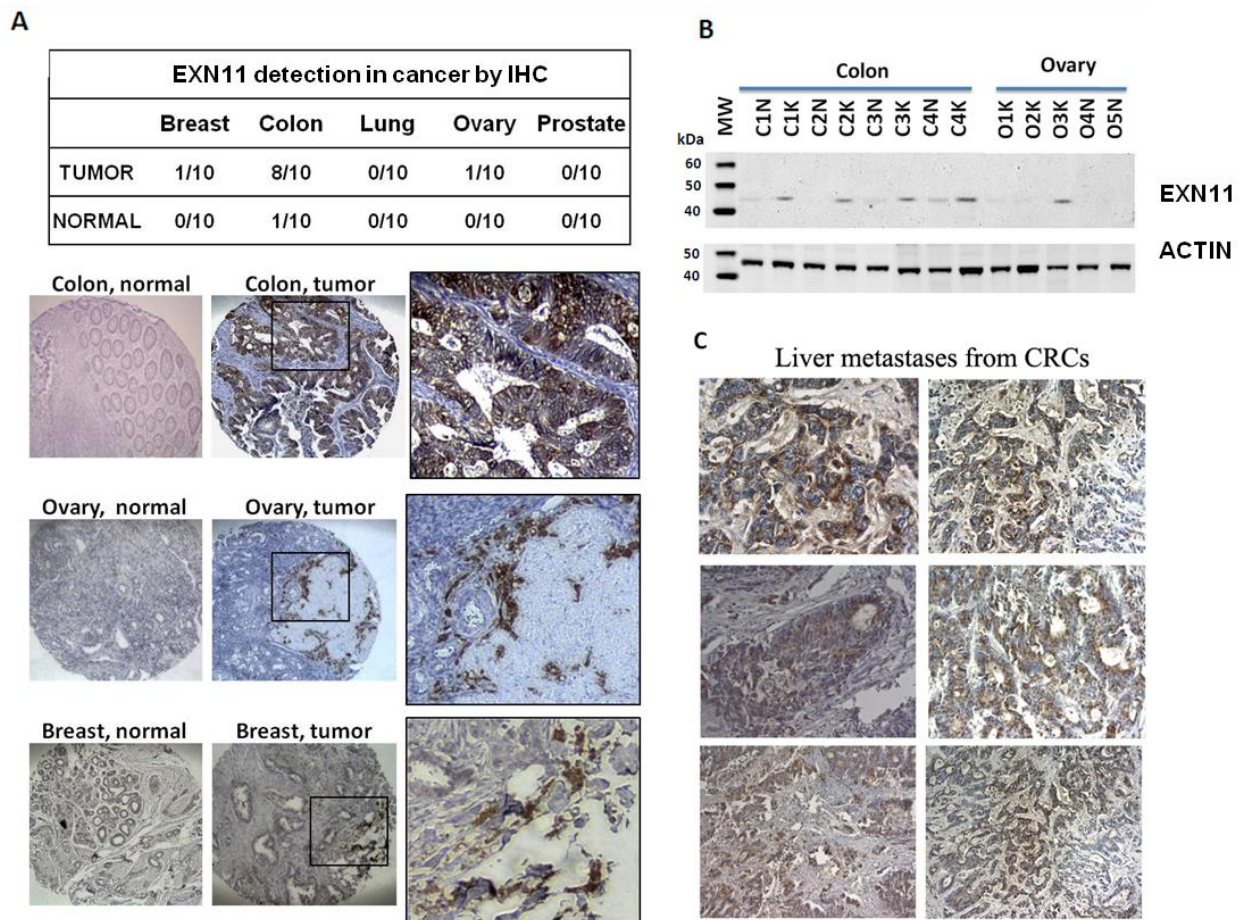
Figure 13. Detection of EXN11 in cancer.
A) IHC analysis of tissue microarrays. TMA were incubated with the anti-EXN11 polyclonal antibody The table reports the frequency of cancer tissues showing differential immunostaining versus corresponding normal samples. Immunostaining images of cancer and normal samples. **B)** WB analysis of clinical samples. Total protein extracts from cryo-preserved colon and ovary biopsies of cancer (k) and normal (n) tissues from patients were analysed by WB with anti-EXN11 or anti-actin antibodies. Arrows mark the EXN11 and actin bands. **C)** IHC analysis of liver metatastasis from CRC, stained with the anti-EXN11 antibody.

3.2.2. EXN11 has a marginal endogenous expression under in vitro standard growth of tumor cell lines

Cancer cells, infiltrating cells and stromal cells secrete endothelial cell growth factors that modulate vascular network formation. Thus, we first assessed whether EXN11 is endogenously expressed under normoxic growth conditions in a panel of human cells by Q-RT-PCR, including: i) human cancer epithelial cells from different organs, such as breast (T47D, MCF7, MDAMB231, SKBR3), colon (Colo205, HCT15, HCT116), ovary (Ovcar3, Ovcar4, Ovcar8), and melanoma (A375, HS294T); ii) the human endothelial cell lines HUVEC, DMVEC and EPC; iii) cancer associated fibroblasts/myofibroblasts and M1-polarized macrophages. As shown in Figure 14A,

EXN11 transcript was successfully amplified from Ovcar8 cells while it was barely detectable in the other samples. In line with the data, WB analysis of total protein extracts (25 µg per lane, derived from approximately $1x10^5$ cells) and culture supernatants (15 µl of 40-fold concentrated supernatants per lane, corresponding to $3x10^5$ cells) of the same cells using anti-EXN11 antibodies showed a faint EXN11 band in the cell culture supernatant of Ovcar8 (Figure 14B), which was not detectable in the other cells tested (Figure 14B represents exemplary data in HCT15 cells). EXN11 expression was clearly detected by the anti-EXN11 antibody in culture supernatants of cell lines transfected with an EXN11 encoding plasmid, loaded in parallel as control.

These results indicate that EXN11 endogenous expression is generally very low or negligible and suggest that specific environmental or regulatory factors might influence its expression.

3.2.3 EXN11 expression is induced by hypoxia in cancer cells

Since tumor angiogenesis is the result of a balance between stimulatory and inhibitory factors, an important aspect is to identify the regulatory or environmental conditions that promote the expression of ANGPL7 by malignant cells. To this purpose we exposed Ovcar8 and A375 cells to a series of stimuli and monitored EXN11 expression in the cell culture supernatant by WB. Cells were either grown for 24 h under hypoxia (1% O_2) or oxidative stress (500 µM H_2O_2), stimuli that induce structural and metabolic cell changes. In parallel, to mimic the effect of tumor microenvironment, cancer cells were also co-incubated for 24 h with the conditioned media from stromal cells known to be involved in cancer progression, such as cancer associated fibroblasts/myofibroblasts and M2-polarized macrophages. WB analysis on the culture supernatant of the stimulated cells showed that EXN11 expression level was specifically up-regulated in response hypoxia, whereas it was unchanged under oxidative stress (Figure 14B) an in any other tested conditions (not shown). EXN11 up-regulation in response to hypoxia was also confirmed by Q-RT-PCR on RNA samples extracted in parallel (Figure 14C). Similar results were obtained in HCT15 colon cells exposed to the same stimuli (data not shown).

3.2.4. EXN11 is associated with exosomes

Exosomes are cell-released vesicles that serve as vehicles of protein and nucleic acids and provide a route of communication between cancer cells and various stromal cells infiltrating the tumor interstitium. Recently, numerous studies have shown that exosomes affect several stages of tumour progression, including angiogenesis. We investigated the presence of EXN11 in the exosomial fraction of cancer cells by WB. Exosomes were purified from the culture supernatant

of Ovcar8 cells by sequential differential centrifugations that yielded five centrifugation pellets, of which P1 represents the cellular pellets, P2-P4 are intermediate supernatant-derived pellets and P5 is the final exosome-enriched pellet and protein extracts of P1 (20 µg/lane, corresponding to approximately 1×10^5 cells), P4 and the final exosome pellet P5 (10 µg/lane, corresponding to 2×10^7 cells) were subjected to WB with antibodies raised against the exosomial marker CD81, and with anti-EXN11 antibodies. CD81 was highly enriched in the exosomial fraction confirming the quality of the preparation. A band of 45 kDa was detected with anti-EXN11 antibodies, indicating that the protein at least partially associates with exosomes (Figure 14D).
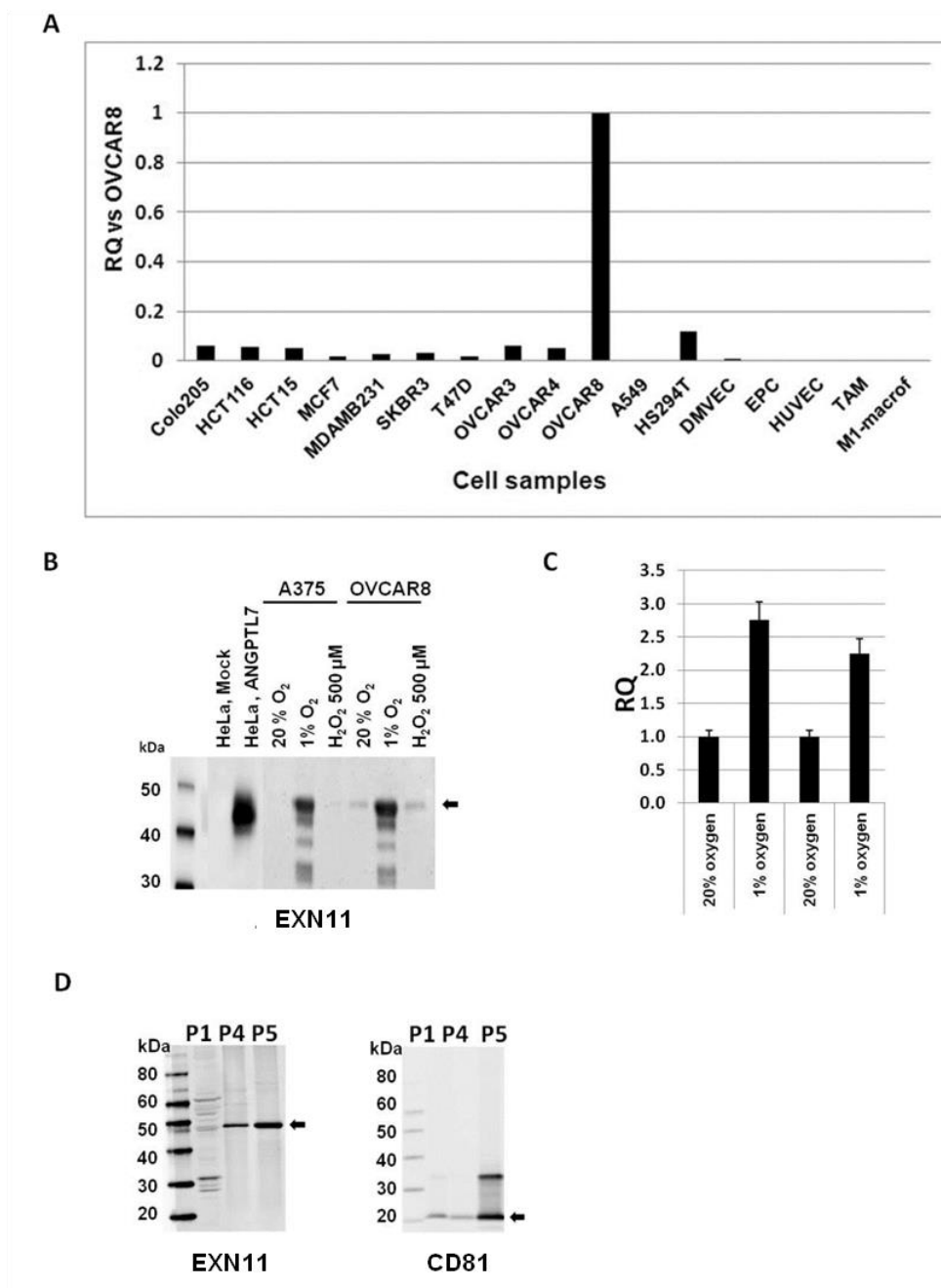
Figure 14. EXN11 expression profile in human cells.
A) EXN11 transcription analysis in human cells. EXN11 relative transcript level (RQ) was quantified in total RNA from different cell lines by q-RT-PCR vs MAPK gene and further normalized to the EXN11 RQ in Ovcar8 cells. **B)** WB analysis of EXN11 expression in response to oxygen availability and oxidative stress. Ovcar8 and A375 cells (5×10^6 cells) were cultured for 24 h under normoxia (20% O_2) or hypoxia (1% O_2), or treated with H_2O_2 500 μM. The culture supernatant (20 ml) from each sample was concentrated 40 fold, and 15 μl were subjected to immunoblot with anti-EXN11 antibodies. **C)** EXN11 transcription in response to oxygen availability. EXN11 RQ was assessed in total RNA from Ovcar8 and A375 cells incubated under normoxia or hypoxia, using MAPK as internal standard. **D)** EXN11 association with exosomes. The exosomial fraction was purified from Ovcar8 cells by sequential centrifugations yielding five centrifugation pellets (P). P1 (cellular pellet), P4 (intermediated pellet), and P5 (exosome-enriched pellet) were subjected to WB with anti-EXN11 or anti-CD81 antibodies.

3.2.5. EXN11 exerts pro-angiogenic activities on differentiated endothelial cells in vitro

The formation of new blood vessels is a tightly regulated multi-step process that begins with the activation and migration of endothelial cells to chemotactic stimuli issued by the surrounding stroma. Extravasation of endothelial cells is followed by intense proliferation, as well as changes in morphology that generate primitive tubular structures from which the new vessel generates. Each of these phases may constitute an aspect of investigation *in vitro (50,51)*. Thus, we investigated the EXN11 angiogenic activity by measuring its influence on the motility and invasiveness of different endothelial cell populations, as well as on their ability to reorganize and differentiate into capillary-like networks. We first assessed the effect of increasing concentrations of purified human recombinant EXN11 (ranging from 0.1 to 5 μg/ml) on migration and invasiveness of the human umbilical vein endothelial cells (HUVECs) using a Boyden chamber assay. As shown in Figure 15A, HUVEC transmigration increased in a concentration dependent manner with respect to non-stimulated cells (approximately four-fold increase already at 0.1 μg/ml of EXN11), reaching a plateau at 1 μg/ml. We next analyzed the EXN11 influence on the ability of HUVECs to organize into a tubular network by the Matrigel morphogenesis assay. As shown in Figure 15B, HUVEC incubated at 1 μg/ml or higher concentrations of EXN11 showed an improved capacity to form capillary networks, compared to untreated cells.
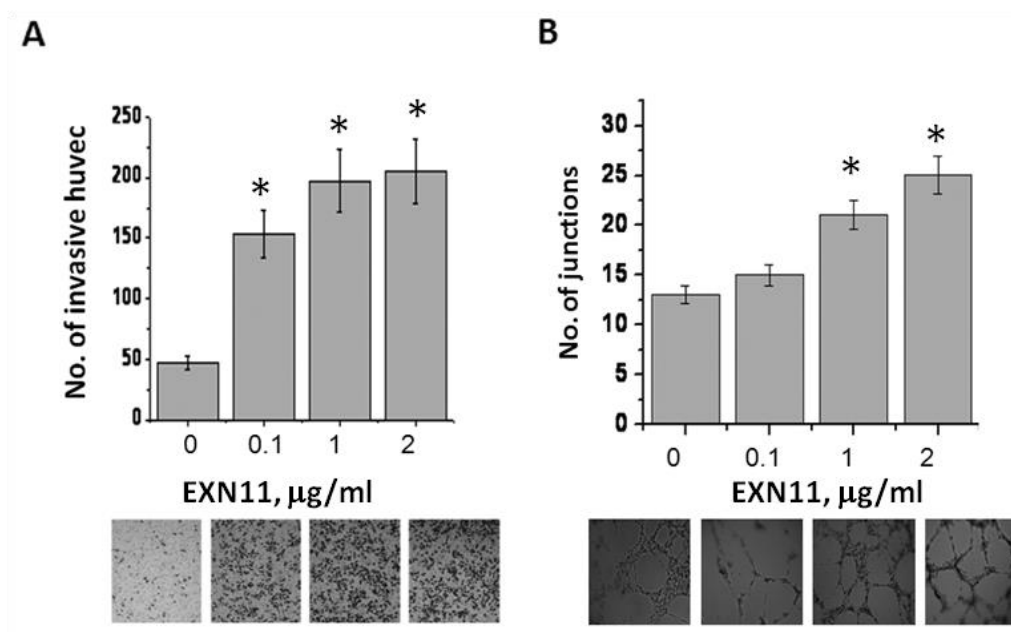
Figure 15. Purified EXN11 has pro-invasive and pro-angiogenetic effects on HUVEC.
A) Boyden Invasion assay. HUVEC (2.5×10^4) were placed onto the upper compartment of the Boyden chamber in presence of medium containing different concentrations of purified EXN11 and incubated overnight at 37°C. Cell invasiveness was evaluated after Diff-Quick staining by counting cells in six randomly chosen fields. **B)** Capillary morphogenesis assay. 2×10^4 HUVEC were plated on Matrigel-coated 96-well plates and the tubular network formation was photographed 7 h after seeding. Results are shown as mean of three independent experiments performed in duplicate ± standard deviation. Asterisks mark EXN11 stimulated cells showing a significant difference ($p < 0.05$) vs untreated cells. Images representing the visual counting of each sample are reported below the graphs.

The pro-angiogenic effects of EXN11 were further confirmed using the conditioned medium of A375 cells transfected with EXN11 (EXN11 CM), on both HUVEC and human dermal microvascular endothelial cells (DMVEC) (the latter represent a more physiological vascular cell model). The concentration of soluble EXN11 in the EXN11 CM was approximately 0.4 μg/ml as opposed to 0.6 ng/ml of EXN11 found in CM of cells transfected with the empty vector (Mock CM). EXN11concentration in CM was estimated by densitometric analysis of WB of EXN11 CM compared to different amounts of purified EXN11, and further confirmed by the Proseek system, a

PCR method based on the proximity ligation principle (not shown). EXN11 CM exerted pro-migratory and pro-invasive effects on both HUVEC or DMVEC (Figure 16), as indicated by the 1.5-2 fold increase in migration and invasiveness with respect to Mock CM. EXN11 CM also stimulated the formation of primitive tubular structures in matrigel tube assays, inducing about 2 fold increase of tubular junctions (Figure 16). Similar phenotypic effects were observed using the CM of Ovcar8 and HCT15 cells transfected with EXN11 (data not shown).
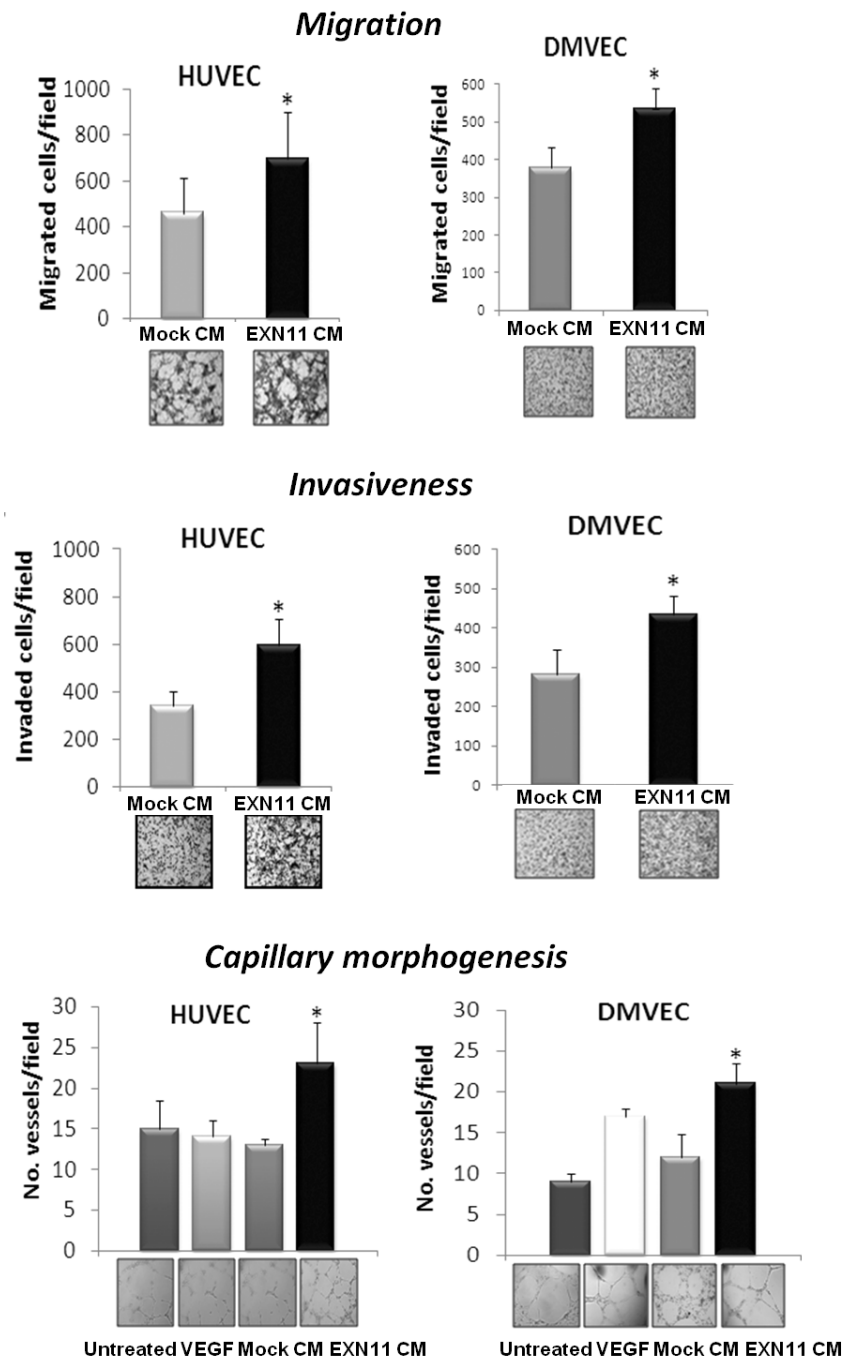
Figure 16. EXN11 secreted by transfected cells has pro-angiogenetic effects on differentiated endothelial cells. For Boyden migration and invasiveness assays 2.5×10^4 HUVECs or 2×10^4 MVECs were placed

onto the upper compartment of the Boyden chamber in presence of medium conditioned from transiently transfected A375 (Mock CM and EXN11 CM) and incubated overnight at 37°C. Capillary morphogenesis assay was carried out placing 2×10^4 HUVECs or DMVECs on Matrigel-coated 96-well plates and the tubular network formation was photographed 7 h after seeding. As controls, cells were also stimulated with VEGF (10 ng/ml). VEGF had a neutral effect on HUVEC and a pro-angiogenic activity on DMVEC. For each experiment, results represent mean and standard deviation obtained from 3 independent experiments performed in duplicated. Asterisk marks a statistically significant difference ($p < 0.05$) vs Mock C.M treated cells. Images of the visual counting of each samples are reported below the graphs.

3.2.6. The human endothelial progenitor cells respond differently to the stimulation induced by EXN11

We then investigated whether EXN11 exerts its pro-angiogenetic effect on EPCs. EPC are bone marrow-derived progenitor cells that, once recruited to neo-vascularization sites into the vascular endothelial lining, differentiate in situ into mature endothelial cells. In adults EPC participate to the assembly of newly-forming blood vessels, and a growing number of studies report that EPC-dependent vasculogenesis plays an important role in cancer progression. Since several anti-angiogenic therapies, or antiangiogenic factors as endostatin, have been ineffective on EPC-mediated vasculogenesis, vasculogenetic factors are becoming potential attractive targets for developing of novel cancer therapeutics. We analyzed the EXN11 CM effect on EPCs migration, invasiveness, and morphogenesis, as described above. As shown in Figure 17, neither cell motility (panels A and B, respectively) nor the ability to generate tubular structures *in vitro* (panel C) were significantly stimulated by treatment with EXN11 CM. This result suggests that EXN11 exerts its angiogenic activity on cells, such as HUVEC and DMVEC, already differentiated towards endothelial lineage, while has little effect on endothelial cell progenitors.
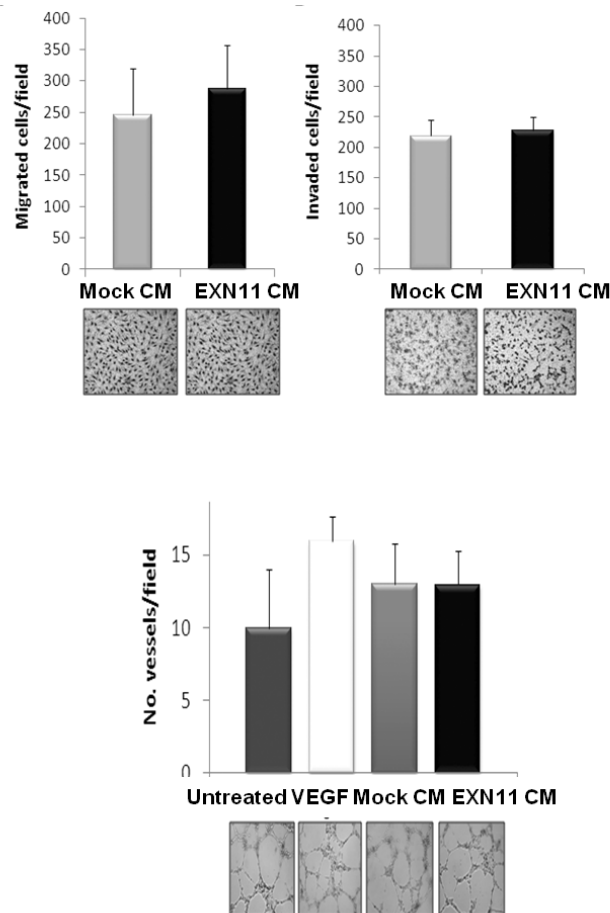
Figure 17. EXN11 secreted by transfected cells does not influence angiogenetic phenotypes of endothelial progenitor cells. For Boyden cell migration and invasiveness assays (A and B, respectively) 20 x 10^4 EPC were seeded into the upper chamber of Boyden chambers with EXN11 C.M. or Mock C.M. Cells were allowed to migrate for 16 h through the filter toward the lower chamber filled with complete growth medium. Capillary morphogenesis angiogenesis (C) was carried out placing 2 x 10^4 EPCs (C) or DMVECs (F) on Matrigel-coated 96-well plates and the tubular network formation was photographed 7 h after seeding. As controls, cells were also stimulated with VEGF (10 ng/ml). Results are representative of 3 independent experiments. Images of the visual counting of each samples are reported below the graphs.

3.2.7. EXN11 promotes vascularization in the mouse matrigel sponge assay

Finally, we confirmed the EXN11 pro-angiogenic activity by measuring its ability to promote vascularization in mice, using the Matrigel sponge assay. Briefly, Swiss male mouse groups were injected subcutaneously into either of the two flanks with unpolymerized matrigel containing purified recombinant human EXN11 or Medium (4 mice per group). Four days after injection mice were killed and the vessel formation was evaluated in the matrigel sponges by visual inspection. 8/8 EXN11-containing matrigel plugs showed the formation of dendritic branched vessels which extended toward the center of the sponge whereas controls showed only sketches of vessels at the periphery of the sponge (Figure 18A), thus confirming the EXN11 ability to act as

angiogenic factor *in vivo.* Quantitation of vessels formed within sponges (Figure 18B) and confirmed the ability of EXN11 to act as angiogenic factor *in vivo.*
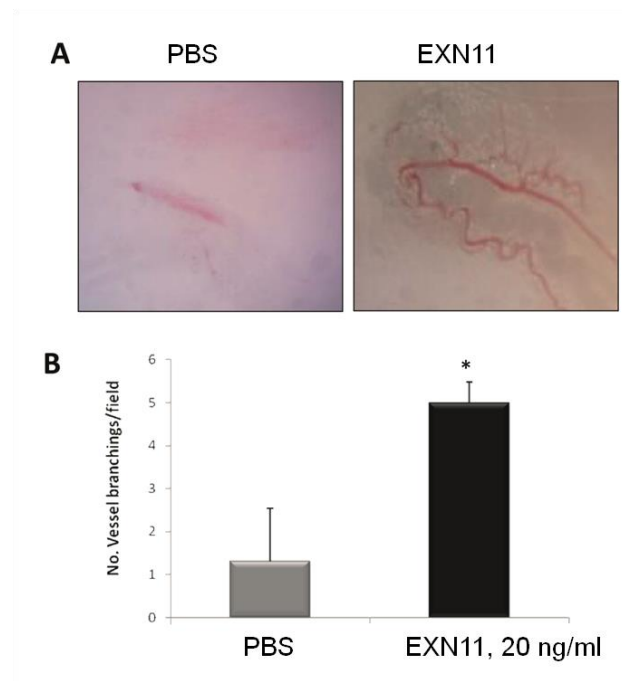
Figure 18. EXN11 stimulates vessel formation in the mouse matrigel sponge assay.
Swiss wild type male mice were implanted into both flanks with the Matrigel sponges containing purified EXN11 (10 μg/ml) or matrigel alone and four days after injection sponges were excised and photographed. The new vessel formation was quantified by visual inspection. A) Microscopic enlargements of EXN11-injected sponges and negative controls. B) Quantification of microvessel density. The effect induced by EXN11 was quantified by counting the vessel branching in EXN11-injected and negative control sponges. Asterisk mark the statistical difference between the samples (P<0.02, determined by Student's T test).

4. DISCUSSION AND FUTURE PERSPECTIVES

Progress in prevention and therapy has led to remarkable decreases in mortality and death rates due to cancer. Basic research has reached amazing advancements in different areas, such as the genetic basis of cancer, understanding the regulation of signaling pathways and their biochemical components, new insights in the communication between cancer cells and normal cells, and the elucidation of the mechanisms of metastasis. The research progress has pushed the research of molecularly targeted therapies, directed against individual signaling components activated in cancer cells, that have improved the success of treatment (1). Notwithstanding the impressive success in the field, there is still a great demand for new validated cancer markers are needed to improve diagnosis, patient's stratification, predict the disease outcome, help physicians to rationalize the use of cancer therapies and monitor their efficacy. Similarly, there is an urging need for novel drug targets, to be used alone or in conjunction with existing therapies.

This study addresses these needs by characterizing two novel cancer markers and potential therapeutic targets previously identified by Externautics, in collaboration with the European Institute of Oncology.

Concerning EXN6, this rather unknown protein that seems to be very attractive for therapeutic applications. Our data show that it is mainly detected in ovary and breast cancers, and with lower frequency in colon and lung cancers. By confocal microscopy and FACS analyses, we showed that it is a surface exposed protein, thus it could be target of novel drugs, such as small molecules and mAbs. Moreover, gene silencing experiments aimed at understanding its role in cancer processes relevant for tumor growth and progression showed that loss of EXN6 expression clearly impairs cell proliferation and invasiveness, strongly suggesting that EXN6 is a key component of cancer signaling pathways. Experiments to understand the molecular pathways in which it is involved are ongoing.

This study also led to the selection of anti-EXN6 mAbs with potential therapeutic applications. Within a group of mAbs that were generated and characterized, five were able to bind the surface of ovary and breast cancer cell lines. Of utmost interest are two mAbs that, after binding on the cell surface, are efficiently internalized by cancer cells. This important property make them amenable to the generation of ADC for EXN6 targeted therapy. Moreover, the evidence that they have limited reactivity in a panel of 33 normal human tissues could predict an acceptable tolerability and safety profile *in vivo*. The mAb elucidation of the mechanism of internalization,

under study, will be a necessary additional information to rationalize the ADC strategy and the selection of the most appropriate linkers and drugs to be conjugated with the antibodies.

In addition to internalization, we also found that one mAb also shows ADCC activity. Thanks to its dual activity, this mAb could be exploited either as naked or as ADC. Efficacy studies in xenograft mouse cancer models are currently ongoing to evaluate its ability to prevent, delay the tumor formation or interfere with its progression is assessed. Moreover, experiments are ongoing to identify of the molecular pathways perturbed by the mAb interaction with cancer cells.

Concerning EXN11, this protein shows high structural homology with ANGPTLs, proteins, with which it shares a coiled-coil domain at the N-terminus and a fibrinogen-like domain at the C-terminus. After approximately a decade from their first identification, most members of this family are emerging as central players of different cell processes and human diseases. Several ANGPTL proteins potently modulate angiogenesis (42-45), though for some of them the effect remains controversial. ANGPTLs are also involved in other processes independently from angiogenesis, such as augmentation of energy expenditure, induction of inflammation, and metabolism (45,46)

Our study provides information on the biological role of EXN11 by showing that i) it is differentially expressed in certain types of cancer, and ii) it acts as pro-angiogenic and vascularization factor. By immune-histochemical analysis of paraffin-fixed samples and WB analysis of fresh clinical samples, EXN11 was found over-expressed in colon cancer and, at lower frequency, in breast and ovary cancers, while it appeared to be expressed at basal level in prostate and lung cancer. Moreover, the protein was also over-expressed in liver metastasis from CRC cancer suggesting that it could be involved in cancer dissemination at distant sites.

Based on its homology to ANGPTLs, we investigated the EXN11 role angiogenesis. Angiogenesis is an essential step during the initial stages of tumor development, in which cancer cells secrete growth factors to promote tumor neovascularization and improve the otherwise inadequate vasculature. Enhanced expression of proangiogenic genes can activate quiescent microvascular endothelial cells, causing them to dissociate from the cell-cell junctions, migrate into the perivascular space, proliferate extensively, and form tubular structures.

We assessed the role of EXN11 in angiogenesis by evaluating its effect on different endothelial cell populations. They include HUVEC and DMVEC, derived from large wall vessels and dermal microvasculature, respectively, and extensively used as models of fully differentiated endothelial cells to assess de novo angiogenesis. Moreover, since recent evidence indicates that the de novo generation of endothelial cells from bone marrow-derived circulating endothelial cell precursors

(EPC) also contributes to the vasculature formation (a process referred to as vasculogenesis) especially under pathological conditions including cancers (52,53) we also investigated the EXN11 effect on EPC.

Overall, our data include EXN11 among the factors capable of regulating vessel organization and maturation of endothelial cells but not of bone marrow-derived undifferentiated precursors. Indeed, we consistently showed that EXN11 enhances migration and capillary network formation ability of HUVEC and DMVEC, whereas it does not influence these phenotypes in EPC, indicating that differentiated and progenitor endothelial cells respond differently to this protein. A hypothesis that needs appropriate investigation is that the EXN11 receptor is expressed only in a final step of differentiation process of endothelial cells. Overall, EXN11, although not effective on EPC-dependent vasculature formation, is very active in promoting vascular organization of cells that have already undergone differentiation towards endothelial lineage, and could represent an attractive therapeutic target for anti-angiogenic tumor therapy.

Since the actual regulation of tumor angiogenesis is the result of a fine balance between stimulatory and inhibitory factors secreted by cancer cells, infiltrating cells and stromal cells in response to environmental stimuli, we elucidated the conditions that promote EXN11 expression. In agreement with previous evidence (54) our profile analysis of EXN11 basal expression in a panel of cancer epithelial, endothelial and stromal cell lines showed that, in general, EXN11 has a very low expression under standard conditions. This evidence further corroborates the notion that EXN11 expression is not a common event in human cancer cells and might be tightly regulated in response to specific parameters. By exposing cancer cell lines to different stimuli reproducing some aspects of cancer environment we found that, similarly to ANGPTL2 and ANGPTL4 (55,56), EXN11 is specifically stimulated by hypoxia, the predominantly stimulus that induces the aberrant expression of pro-angiogenic factors. We also observed that other environmental stimuli, like exposure to cancer-associated fibroblasts or M2 polarized macrophages, known to rapidly modulate other angiogenic factors as VEGF, FGF-2 or IL-8, are ineffective in eliciting EXN11.

The regulatory mechanisms underlining the EXN11 response to hypoxia and angiogenesis need to be elucidated. Bioinformatic annotations of human EXN11 promoter available in public web databases (http://www.genecards.org) do not reveal canonical core binding sites of the hypoxia-inducible factor 1-alpha (HIF-1a), the major regulator of the transcriptional response to oxygen availability, suggesting that HIF-1 does not exert a direct regulation on EXN11 expression.

Recently, an integrative approach combining both computational and experimental strategies to HIF-1-target genes did not categorize EXN11 among the genes involved in the core response to hypoxia (57). Instead, the EXN11 promoter analysis identified two major cis-regulatory elements. In particular, four Lymphoid-Enhancing Factor/T-Cell Factor LEF (LEF/TCF)-binding sites are present in the promoter region (58). LEF/TCF proteins belong to the family of architectural transcription factors that control developmental processes and have an important role in oncogenesis. Since LEF/TCF proteins are typically activated by the WNT/ß-catenin signaling pathway, a key player in oncogenesis and also involved in many aspects of normal and pathological angiogenesis (59), we speculate that WNT/ß-catenin exerts a control on EXN11 expression in cancer. Interestingly, the promoter of the human EXN11, but not the mouse ortholog, also contains four interferon-stimulated response elements (ISREs) suggesting that Interferon could stimulates the transcriptional activity of the human promoter in vitro. The possible existence of a cross talk between LEF/TCF and IFN on EXN11 expression deserves further dedicated studies.

Another open question is how EXN11 influences cancer angiogenesis. Interestingly, here we showed that this protein, similarly to ANGPTL4 (www.exocarta.org), is at least partially associated with exosomes released by cancer cell lines. Thus, an interesting hypothesis, currently under investigation, is that, like other ANGPLTs, EXN11 could be released in the circulation of oncologic patients, and could function in an endocrine manner, both in the soluble and in exosome-associated forms.

A final aspect, beyond the purpose of this study, is related to the EXN11 receptor molecule on target cells. Most members of the ANGPTL family are currently considered orphan ligands because information regarding their potential binding partners is lacking. To date, integrins have been regarded as major functional interactors of ANGPTLs. For instance, $\alpha 5\beta 1$ integrin is a receptor for ANGPLT2 and ANGPTL4 (46,56). In an elegant study Marchiò et al., by screening a phage displayed random peptide library, found that hepatic ANGPTL6 protein binds a complex of E-cadherin and $\alpha 6$ integrin on the surface of CRC by involving a binding site mimicking the structure of peptide CGIYRLRS, and demonstrated that such interaction drives liver homing and colonization by CRC cells (60). Moreover, these authors provide a possible molecular mechanism for CRC metastasis by showing that that the angiopoietin-like 6-mimicking peptide has anti-metastatic effects in preclinical models of human CRC. They also describe different proteins

sharing similarity with the metastasis-specific peptides, and speculate that the angiopoietin-like 6/α6 integrin/E-cadherin system is part of an interconnected network, in which several proteins interact with different molecular partners based on their availability, combination and affinity. Since similar peptide motifs are also found in EXN11 sequence, an intriguing hypothesis is that also this protein participates to the same functional complex with E-cadherin and α6 integrin. Experiments to elucidate this aspect are in progress.

5. MATERIALS AND METHODS

Chemicals, reagents and cell cultures

Unless specified, all reagents were obtained from Sigma. Restriction enzymes and DNA modification enzymes were from New England Biolabs. DNA and RNA extraction kits were from QIAGEN. Antibodies for WB analysis were from BD Biosciences. VEGF-A was purchased from PeproTech. Matrigel Basement Membrane was from BD Biosciences. Human epithelial cells were obtained from the ATCC collection and cultured under ATCC recommended conditions. Human umbilical vein endothelial cells (HUVEC) were purchased by the European Collection of Cell Cultures. Microvascular endothelial cells (DMVEC) and endothelial progenitor cells (EPC), were kindly provided by Prof. M. Del Rosso of Department of Clinical Physiopathology, University of Florence. DMVECs have been isolated from biopsy samples of skin of hands of healthy patients, while human UCB samples of health newborns were used as a source of EPCs, as previously described (61,62). All endothelial cell lines were cultered on gelatin 1%-coated dishes in complete endothelial cell growth medium (EGM-2 BulletKit, Lonza), which includes endothelial basal medium plus the SingleQuots Kit (hydrocortisone, human fibroblast growth factor B, VEGF, LongR3 insulin-like growth factor 1, ascorbic acid, human epidermal growth factor, GA-1000, and heparin), supplemented with antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin) and 2% FBS for HUVECs or 10% FBS for DMVEC and EPC. Endothelial cell types were used between the third and tenth passages *in vitro*. A375 human melanoma cells were purchased from ATTC. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 4mM L-glutamine and antibiotics (100 UI/ml penicillin, 100 µg/ml streptomycin). *Escherichia coli* BL21(DE3) was grown aerobically in Luria broth (LB) medium (Difco) at 37°C. When appropriate, ampicillin (Amp, 100 µg/ml) and isopropyl-β-D-galactopyranoside (IPTG; 1mM) were added to the medium.

Generation of anti-EXN11 and antiEXN6 mouse polyclonal antibodies.

Suitable fragments of the EXN11 and EXN6 coding regions (CDS) were selected for cloning / expression using bioinformatics tools. The leader sequence for secretion was replaced with the ATG codon to drive the expression of the recombinant proteins in the cytoplasm of *E. coli*. For cloning, cDNAs were generated by reverse transcription from total RNAs (Euroclone) of human tissues (bone marrow, fetal brain, placenta and testis) and pooled. Gene fragments were amplified from cDNA pools using specific primers (designed using Primer3 Software, Premier Biosoft International) so as to fuse a 10 histidine tag sequence at the 3' end, annealed to in house

developed vector (p2N) derivative of vector pSP73 (Promega) adapted for the T4 ligation independent cloning method (10) and used to transform *E.coli* NovaBlue cells recipient strain. *E. coli* transformants were plated onto selective LB plates containing 100 μg/ml ampicillin (LB Amp) and positive *E.coli* clones were identified by restriction enzyme analysis of purified plasmid followed by DNA sequence analysis. EXN11 and EXN6 recombinant proteins were purified from the inclusion bodies. Briefly *E.coli* BL21(DE3) cells harboring the plasmid were inoculated in 50 ml of ZYP-5052 growth medium and grown at 37°C for 24 hours. Afterwards, bacteria were harvested by centrifugation and lysed into 10 ml of B-Per Reagent (Pierce) containing 1 mM MgCl2, 10 units/ml of DNAse I (Sigma), and 1 mg/ml Lysozyme (Sigma). After 30 min at room temperature under gentle shaking the insoluble inclusion bodies were collected by centrifugation at 30.000 x g for 40 min at 4°C. To solubilize inclusion bodies, the pellet was resuspended in 10 ml of 6M guanidine hydrochloride, 1 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Pierce), 40 mM Tris-HCl pH 8. After clarification by centrifugation at 30.000 g for 30 min, the supernatant was loaded on 0.5 ml columns of Ni-activated Chelating Sepharose Fast Flow resin (GE-Healthcare). After wash of the resin with 10 column volumes of 6M urea, 60 mM imidazole, 0.5M NaCl, 1 mM TCEP, 50 mM TRIS-HCl pH 8, recombinant proteins were eluted with the same buffer containing 500 mM imidazole. Proteins were analysed by SDS-Page and their concentration was determined by Bradford assay using the BIORAD reagent (BIORAD) with a bovine serum albumin standard according to the manufacturer's recommendations.

To generate anti-EXN11 and EXN6 polyclonal sera, the purified proteins were used to immunize CD1 mice (6 week-old females, Charles River laboratories, 5 mice per group) subcutaneously with 3 protein doses of 20 micrograms each, at 2 week-interval. Freund's complete adjuvant was used for the first immunization, while Freund's incomplete adjuvant was used for the two booster doses. Two weeks after the last immunization animals were bled and sera collected from each animal were pooled.

Immunohistochemistry

The capability of the anti-EXN11 and anti-EXN6 polyclonal antibodies to recognize their target proteins in tumor samples was assessed by immunohistochemistry. Tissue Micro Array (TMA) were generated using formalin-fixed, paraffin-embedded samples for CRC (adenocarcinoma), ovarian cancer (serous and endometroid), breast cancer (ductal and invasive lobular carcinoma), lung cancer (adenocarcinoma, squamous cell carcinoma), prostate cancer (acinar carcinoma), that were selected from the archives at the IEO (Istituto Europeo di Oncologia, Milan, Italy). Normal

tissues were defined as microscopically normal (non-neoplastic) and were generally selected from specimens collected from the vicinity of surgically removed tumors (63). Patients were consecutive cases selected based on tumor size (tumors having a maximum diameter greater than 2 cm were selected). Corresponding whole tissue sections were examined to confirm diagnosis and tumour classification, and to select representative areas in donor blocks to be cored. Briefly, each TMA slide included tumor tissue cores representative of 5 different well pedigreed patients for each cancer type, and an equal number of matched normal tissue cores from the same patients in duplicate. The direct comparison between tumor and normal tissues of each patient allowed the identification of antibodies that differentially stain tumor cells and provided indication of target expression in the tumor under investigation. TMA production was performed essentially as previously described (63). Briefly, a hole was made in the recipient TMA block. A cylindrical core tissue sample (1 mm in diameter) from the donor block was acquired and deposited in the recipient TMA block. This was repeated in an automated tissue arrayer "Galileo TMA CK 3500" (BioRep - Milan) until completion of the TMA design. TMA recipient blocks were baked at 42°C for 2 h prior to sectioning. Two- to 3-micron thick sections were cut from the TMAs and placed onto poli-L-lysinated glass slides for immunohistochemical analysis. Moreover, 20 clinical specimens of liver metastasis from CRC, selected from the archives at the Istituto di Candiolo - IRCCS (Candiolo, Italy) were analyzed by traditional IHC methods.

Automated immunohistochemistry was performed as previously described (63). Briefly, the glass slides were heated for 30 min at 60°C, de-paraffinized in xylene (2 x 15 min) using the Bio-Clear solution (Midway. Scientific, Melbourne, Australia), and re-hydrated in graded alcohols. For antigen retrieval, slides were immersed in 0.01 M Na-citrate buffer, pH 6.0 at 99°C for 30 min, placed in an automatic immunostainer (Autostainer (R) Dako, Glostrup, Denmark)) and endogenous peroxidase was initially blocked with 3% H_2O_2, for 5 min. Slides were then blocked in Dako Wash Buffer containing 5% bovine serum albumin (BSA) and subsequently incubated with the anti-EXN11 mouse polyclonal antibodies for 30 min (dilution 1:200 in Dako Real ^{TM} dilution buffer). After washing with Dako wash buffer, slides were incubated with the goat anti-mouse peroxidase conjugated Envision(R) detection reagent (Dako) for 30 min at room temperature. Finally, diaminobenzidine and the substrate chromogen (Dako) was used to visualize the reaction and Harris hematoxylin (Sigma Aldrich) was used for counterstaining. The slides were mounted with Pertex(R) (Histolab). Staining results were evaluated by a trained pathologist at the light microscope, and an IHC score was assigned corresponding to the percentage of immunostained cells (from 0 to 100%) multiplied by the intensity of staining (from 1 to 3).

Individual values and combined scores (from 0 to 300) were recorded in a custom-tailored database, which also included the subcellular localization of the immunoreactivity (membranous, cytoplasmic, nuclear). Digital images of the immunocytochemical findings were taken at a Leica DM LB light microscope, equipped with a Leica DFC289 color camera.

WB analysis of clinical samples

Cryo-preserved normal and cancerous samples (10 mg) selected from the IEO archives from breast, ovary and colon were homogenized by mechanic tissue disruption in 1 ml buffer containing 40 mM TRIS-HCl, 1 mM Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP, Pierce) and 6M guanidine hydrochloride, pH 8. After clarification 15' at 14,000xg, the protein concentration was quantified by Bradford assay (BIORAD). After, 400 µg of protein extracts were methanol precipitated, washed with 70% ethanol and resuspended in Laemmli buffer containing DTT. 20µg of total protein extracts were loaded on pre-cast SDS-PAGE gradient gels (NuPage 4-12% Bis-Tris gel, Invitrogen) under reducing conditions, followed by electro-transfer to nitrocellulose membranes (Invitrogen) according to the manufacturer's recommendations. The membranes were blocked in blocking buffer composed of 1x PBS-0.1% Tween 20 (PBST) added with 10% dry milk, for 1 h at room temperature, incubated with the anti-EXN6, the anti-EXN11 pAbs or with anti-actin Ab diluted 1:1000 in blocking buffer containing 1% dry milk and washed in PBST-1%. The secondary HRP-conjugated antibody (goat anti-mouse immunoglobulin/HRP, Perkin Elmer) was diluted 1:5000 in blocking buffer, and chemiluminescence detection was carried out using a Chemidoc-IT UVP CCD camera (UVP) and the Western Lightning^{TM} Cheminulescence Reagent Plus (Perkin Elmer), according to the manufacturer's protocol.

Cell transfection

For transfection experiments, the EXN11 and EXN6 encoding plasmids were generated by PCR amplifying the full length gene coding regions with specific primer pairs, and the PCR products was cloned into plasmid pcDNA3.1D (Invitrogen) and sequence verified. For transfection, the indicated cell lines were plated in 100-mm cell culture dishes in 6-well plates ($1x10^6$ or in 400,000 per well, respectively) were transiently transfected with 4 micrograms of the EXN11 or the EXN6 encoding plasmids and with the empty vector as negative control (mock) using the Lipofectamine-2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. The enhanced green fluorescent protein (EGFP) was co-transfected with empty vector (Mock) or plasmid DNA

encoding EXN6 or EXN11. The efficiency of transfection was valuated thereby fluorescent microscopy. After 24 hours, cells and culture supernatants were collected for expression analysis.

Gene silencing,

The indicated cancer cell lines ($2x10^5$ cells in 6wells) were transfected with 10 mM of specific EXN6 siRNAs or irrelevant siRNA (QIAGEN) using the HiPerfect transfection reagent. After 48 or 72 hours, cells were collected for expression or phenotypic analyses.

Transcription analysis

The marker expression was analyzed at RNA level in transfected and untreated cell lines by Q-RT-PCR. Total RNA was extracted from the indicated cell lines and samples using the RNAeasy mini kit (QIAGEN) following the manufacturer's protocol and 5 μg of it were reverse transcribed using Superscript III Reverse Transcriptase (Life Technologies) with oligo dT. Triplicate cDNA samples from each cell line (equal to 50 ng RNA/sample) were subjected to real-time Q-PCR using the Quantitect SYBR Green PCR kit (QIAGEN), and commercially available primers and analysed with the One-Step Plus Q-RT_PCR equipment (Applied Biosystems). To control slight variations in the amount of RNA loaded into the PCR reactions, the difference in Ct (ΔCt) between samples were normalized by detracting the average Ct values of the housekeeping genes GAPDH.

WB analysis

For WB analysis of cancer cell lines, cell monolayers were detached with PBS-0.5 mM EDTA and lysed by several freeze-thaw passages in PBS-1% Triton. Total protein extracts (25μg corresponding to approximately $2+10^5$ cells) and concentrated supernatants (normalized on number of cells), were loaded on pre-cast SDS-PAGE gradient gels (NuPage 4-12% Bis-Tris gel, Invitrogen) under reducing conditions and subjected to WB with anti-EXN11 and anti-EXN6 polyclonal and mAbs, as described.

Surface localization analysis

FACS

For flow cytometry analysis of EXN6 localization, cells ($2x10^4$ per well) were pelletted in 96 U-bottom microplates by centrifugation at 200 x g for 5 min at 4°C and incubated for 1 hour at 4°C with the appropriate dilutions of anti-EXN6 antibodies. The cells were washed twice in PBS-5%

FCS and incubated for 20 min with the appropriate dilution of R-Phycoerythrin (PE)-conjugated secondary antibodies (Jackson Immuno Research, PA, USA) at 4°C. After washing, cells were analysed by a FACS Canto II flow cytometer (Becton Dickinson). Data were analyzed with FlowJo 8.3.3 program.

Confocal microscopy

Cells were plated on glass cover slips and after 48 h were washed with PBS and fixed with 3% paraformaldheyde solution in PBS for 20 min at RT. Then, after extensive washing in PBS, cells were incubated with the appropriate antibodies overnight at 4°C (1:200), before or after permeabilization with 0.01% BriJ96® (Fluka). Cells were then stained with Alexafluor 488-labeled goat anti-mouse antibodies (Molecular Probes). DAPI (Molecular Probes) was used to visualize nuclei. The cells were mounted with glycerol plastine and observed under a laser-scanning confocal microscope (LeicaSP5).

mAb Internalization

For Flow cytometry analysis of mAb internalization T47D and Ovcar3 cells were incubated with the anti-EXN6 mAbs or irrelevant mAb control (10 micrograms/ ml) for 30' at 4°C to allow antibody binding on the cell surface. Then cells were washed with PBS-5% FCS to remove unbound antibody and shifted to 37°c for 1 hour. At time points, cells were incubated for 20 min with the appropriate dilution of R-Phycoerythrin (PE)-conjugated secondary antibodies at 4°C. After washing, cells were analysed by a flow cytometer and the data were analyzed, as described.

For confocal microscopy analysis the same cells were plated on microscope cover-slips and after 48 h were washed with PBS. Cells were incubated with the mAbs for 1 hour at 4°C (10 micrograms/ ml) and subsequently shifted at 37°C. Cells were then fixed with 10 minute incubation with 90% cold methanol and stained with Alexafluor 488-labeled goat anti-mouse antibodies and DAPI, as described.

FabZAP assay

T47D cells were plated in 96w plates (2000 cells/w) and incubated with the anti-EXN6 mAbs or an irrelevant mAb control at different concentrations (from 1 to 40 µg/ml) for 30' at 4°C to allow antibody binding on the cell surface. Then cells were washed with PBS-5% FCS to remove unbound antibody, added with FabZAP (Advanced Targeting System) at the conditioned suggested conditioned and shifted to 37°c and 5% CO_2 for 72 hours. Residual viability was measured by the MTT assay.

EXN11 expression stimulation in human cells

The microenviroment influence on EXN11 expression in cancer cells was investigated by exposing A375, OVCAR8 and HCT15 cells to different stimuli. The effect of oxygen availability and oxidative stress cells was assessed on cells maintained for 24 h in hypoxia condition (1% O_2), or treated with H_2O_2 500 μM for 24h and analyzed by WB. The effect induced by the conditioned media (CM) of cancer associated fibroblasts/myofibroblasts and M1-polarized macrophages on A375, OVCAR8 and HCT15 cells was also assessed. Briefly, human monocytes were obtained from normal donor buffy coat by gradient centrifugation using Ficoll (Histopaque-1077). Non-adherent cells were removed and purified monocytes were incubated for 7 days in RPMI 1640 supplemented with 10% FBS and 50 ng/ml macrophage-colony stimulating factor (M-CSF) to obtain macrophages. M0 cells were obtained by treating with serum-free medium for 48h. M1 macrophages were polarized by stimulating overnight with LPS (100 ng/ml) (Peprotech) and IFNγ (100 ng/ml) (Peprotech). HDFs were grown to sub-confluence and treated for 24 h with 10 ng/ml rTGF-b1 in normoxic (20% oxygen) or hypoxic conditions (1% O2). Fresh serum-free medium was added for additional 24 h before collection of CM. Total RNA and protein extracts were extractd for transcription and WB analysis, respectively.

Exosomes preparation and analysis

Exosomes from cell culture supernatants were isolated by differential centrifugation as described (64). Briefly, $1x10^8$ OVCAR8 cells were cultured in 400 ml of serum-free medium (PFHM-II Gibco-Life Technologies) for 24 h and then centrifuged at 200xg for 10 min (pellet P1). The supernatant was collected and centrifuged twice at 500g for 10 min (pellet P2). The second supernatant was sequentially centrifuged at 2,000xg twice for 15 min (pellet P3), once at 10,000xg for 30 min (pellet P4), and once at 70,000xg for 60 min (pellet P5), using a SW28 rotor (Beckman instruments, Inc.). The cellular pellet P1 was solubilized in 1 ml of C-RIPA buffer (50 mM Tris-Hcl pH7.5, 150 mM NaCl, 1% Nonidet P-40; 2mM EGTA, 1 mM orthovanadate, 0.1% SDS, 0.5% Na-deoxycholate, 1 mM phenyl-methane-sulphonylfluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) while each of the supernatant-derived pellets P2–P5 were solubilized in 0.5 μl of the same buffer. After clarification, the protein concentration of each sample was determined by Bradford. 20 μg of P1 extract and 10μg of P2-P5 extracts (corresponding to approximately $2x10^5$ and $2x10^7$ cells, respectively) were loaded on SDS-PAGE (4-12%) and WBting with the anti-

EXN11 polyclonal antibody. As quality controls of the exosomial preparation, the blots were also incubated with antibodies targeting the exosomial marker CD81.

Preparation of conditioned media (CM) for phenotypic assays

CM for phenotypic assays were mainly obtained from A375 transiently transfected with empty vector (Mock CM), or with plasmid encoding EXN11 (EXN11 CM). 24 h after transfection, medium was removed and replaced with endothelial basal medium (EGM-2 deprived of FBS and all growth factors) and incubation was prolonged for additional 24 h. Then medium was harvested, clarified by centrifugation, and used freshly for conditioning endothelial cell lines.

Quantification of EXN11 secreted in cell CM

The EXN11 concentration in EXN11 CM or mock CM was first measured in solution with a mouse polyclonal anti-EXN11 antibody and the Proseek2 kit (Olink Bioscience, Uppsala, Sweeden), an assay based on the antibody-based proximity extension assay (65). EXN11 quantification was also confirmed by densitometric analysis of immunoblot. Different amounts of EXN11 CM were subjected to WB and the intensity of the EXN11 band was compared with the corresponding band of defined amounts of purified protein. Both analysis indicated that EXN11 concentration was approximately 0.5-1 μg per ml in EXN11 CM and it was undetectable in mock CM.

Capillary morphogenesis assay

The effects of EXN11 on the ability of endothelial cells to reorganize and differentiate into capillary-like network, were assessed thereby Matrigel morphogenesis assay. Briefly, 50 μl of Matrigel matrix growth factor reduced (1 mg/ml) was added into wells of a 96-well plate and polymerized for 1 h at 37°C. Endothelial cells, after 24 h of incubation in basal EGM-2 medium, were washed once with PBS, harvested by trypsinization and collected by centrifugation. Then, cells were resuspended in 200 μl of EGM-2 containing different concentrations of purified EXN11 or conditioned medium (Mock CM or EXN11 CM) and placed into Matrigel-coated wells ($2x10^4$ HUVEC, or DMVEC, or EPC/well). Cells diluted in EGM-2 basal medium supplemented with VEGF 10 ng/ml were used as positive control. After 6h-incubation on Matrigel at 37°C, network formation was observed using a phase contrast microscope equipped with CCD optics and a digital analysis system. Each experiments was carried out in duplicate. Results are expressed as

mean of joint numbers counted in four randomly chosen fields for well, and averaged from at least 3 independent experiments.

MTT assay

The MTT assay was used as a to assess cell viability and proliferation in cancer cell lines transfected with EXN6 siRNA. After 48 hours cells were serum starved and seeded into 96-well plates (2×10^5 cells/ml) in the presence of medium, with or without 2%FCS and EGF. The MTT assay was performed using a commercially available MTT assay kit (Roche Diagnostics, Indianapolis, IN) according to manufacturer's instructions. The remaining viable cells with MTT dye uptake were determined by measuring the optical density at 490 nm in an enzyme-linked immunosorbent assay reader. Values shown were averaged of at least six measurements, and represented three replicated experiments.

ADCC-reporter gene assay

The T47D cell line was seeded at 10000 cells per well in a 96 well opaque tissue culture plate. Anti-EXN6 mAbs antibody and irrelevant isotype controls (IgG1, IgG2a) were serially diluted and incubated with the T47D cells for approximately 1 h at 37°C, 5% CO2. Following incubation, Jurkat NFAT luciferase reporter cells, were added to the T47D/antibody mixture at 30000 cells per well. The mixture was incubated for approximately 4 h at 37°C, 5% CO2, and then measured for luciferase production using a luminescent substrate (Promega Steady Glo).

Matrigel sponge assay

The mouse *m*atrigel sponge assay was used to confirm the ability EXN11 to promote vascularization on phenotype of different endothelial cell lines. For this purpose, recombinant human EXN11 was diluted in unpolymerized liquid Matrigel matrix containing 20 ng/µl of growth factors in the presence of heparin 50 U/ml. 350 µl of Matrigel suspension were injected subcutaneously into the flanks of 6 week-old Swiss wild type male mice (Charles River) using a cold syringe (4 animals group, 2 injections/animal) and allowed to polymerizes at body temperature, forming a solid gelatinous sponge. Four days after injection, animals were killed and the sponges were removed and photographed to quantify the new vessels formed within the sponge. Mice inoculated with the same volume of Matrigel matrix alone were used as negative controls. Four sponges were inoculated for each experimental condition.

Statistical analysis

Data are presented as means ± SD from at least three independent experiments. Statistical analysis of the data was performed using two-tailed Student's t-test. P-values ≤ 0.05 were considered statistically significant.

6. ACKNOWLEDGEMENTS

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