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Molecular and functional characterization of the human RNASET2 oncosuppressor gene

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INTRODUCTION

THEORIES ON THE ORIGIN OF HUMAN CANCER

Cancer represents a significant burden of disease, as it represents the second leading cause of death worldwide. Each year, millions of new cases are expected to occur, with the most common sites of tumor onset being lung, colorectal, breast and prostate.

Epidemiologically, it is widely acknowledged that only 5% of human cancers shows strong familial aggregation patterns that are compatible with inheritance of high susceptibility alleles through the germline, whereas most cancers (95%) are better described as a “sporadic” disease, whose pathogenesis is likely linked to lifestyle-related factors or other environmental factors (physical, chemical and biological carcinogens), although genetic susceptibility factors are also likely involved.

According to R. A. Willis definition, which dates back to 1960, a tumor "*is an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change*". [1].

Nowadays, cancer is the general term used to describe a large group of diseases striking almost every human tissue and organ and involving abnormal cell growth endowed with the ability to spread beyond their original boundaries and invade other organs outside the site in which they arose.

However, despite decades of intensive research have unveiled countless features of cancer, from organ to cell and further down to the molecular level, the processes underlying cancer initiation and progression are still a matter of intense debate. Indeed, a number of theories have been put forward to try to explain how cancer arises.

In broad terms, the most acknowledged models could be gathered into a simpler scheme of two types: (i) according to the first model, successive waves of genetic changes in a target cell population lead to malignancy; (ii) by contrast, a second model predicts that concurrent

changes in tumor microenvironment along with changes in target cell population are necessary for cancer development [2].

Solid tumors generally contain a parenchyma of proliferating neoplastic cells and a supporting tissue, or stroma. Since the tumor size typically increases with time, it has long been hypothesized that the underlying cause of tumor formation must have been excessive cell proliferation in the parenchyma. Therefore, for many years cancer has been mainly interpreted as a cell-based disease, as described by the somatic mutation theory (SMT), first enunciated in 1914 by Theodor Boveri and still prevailing in the cancer research community.

The SMT is based on the following premises: 1) cancer is derived from a single somatic cell that progressively accumulates multiple genetic (and epigenetic) alterations, whence the monoclonality concept; 2) these mutations occur in genes that control cell proliferation, cell death or DNA damage repair and 3) as implicitly inferred, the default state of cell proliferation in multicellular organisms is *quiescence* [3].

This theory gained a progressively increasing credibility when many experimental evidences led to the discovery of oncogenes and tumor suppressor genes over the last three decades. Indeed, cancer cells are well known to bear countless mutations in genes that are physiologically involved in regulating cell proliferation and cell death, and these mutations are considered the leading cause for the wide range of human cancers that affect millions of people worldwide. The “normal” alleles of these genes came to be called proto-oncogenes, to emphasize their intrinsic tumorigenic potential, while the mutated, cancer-causing forms were accordingly called oncogenes. Since oncogenes actively promote cell proliferation, mutations converting proto-oncogenes to oncogenes typically increase or deregulates the activity of the encoded protein or, alternatively, raise the expression of the normal gene to unphysiological levels. Such mutations have therefore been given the attribute of “gain-of-function mutations”, by which only one copy of these genes needs to be mutated in order to promote cancer [4].

On the other hand, tumor suppressor genes (TSGs) encode for proteins that physiologically inhibit cell proliferation, or act as “brakes” for the cell cycle by regulating apoptotic signals.

Since they normally inhibit the formation of tumors, mutations in these genes contribute to the development of cancer by inactivating such inhibitory function. The genetic changes striking

this second class of tumor-related genes are thus typically “loss-of-function” mutations and must usually affect both copies of the gene in order to drive cancer development [4].

Given these premises, the SMT’s view of tumorigenesis entails a process analogous to a Darwinian process, in which accumulating genetic alterations lead to progressive acquisition of novel capabilities that enable the tumor cells to growth increasingly fast, evade cell death and finally proceed to metastatic dissemination. In 2000, Hanahan and Weinberg, two strong exponents of the SMT theory, proposed that for a full-blown cancer to arise, cancer cells have to acquire the following six essential capabilities that are now widely known as the “hallmarks of cancer”:

1) self-sufficiency in growth signals
2) insensitivity to antigrowth signals
3) resistance to cell death
4) limitless replicative potential,
5) sustained angiogenesis
6) tissue invasion and metastasis [5].

Later on, growing experimental evidences reported the occurrence of a significant cross-talk between the cancer cells and the surrounding stroma, supporting the hypothesis that the tumor microenvironment plays an essential role in cancer development and progression as well. Accordingly, in 2011 Hanahan and Weinberg updated their theory by further adding two novel cancer hallmarks: reprogramming of energy metabolism and escaping immune destruction [6].

In the last decade, an independent theory for cancer development has been proposed under the name of tissue organization field theory (TOFT) [3]. When compared to the SMT, this theory is based on drastically different premises: 1) carcinogenesis is a problem of tissue rather than cell organization and is therefore somehow attributable to a defect in organogenesis (whence the “development gone awry” concept) and 2) *proliferation* rather than quiescence is the default state of all cells within an organism [3].

Of note, a dysfunctional interaction between the stromal and parenchymal compartment lies at the core of the TOFT: a single or multiple carcinogenic exposure acts by altering the reciprocal biochemical, biophysical and biomechanical communication network between the epithelial cells that will give rise to most cancers and the surrounding stromal cells. As a consequence, the proliferation and motility restraints imposed by the normal tissue architecture on the relevant tissue are lost, and hyperplasia/displasia occur.

This model largely relies on the presence and the key role of a wide range of stromal cell types in the tumor surroundings, such as cancer-associated fibroblasts (CAFs), endothelial cells, pericytes, tumor-associated macrophages (TAMs) and progenitor cells of the tumor stroma. According to the TOFT, all these cell types could display either a cancer-promoting or -inhibitory activity. For instance, altered epithelial cells may trigger stromal reactions that in turn confer reciprocal signal exchanges in tumor epithelia to promote further carcinogenic processes. Ultimately, such aberrant reciprocal tumor–stroma interaction culminates in increased migratory, invasive, and metastatic behavior of cancer cells.

Hence, under the TOFT paradigm carcinogenesis and tumor progression are thought of not just as cell-autonomous, cancer cell-centered processes, but rather as a complex phenomenon involving heterotypic multicellular interactions within the newly formed cancer tissue.

Under such view, cancer is therefore basically equated to an ecological/community system whose participants are exchanging wrong information among them.

However, both cancer theories had to be somehow integrated with recent experimental data based on the increased understanding of stem cell biology, which reported several similarities between cancer cells and normal stem cells, thus leading to the notion that cancer may arise from the accumulation of mutations within normal, tissue-resident stem cells. This subset of cells, termed cancer stem cells (CSCs), were identified within several cancer types and appears to selectively possess tumor-initiating properties and to be inherently drug resistant and are hence predicted to contribute to both cancer development and relapse. These findings ultimately led to postulate a variant SMT model, the cancer stem cell (CSC) theory, which can potentially account for a typical feature often exhibited by tumors: their functional

heterogeneity [7]. However, the contribution of the stromal compartment to tumor growth and development is nowadays a widely accepted notion in the cancer research community and, in this context, the role of the immune system in cancer has been given an ever-growing attention.

THE ROLE OF THE HUMAN RNASET2 IN CANCER

Irrespective of the origin of cancer, almost all human tumors show consistent chromosomal aberrations and genomic instability is actually considered a typical feature for many human cancers [8]. Indeed, almost all solid tumors and several hematological malignancies display various degrees of karyotype changes, including chromosome translocations, deletions and duplications as well as more subtle aberrations such as small-scale rearrangements, deletions and amplifications. Of note, the functional relevance of these chromosomal rearrangements in the tumorigenic process has long been acknowledged, since a wide range of allelic analyses on tissue cancer samples have reported frequent loss-of-heterozygosity (LOH) events on specific chromosomal regions, supporting the hypothesis of the presence of tumor-suppressor genes (TSGs) or other genes related to tumor pathogenesis in the regions underlying LOH [9].

In this context, human chromosome 6 has been intensely investigated to find putative TSGs, since several studies have consistently reported the occurrence of chromosomal anomalies, mostly in the peritelomeric region of this chromosome [10]. Indeed, in a wide range of solid and hematological neoplasia (carcinomas of the ovary, breast, uterus, melanoma, non-Hodgkin B-cell lymphoma and acute lymphoblastic leukemia) rearrangements and deletions in this chromosomal region have been found. By focusing on this chromosome, our research group has mapped, cloned and characterized the *RNASET2* gene (the only human member of the highly conserved Rh/T2/S-glycoprotein family of extracellular ribonucleases) from the 6q27 region [10].

Ribonucleases (RNases) represent an important class of enzymes found in almost all organism which participate in many cellular functions, from DNA replication to control of gene expression and defense against microorganisms [11]. In recent years, enzymes and other proteins affecting RNA fate and turnover are becoming increasingly important to better understand the basic processes underlying cell proliferation, differentiation, apoptosis, and their alterations with putative implications in cancer development.

RNases can hydrolyze single-stranded RNA, double-stranded RNA, and RNA-DNA hybrid molecules [12]. Among those that hydrolyze single-stranded RNA are the transferase-type RNase. This set of RNases are secreted or localized inside cellular structures associated with the secretory pathway, but normally not associated with the presence of RNA. They have been ranked in several ways on the basis of their base specificity, structure, function, optimal pH, and origin but, in broad terms, they are currently classified as alkaline RNases (RNase T1 family and RNase A family) and acid RNases (RNase T2 family) [12].

T2 ribonucleases were first classified by their similarity to the first acid RNase purified from *Aspergillus oryzae* [13] and, unlike members of the A and T1 family, are widely distributed among taxa (viral surface proteins, bacteria, fungi, plants and higher animals). All members of the T2 family show a defined feature in their primary structure, represented by two characteristic motifs called CAS I and II (conserved active-site segments), endowed with the catalytic function of these enzymes. Of note, a key feature of Rh/T2/S ribonuclease family members is their highly disparate physiological functions, their extreme evolutionary conservation and their subcellular localization, which also varies and includes compartments where RNA is not expected to be readily available, suggesting that these enzymes could have other roles that might be independent from their catalytic activities [12].

As mentioned above, the only human member of the T2 family is called *RNASET2* and is present in the human genome on chromosomal region 6q27 as a single copy gene, organized into nine exons and eight introns. Human RNASET2 is a 256-residues long protein with a predicted molecular weight of ~30 kDa. The protein is composed of a signal peptide for secretion at the N-terminal, two CAS I/II catalytic sites and three putative N-glycosylation sites, which increase the protein's molecular weight by about 6 kDa (Figure 1).

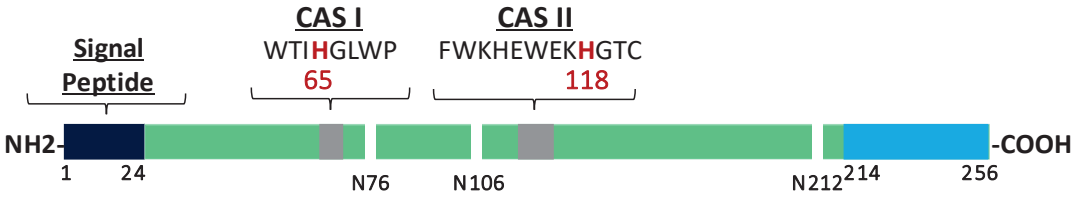

Figure 1: Schematic representation of the human RNASET2 protein
The human RNASET2 protein is composed by 256 aminoacids. Blue box: signal peptide for secretion; Gray boxes: Conserved Active Sites (CAS I-II); Blank boxes: N-glycosilation sites.

Within the cell, RNASET2 is present in three forms of different molecular weight, namely 36, 31 and 27 kDa. The 36 kDa isoform represents the full-length and secreted form, whereas the other two isoforms represent intracellular forms derived from proteolytic cleavage occurring at the C-terminal end. A subcellular fractionation assay on RNASET2-overexpressing Hey4 ovarian cancer cell line suggested that RNASET2 is produced as the full-length form, which is detected in the secretory pathway, whereas the proteolytic cleavage forms are likely originated during transport/delivery to intracellular Processing Bodies (PBs) and lysosomes. All three forms are similarly glycosylated [14].

Due to the chromosomal location of the human *RNASET2* gene in a region frequently rearranged in tumors, the putative tumor suppressive role of this gene has long been investigated by our research group.

Using ovarian carcinoma as an experimental model, the *RNASET2* gene has been first ascribed to the class II tumor suppressor genes family, since its structure was reported to be structurally intact, but frequently hypoexpressed or silenced in cancer tissues [15].

To better define the role of *RNASET2* as a tumor suppressor, both ovarian carcinoma (the human Hey3Met2 cell line) and malignant melanoma (the human SK-MEL 28 cell line) were used as experimental models. Strikingly, *in vivo* xenograft assays carried out in nude mice with *RNASET2*-overexpressing clones derived from both cell lines showed a marked RNASET2-mediated suppression of tumorigenic and metastatic potential *in vivo* [16,17].

Additionally, the analysis of Hey3Met2 human ovarian cancer cells overexpressing a catalytically inactive RNASET2 mutant protein (in which two key histidine residues within the

CAS sites were replaced by phenylalanine residues) showed that *RNASET2*-mediated tumor suppression is carried out independently from its ribonuclease activity (see Figure 2).

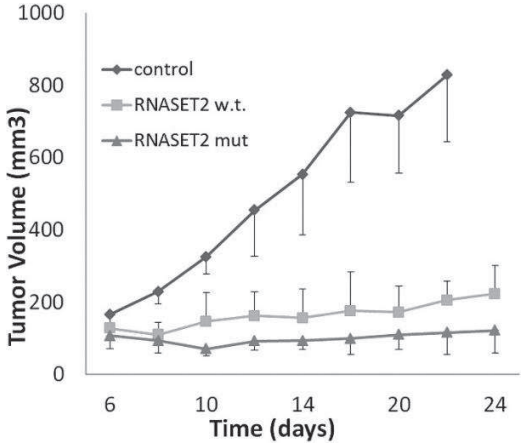

Figure 2: Image from Acquati F. Microenvironmental control of malignancy exerted by RNASET2, a widely conserved extracellular RNase. Proc Natl Acad Sci U S A. 2011 Jan 18;108(3):1104-9.
Expression of RNASET2 in the human Hey3Met2 cell line is able to suppress tumor growth *in vivo* following inoculation of nude mice with these clones. The expression of a catalytically inactive RNASET2 protein still suppresses the tumorigenic potential.

Moreover, a close histological examination of xenograft tumors sections showed that the tumor suppressive role carried out by the RNASET2 gene was associated with a consistent infiltrate of host cells belonging to the M1 subclass of macrophages, which is known to have antitumorigenic properties.

Indeed, macrophages regulate numerous functions related to tissue remodeling, homeostasis, inflammation and disease. These pleiotropic features of macrophages appear to be related to their high plasticity, since a wide range of functional states have been described for this cell type, with the well-known M1- and M2-polarized populations representing the two extremes of this range. Of note, M1-polarized macrophages are known to actively contrast tumor growth by secreting several inflammatory cytokines, whereas M2-polarized macrophages (also known as Tumor-Associated Macrophages or TAMs) are endowed with pro-tumoral activities [18].

To further define the role of host macrophages in *RNASET2*-mediated tumor suppression, a subsequent xenograft model based on Rag/γ-chain double knock-out mice ($Rag2^{-/-}\gamma_c^{-/-}$), which

lack both lymphocytes and NK cells, was exploited to evaluate if the population of host-derived cells infiltrating RNASET2-expressing tumors could be functionally responsible for RNASET2-mediated suppression of tumorigenicity. Indeed, when mice were pretreated with the macrophage-depleting agent clodronate before inoculation of RNASET2-expressing Hey3Met2 cells, the tumor suppressing activity of wild-type RNASET2 turned out to be largely impaired (Figure 3) [19].

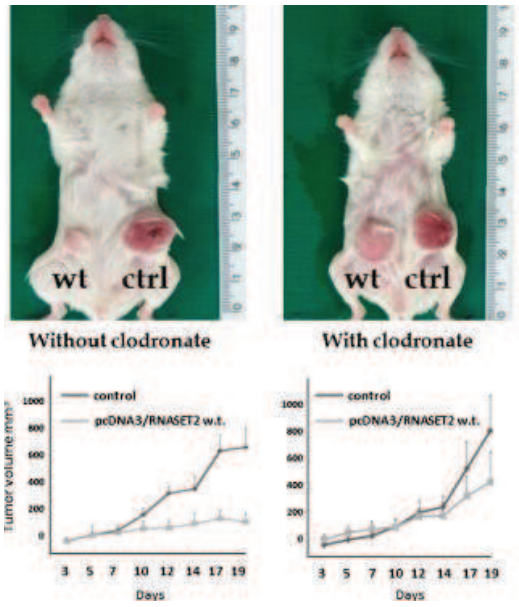

Figure 3: Image from Acquati F. Microenvironmental control of malignancy exerted by RNASET2, a widely conserved extracellular RNase. Proc Natl Acad Sci U S A. 2011 Jan 18;108(3):1104-9.
Rag/γ-chain mice were used to further analyze the functional role of host macrophages in the RNASET2-mediated tumor suppression. Mice either mock-treated or treated with clodronate (a macrophage-depleting agent) before inoculation with RNASET2-expressing Hey3Met2 clones were compared. Representative images of animals are presented with tumor growth kinetics that show clear increase in tumorigenicity for treated mice.

These results are in keeping with an extensive body of evidence from the literature, which strongly suggests a role for T2 RNases in the modulation of innate immune response. As an example, the T2 ribonuclease omega-1 secreted from *Schistosoma mansoni* eggs has been found to be the major soluble factor involved in priming dendritic cells to promote Th2 lymphocyte differentiation [20]. Moreover, preliminary results on the invertebrate experimental model *Hirudo verbana* (the common leech) also showed a marked recruitment of

cell macrophages induced by RNASET2 injection in the body wall (Baranzini *et al*, manuscript in preparation).

The antineoplastic features of RNASET2 were also confirmed by a complementary *in vivo* experimental model established in our lab. *RNASET2* was knocked-down by RNA interference in the OVCAR3 human ovarian cancer cell line, which shows high endogenous levels of this protein. In agreement with the data previously obtained with the Hey3Met2 cell line, silencing of *RNASET2* expression in OVCAR3 cells caused an increased in cancer growth rate *in vivo,* coupled with a significant decrease of M1-polarized macrophage infiltration as a distinct feature of the *RNASET2*-silenced tumors [21].

Noteworthy, we also demonstrated that the RNASET2 protein behaves as a strong *in vitro* chemoattractant for human monocytes and it can bind the monocyte-macrophage cell surface in a dose-dependent manner, which suggested the occurrence of a receptor-mediated interaction [21].

Taken together, these data strongly suggest that the tumor microenvironment (in particular the monocyte-macrophage cell population) seems to play a crucial role in RNASET2-mediated tumor suppression *in vivo*. Within this frame, we defined *RNASET2* as a gene endowed with a marked *non-cell-autonomous* oncosuppressive role.

However, in light of the very disparate functional roles that have been attributed to members of the T2 RNase family [22], the occurrence of an independent *cell-autonomous* role for *RNASET2* cannot be formally ruled out. Therefore, based on both the extreme conservation of T2 ribonucleases throughout most *phyla* and the presence of an intracellular pool of RNASET2, we hypothesized that, besides its previously reported role in microenvironmental-mediated tumor suppression, RNASET2 could be endowed with an ancestral *cell-autonomous* activity that might be related to cancer suppression as well.

In particular, drawing from the evidences that several members of the T2-Rnase family appear to be activated upon stress conditions [23] and that the RNASET2 protein re-localize to processing bodies (PBs) in conditions of metabolic stress, we started to investigate whether human *RNASET2* might play a role in stress-response in mammalian cells. Strikingly, by

challenging ovarian cancer cell lines with different stress-inducing chemicals or treatments, a significantly increase of RNASET2 protein levels, in both intra- and extracellular compartments, was observed in response to most applied stresses [24].

Of note, several cancer-related parameters were found to be affected *in vitro* in *RNASET2*-silenced OVCAR3-cells, supporting the occurrence of a cell-autonomous oncosuppressive role for this gene.

Moreover, a marked RNASET2-mediated remodeling of the actin cytoskeletal organization was observed as well, a feature which is clearly related to cancer due to its implications in cell motility and adhesion [24].

Again, the observed cytoskeleton-remodeling ability of a tumor suppressing T2 ribonuclease is in keeping with previous works, since a strong antitumor activity has been described for a fungal member of T2 family ribonucleases, named ACTIBIND due to its ability to bind actin [25].

Taken together, these data prompted us to consider *RNASET2* as a protein endowed with a marked pleiotropy, which refers to the phenomenon by which a single gene can affect multiple biological processes and, by consequence, several traits [26].
Particularly, we ascribe RNASET2 to the growing family of "moonlighting proteins", which describes a class of multifunctional proteins that are involved in several complex biological processes such as DNA replication, transcription and tumor suppression, but that were previously identified for totally unrelated functions. Of note, a typical feature of these proteins is that their function can vary widely as a consequence of their cellular localization and cellular context and this seems to be a feature belonging to RNASET2 as well [27,28].

In the light of these findings, it is therefore of great interest to carry out further investigations in order to shed more light on *RNASET2's* modes of action in the context of cancer suppression. To this end, an interesting goal would be to develop new experimental models to better understand how the crosstalk between cancer-cell derived RNASET2 and cells belonging to

innate immune system operates *in vivo*. To this aim, an *in vitro* cellular model to investigate how RNASET2 affects the macrophage polarization pattern could be highly informative.

Moreover, the oncosuppressive role of *RNASET2* in the context of a competent immune system would be worth investigating in a proper *in vivo* experimental model as well. This task could be best achieved by developing a syngeneic mouse model where *RNASET2* expression is experimentally modulated.

Finally, it would be extremely important to confirm (and possibly extend) the role(s) of *RNASET2* in other cellular models representing cell types known to be susceptible to cancer development following rearrangements of the 6q27 chromosomal region, such as the mammary epithelium [29]. In this context, the MCF7 breast cancer cell line represents a widely used experimental model, derived from a pleural effusion taken from a patient with metastatic breast cancer [30]. This cell line has been thoroughly used for *in vitro* breast cancer studies since it has retained several features that are peculiar to the mammary epithelium. Moreover, these cells might be exploited to compare in a single experimental model the effect of *RNASET2* in the context of two independent experimental settings (2D and 3D cell culture).

Indeed, it is widely acknowledged that a fully formed organ is significantly more complex than cells kept in culture monolayers. 3D cultures are beginning to bridge the gap between these two experimental models, by retaining some of the architectural features that are usually lost when the structure of organs and tissues is destroyed by dissociating cells and culturing them in 2D. Of note, 3D models are known to faithfully recreate some key aspects of the tissue microenvironment and, in some cases, to provide a more comprehensive and relevant biological information that is very difficult (if not impossible) to recapitulate from 2D models [31].

As a matter of necessity, 3D models of the mammary gland acinus have been developed for almost 30 years. Under proper culture conditions, mammary epithelial cells form polarized spheroid structures (also called acini) which consist of a central lumen, a single layer of polarized luminal epithelial cells surrounded by myoepithelial cells and a basement membrane. Interestingly, disruption of the normal acinar architecture is an early hallmark of mammary

epithelial cell transformation, since in the early stages of breast cancer development an increased proliferation rate of mammary epithelial cells is coupled to a loss of acinar organization. This is the reason why three-dimensional culture conditions are increasingly being used to address the molecular mechanisms by which oncogenes or tumor suppressor genes could influence mammary epithelial cell transformation [32].

The three tasks above described have been experimentally addressed in this PhD thesis.

AIMS OF THE PROJECT

The *RNASET2* gene maps to human chromosome 6q27, a region that has been consistently found rearranged in many solid and hematological tumors. This gene encodes for the only human secreted acid ribonuclease of the T2 family.

Our previous experimental data proved a role in the control of tumorigenesis carried out *in vivo* by this gene in two independent human ovarian cancer cell models. Moreover, RNASET2-mediated tumor suppression *in vivo* turned out to involve the recruitment into the tumor mass and possibly activation of innate immune cells belonging to the monocyte/macrophage lineage. Indeed, a preliminary *in vivo* functional characterization showed that RNASET2 specifically recruits M1 macrophages, which are known to carry out a cancer antagonizing role. These data allowed us to hypothesize a non-cell autonomous oncosuppressive role for extracellular *RNASET2*. [16,19,21]

In recent investigations, *RNASET2* was also shown to behave as a stress response gene, since cells cultured under a wide range of stress condition showed a trend for an increase of RNASET2 expression and secretion. Specifically, by challenging three independent ovarian cancer cell lines with hypoxic conditions, which represent a general hallmark of many cancer tissues, a clear increase in RNASET2 expression and secretion was observed, thus supporting the notion of a non cell-autonomous role for RNASET2 reminiscent of that related to “alarmins”, a wide family of extracellular danger-signaling molecules involved in stress response at the tissue level [24].

Finally, a cell-autonomous role in tumor suppression has been reported for the *RNASET2* gene as well, likely involving its ability to affect the cell cytoskeleton [20,24].

Given these premises, which strongly suggest a highly pleiotropic oncosuppressor role for *RNASET2*, we decided to further characterize this gene from a functional point of view.

Within this conceptual frame, in the attempt to gain more insights into the functional interaction between this protein and cells from the monocyte/macrophages lineage, the first aim of my work was to **analyze the effect of RNASET2 protein in the recruitment and polarization pattern of macrophages in both *in vitro* and *in vivo* systems**.

The human monocyte-like THP-1 cell line was used to investigate whether RNASET2 could affect the macrophage polarization pattern. Known M1/M2 markers were analyzed by qPCR following experimental differentiation and polarization of the THP-1 parental cell line, which shows high endogenous RNASET2 levels, and at the same time we analyzed the same cell line devoid of the RNASET2 gene through silencing [33]. Moreover, to verify the evolutionary conservation of the role of RNASET2 in innate immune system regulation, its ability to recruit and possibly activate macrophages was investigated using the medicinal leech as an experimental model.

Furthermore, since our previous *in vivo* data on RNASET2-mediated tumor suppression were obtained in nude mice, a second aim of my PhD project was to investigate the oncosuppressive role of this gene in a **syngeneic mouse model**. This could provide a more detailed knowledge about the interplay of RNASET2 with the tumor microenvironment in a completely immunocompetent *in vivo* experimental model instead of an immunosuppressed one. Additionally, a syngeneic model could also rule out any possible immunological influence of the human protein on the mouse immunological set up that might have arisen using xenograft-based *in vivo* assays.

To this end, we analyzed the expression of the murine ortholog of RNASET2 in different mouse cancer cell lines and chose the C51 and TSA cell lines (representing colon and mammary mouse cancer cells, respectively) to overexpress the murine RNASET2 protein and evaluate how such overexpression affected several cancer-related parameters *in vitro* and *in vivo*.

As mentioned before, chromosomal anomalies of the long arm of chromosome 6 have long been reported for several cancer types, including breast cancer. Therefore, the third task of my work has been to further **address the oncosuppressive role of RNASET2 in the context of mammary carcinogenesis**. In particular, in line with a recent view of cancer development and progression as a process involving an altered tissue organization pattern (by which cancer would represent a disease of “development gone awry”), we decided to **investigate the**

pleiotropic roles of RNASET2 as a tumor suppressor gene in the context of breast morphogenesis.

To this end, the human MCF7 breast cancer cell line was chosen as an experimental model.

Furthermore, recent data gathered in collaboration with another research group showed that, when four different cell populations of healthy mammary gland (namely luminal terminally differentiated cells, myoepithelial basal-like cells, stem cells and EMT cells) were sorted by cytofluorimetry, a striking gradient of *RNASET2* expression was observed, with more differentiated cells from the luminal population showing the highest expression level whereas other cell populations showed a progressively decreasing expression, which reached a minimal value in the stem cells population.

On the basis of these data, we silenced the expression of RNASET2 in the luminal breast cells population to analyze putative changes in their ability to form differentiated organoid structures in 3D. Organoids represent an important bridge between traditional 2D cultures and *in vivo* models, as they are more physiologically relevant than monolayer culture models, so this culture system is expected to provide a more reliable experimental model to investigate the role of RNASET2 in mammary morphogenesis and tumorigenesis [31].

MATERIALS AND METHODS

CELL CULTURES

Adhesion growth: Hey3Met2 (human ovarian cancer). Culture medium: DMEM-F12 + 10% FBS + 1% L-glutamine.

MCF7 (human breast cancer). Culture medium: RPMI, 10% FBS, 1% L-Gln,10 µg/ml insulin.

C51 (mouse colon cancer) and TS/A (mouse mammary cancer). Culture medium: DMEM + 10% FBS + 1% L-glutamine.

Stably-transfected cell clones

Culture medium: basic medium + selective antibiotic. MCF7: 600 µg/ml G418; C51: 400 µg/ml G418; TS/A: 300 µg/ml G418.

Suspension growth: THP-1 (human acute monocytic leukemia). Culture medium: RPMI-1640 + 10% heat-inactivated FBS + 1% L-glutamine.

RNASET2 Knockdown in THP-1 Cells

The recombinant pSicoR expressing vector bearing a RNASET2-targeting shRNA [21] was stably transfected into THP-1 cells with the Cell Line Nucleofector Kit V (Amaxa). The efficiency of RNASET2 knockdown was evaluated by Western blot analysis before every experiment. Cells were maintained under selection (0.75 µg/ml puromycin) throughout all the experiments to ensure the stability of the pool.

Culture conditions: 37°C, 5% CO2, humidified atmosphere.

All cell lines are routinely screened for the absence of mycoplasma contaminations by performing a nested PCR.

Differentiation and polarization protocol of THP-1 cells

THP-1 cells were differentiated into M0 macrophages by addition of 5 ng/ml Phorbol 12-myristate 13-acetate (PMA) for 48 hours in MT6 wells at a concentration of $0.7x10^6$ cells/ml. Following monocyte differentiation into macrophages for 48 h, the medium was changed after three washes and macrophages were polarized into either M1 or M2 as follows: M1 polarization was achieved by treatment with 100 ng/ml LPS (from Salmonella abortus equi S-form – EnzoLifeSciences) and 20 ng/ml IFN-γ (Bio Basic – RC217-17), whereas M2 polarization was induced by treatment with 20 ng/mL IL-4 (Peprotech – 200-04).

THP-1-derived macrophages were allowed to polarize for 18 h (M1) or 48 h (M2). Three different M1 polarization regimens were used: 20 ng/mL IFN-γ alone, 100 ng/ml LPS alone or a combination of the two (IFN-γ+LPS), and a single for M2 polarization: 20 ng/mL IL-4 treatment. A non-induced macrophage control population, cultured without polarizing cytokines, was used as control and referred to as M0. Endotoxin-free recombinant RNASET2 protein was used at a 0.22 μM concentration. Control macrophages were treated with the same endotoxin-free vehicle (PBS) used for recombinant RNASET2 storage. [33]

The recombinant protein was produced in the yeast *Pichia pastoris* expression system and purified as previously reported [34].

Transfection

For transfection experiments, cells were plated in 6-well plates the day before experiment and were incubated overnight at 37°C (in a humidified 5% CO2 incubator) to reach 80% confluence the day of transfection, which was performed with Lipofectamine 2000 according to the manufacturer's protocol.

IN VITRO ASSAYS

Cell proliferation assay

The CellTiter® 96 Non-Radioactive kit (Promega) was used to determine viable cell number based on the cellular conversion of a tetrazolium salt into a formazan product. The absorbance of the solubilized formazan product (directly proportional to the number of cells) was recorded using a 96-well plate reader daily over a 7 or 10-days period. Samples were seeded in triplicates.

Colony-formation assay

50, 100 or 150 cells/well were plated in six-wells culture plates. Following a 14 days incubation, clones were stained with 1% methylene blue/50% ethanol and manually counted.

Apoptosis assays

Cells were plated in 6-well plates the day before experiment and were incubated overnight at 37°C (in a humidified 5% CO2 incubator). The day of the experiment they were challenged with either 100 nM Cisplatin or 200 μM $CoCl_2$. Treatments were conducted in technical triplicates.

24-hours after the treatment, $5x10^5$ cells were fixed in 0.5 ml of ice-cold 100% ethanol, centrifuged, washed with PBS, resuspended in the dying solution (50 µg/ml Propidium Iodide, 20 µg/ml RNase in 1X PBS) and analyzed with FACSCalibur flow cytometer.

Mammosphere assay

For mammosphere formation, cells were enzymatically and mechanically detached and dissociated and 1,000 cells/well (96-wells plates) or 25,000 cells/well (6-wells plates) were seeded into ultralow attachment plates and cultured for 1 or 2 weeks in their grown medium supplemented with 1x B27 and 0.75% methylcellulose (viscosity 4,000 cP). Cell cultures were maintained in a humidified 5% CO_2 incubator.

GENE EXPRESSION ANALYSIS

Total RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen) and reverse transcribed into complementary DNA (cDNA) with random primers according to the instructions of the High Capacity cDNA Reverse Transcription Kits (Applied Bioystems). Quantitative PCR, using gene-specific primers, was performed on CFX Connect (Biorad) with the Power SYBR-green PCR Master Mix (Applied Biosystems), following the manufacturer's instructions. Amplification reactions were performed in triplicate. Results were normalized using the house-keeping gene GAPDH and the $\Delta\Delta$cyclethreshold method and are expressed as relative fold over the control group.

List of primers used for the three different projects:

Primer name	Forward (5'-3')	Reverse (5'-3')
β-actin	CCAAGGCCAACCGCGAGAAGATGAC	AGGGTACATGGTGGTGCCGCCAGAC
EPCAM	AGGAAGAATGTGTCTGTGAAAACTACA	TGAAGTACACTGGCATTGACGAT
Beta-casein	GCCACTTGCCCCAGTTCAT	ACAAAGACGGAAAAGGCATCA
CD44	CAGACCTGCCCAATGCCTTTGATG	CTTTCTGGACATAGCGGGTGCC
CD24	TGCTGCTGCTGGCACTGCTCCTA	CAGAGTTGGAAGTACTCTGGGAGG
ALDH1A1	TCCTTGGAAATCCTCTGACCCCAG	GGCCCCTTCTTTCTTCCCACTC
3xFLAG for qPCR	AAAGACCATGACGGTGATTATAAAG	ACTTGTCATCGTCATCCTTGTA
CXCL10	GACATATTCTGAGCCTACAGCA	CAGTTCTAGAGAGAGGTACTCCT
TNF	TGCACTTTGGAGTGATCGG	TCAGCTTGAGGGTTTGCTAC
CCL22	CTCCAACCTCCAATACCCA	CATAGCATGAATTTGATTGTCAGC
MRC1	GGTTTTGGAGTAATATTCACTGTTCT	TCCATCTTCCTTGTGTCAGC
GAPDH	CTCTCTGCTCCTCCTGTTC	GCGCCCAATACGACCAA
CCL17	GCCATCGTTTTTGTAACTGTGC	CAAGACCTCTCAAGGCTTTGC

WESTERN BLOT ANALYSIS

Adherent cells were mechanically scraped in PBS + 5mM EDTA and resuspended in lysis buffer (0.5% Igepal, 0.5% Triton X-100 in PBS + 5mM EDTA) supplemented with protease inhibitors cocktail (PMSF, benzamidine, aprotinin, and leupeptin). Suspended cells were collected, washed and resuspended in lysis buffer. Quantification of total proteins was performed with Bradford reagent (BIORAD), using bovine serum albumin as standard. For the SDS-PAGE analysis, 30-70 µg of intracellular lysate were loaded. Immunoblot analysis was performed using standard procedures and detected with a chemiluminescent substrate (WESTAR ETA C ULTRA 2.0, Cyanagen).

Antibodies: Polyclonal rabbit anti-RNASET2 (Davids Biotechnologie GmbH, Regensburg, Germany); monoclonal rabbit anti-FLAG (SIGMA ALDRICH); HRP anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, USA); mouse monoclonal anti-α-tubulin (SIGMA ALDRICH); HRP anti-mouse IgG (ABNOVA).

ACTIN CYTOSKELETON STAINING

Cells were grown on coverslips for 24 hours and then processed for immunostaining. Cells were fixed in 3% paraformaldehyde and then permeabilized using Triton X-100. Phalloidin-TRITC dye (SIGMA) incubation was performed in diluted blocking solution (3% BSA in PBS). Coverslips were mounted on microscope slides using Vectashield mounting medium (Vector). Fluorescence/confocal microscopy images were acquired.

CLONING OF THE HUMAN RNASET2 CODING SEQUENCE IN THE INDUCIBLE EXPRESSION VECTOR

The inducible expression vector bearing the mouse Rnaset2 cDNA was bought from the VectorBuilder company (figure 4).

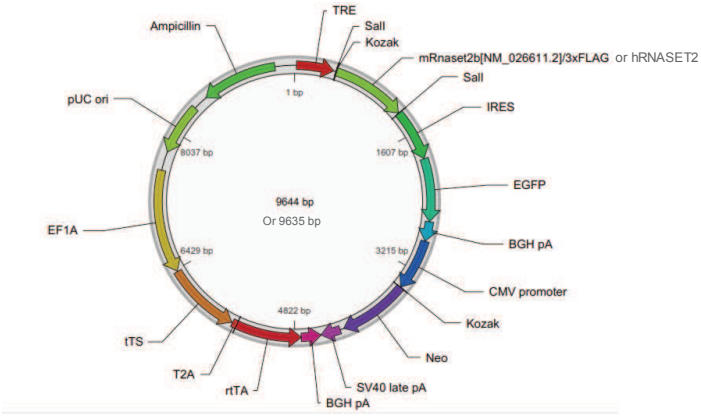

Figure 4. Inducible vector map with the cDNA for mRnaset2 or hRNASET2

In order to have a control for comparison of transfection and expression efficiency, we decided to clone the wild-type human RNASET2 coding sequence inside the same plasmid.

A construct already available in our lab was used as a template for a PCR reaction with the following primer pair:

44-SalI Fw

5' TACGCGTCGACGCCACCATGCGCCCTGCAGC 3'

44-SalI Rev

5'AACGCGTCGACCTACTTGTCATCGTCATCCTTGTAATCGATATCATGATCTTTATAATCACCGTCATGGT CTTTGTAGTCATGCTTGGTCTTTTTAGGTGGGGGA 3'

The amplification product was digested with SalI, gel purified and cloned into the vector builder plasmid, devoid of the mouse gene, before transforming in the DH5α *E. coli* strain. Plasmid DNA was purified and then sent to sequencing (BMR, Padova, Italy), before using it.

CLONING OF THE DIFFERENT FULL OR CHIMERICH CDNA IN THE PCDNA3 PLASMID FOR THE SWAP APPROACH

The pcDNA3 plasmid was chosen as backbone. The inducible vector bought from the VectorBuilder company was used as a template for a PCR reaction with the following primer pair for inserting the 3xFLAG sequence into the pcDNA3 vector:

3xFLAG_version2 Rev: 5' AGAGGTTCTAGACTACTTGTCATCGTCATCCTTGTAATC 3'

3xFLAG_version2 Fw: 5' AACTGGCTCGAGGACTACAAAGACCATGACGGTGA 3'

The amplification product was digested with XhoI and XbaI, gel purified and cloned into the pcDNA3 vector before transforming in the DH5α *E. coli* strain. Plasmid DNA was purified and then sent to sequencing (BMR, Padova, Italy), before using it for the following steps.

Different constructs, already available in our lab, bearing the human and mouse RNASET2 gene were used as templates for PCR reactions with the following primer:

hRNASET2 Fw: 5' ACCTTGGGATCCACCATGCGCC 3'

hRNASET2 Rev: 5' ACCTGAGAATTCATGCTTGGTCTTTTTAGGTGGG 3'

mRNASET2A Fw: 5' TGCTAAGGATCCACCATGGCGCCG 3'

mRNASET2A Rev: 5' ACCTGAGAATTCATGTTGGGTCTTTGTAGGTGGA 3'

mRNASET2A truncated Rev: 5' ACCTGAGAATTCCTGCTCCCCTGGCTCA 3'

hRNASET2 overlap Fw: 5' TGAGCCAGGGGAGCAGCCGTCCCCCAAGCAG 3'

hRNASET2 overlap Rev: 5' TTCCTGCCTGGAGGACAGCTGCTCCCCCGGCT 3'

mRNASET2A overlap Fw: 5' AGCCGGGGGAGCAGCTGTCCTCCAGGCAGGAA 3'

mRNASET2A overlap Rev: 5' CTGCTTGGGGGACGGCTGCTCCCCTGGCTCA 3'

The amplification products were digested with EcoRI and BamHI, gel purified and cloned into the pcDNA3 vector (together with a synthetic oligo coding for a TEV protease recognition site with EcoRI and XhoI sticky ends) before transforming in the DH5α *E. coli* strain. Plasmid DNA was purified and then sent to sequencing (BMR, Padova, Italy), before using it for the transfections.

A schematic representation of the pcDNA3 vector and all cDNAs cloned is shown in figure 5.

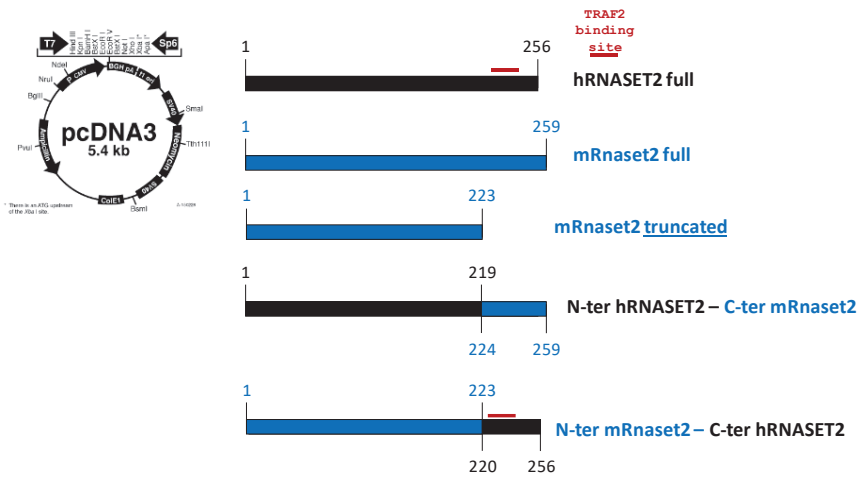

Figure 5. pcDNA3 plasmid vector and schematic representation of cloned cDNAs

The pcDNA3 (Invitrogen) was used as backbone. Full-length isoforms of humand and mouse RNASET2 were cloned together with the two "swapped" cDNAs broken at the 219 aa. (for the human) or 223 aa. (for the mouse). Finally, also a truncated version of mouse Rnaset2 was cloned.

ANIMAL TUMOR MODELS

Housing, treatment, and killing of animals followed national legislative provisions for the protection of animals used for scientific purposes and the ministry of health approved the protocol.

C51 Empty cl. 10, C51 mRnaseT2 truncated cl. 6 and C51 mRnaseT2 full cl. 40 (0.2×10^6) cells were subcutaneously (s.c.) implanted in 7-week-old immunocompetent syngeneic BALB/c mice (Envigo). Tumor growth was daily monitored. The tumor volume was determined using the formula: $(d)^2 \times D \times 0.52$, where d and D are the short and long dimension (cm) of the tumor, respectively, measured with a calliper. Euthanasia was performed when the tumor reached a volume of <1.5 cm3 or when there was a tumor ulceration.

3D EXPERIMENTS

Patient collection

Human mammary organoids were isolated from tissues obtained from informed healthy patients, undergoing mammoplasty reduction procedure. The enrolment was performed at the University of Athens, Greece, at the Department of Breast Cancer Surgery, Hospital "Agios Savvas" following the approval of Clinical Ethical Committee released by the Ministry of Health of Athens (CEC n. 01072016). Patient consent for the use of their specimens in research was obtained. The consent document informs the patients that the donated specimens will be used to study mammary SC differentiation and tumor initiation and progression. The research was performed in compliance with the Helsinki Declaration for experiments involving humans (The Code of Ethics of the World Medical Association).

Isolation of human mammary organoids

Mammary tissue, handled using the appropriate biosafety precautions, is transferred kept on ice, from operating room into a clean, sterile tissue culture hood. Adipose tissue is removed with scissors and tissue is cut in pieces of approximately ∼3–5 mm as described in Piscitelli Et al 2015 [35]. 1–2 g of tissue is transferred to a 15-ml conical polypropylene tube filled with 10 ml collagenase (Sigma) solution. Tubes are incubated overnight on a rotator at 37 °C until the tissue fragments are dissociated. The following day tubes are removed from the incubator and

organoids, composed of luminal epithelial and myoepithelial cells are collected to a fresh tube by filtration. Organoids are washed with 10 ml PBS and centrifuged (300xg at 4 °C) for 5 min three times.

Lentiviral particle production

GFP reporter vector construction (pCDH-CMV-MCS-EF1-copGFP from SBI) and lentiviral particles were generated as described in Pelucchi et al. 2013 [36].

Lentiviral-mediated transduction of shRNASET2-GFP into human breast organoids.

10 organoids are transferred to one Eppendorf tube or to well of a six-well plate. 10^5–10^7 colony-forming units of lentiviral particles plus 8 µg/ml of polybrene are added to the organoids, and the tube or the plate are spin at 32°C at 300g for 2hrs. Organoids are placed at 37°C for 30 min. 1 ml of pre-warmed organoid medium is added and organoids are incubated overnight at 37 °C. The following day organoids are expanded for GFP expression.

3D-organoid expansion

Organoids are expanded by embedding them in 100 µl ice-cold Growth Factor Reduced Matrigel (BD Biosciences) at the concentration of 5-8 organoids /well in 48-well-plates (Greiner, Twin-Helix). Organoid medium (Ham's F12/DMEM-GlutaMAX (1:1) containing 1X NEA-MEM (Invitrogen), 1 µg/mL insulin, 0.5 µg/mL hydrocortisone, 10 ng/mL EGF (Sigma), and 4 ng/mL bFGF (ImmunoTools) is added to the solidified Matrigel and then replaced every 3 days. Organoids are generated and re-passaged every 7-10 days. Cells during organoid formation assay were monitored with a fluorescence microscope (Olympus IX51) to characterize cell morphology and cell number.

Matrigel dissociation for intact organoid recovery

Disaggregation of Matrigel consists in several incubations of the organoids for 10 min each in ice, followed by a centrifugation at 0.4 g for 5 mins. This step is repeated until complete disruption of Matrigel.

RESULTS

Part I: RNASET2 effect on *in vitro* macrophages polarization

Our previous experimental evidences allowed us to describe *RNASET2* as a tumor suppressor gene, acting primarily in a non cell-autonomous manner in the context of an *in vivo* model of human ovarian carcinoma. Indeed, *RNASET2*-overexpressing tumors showed a marked decrease in their tumorigenic potential associated with a high infiltration by murine macrophages in xenograft-based assays. Moreover, *in vivo* host macrophages depletion in nude mice before human cancer cell inoculation led to a massive decrease of the observed *RNASET2*-dependent tumor suppressive activity [19,21]. Finally, using a human leukemic monocytes-derived cell line as an experimental model, we also reported a strong chemotactic role carried out by recombinant RNASET2 in *in vitro* migration assays [21].

Taken together, these data led us to hypothesize that cancer cell-derived RNASET2 protein acts by actively recruiting to the tumor mass cells from the monocytes-macrophages population endowed with anticancer properties, as suggested by the M1 polarization pattern observed in macrophages infiltrating *RNASET2*-overexpressing tumors *in vivo.*

These data prompted us to investigate the putative role of the RNASET2 protein not only in monocyte/macrophage recruitment, but also in macrophage polarization. This issue is of key relevance, since tissue macrophages have long been known to carry out either pro- or anti-tumoral activities based on their polarization pattern [18].

To address this issue, we therefore decided to establish a suitable *in vitro* experimental model that might help us to better define the mechanism by which RNASET2 could affect this cell population's functional properties *in vivo*.

To this aim, we chose the THP-1 human cell line, a well-established cell model which shares many properties with normal human monocytes and has been frequently used for investigations on macrophages' differentiation and polarization pattern.

Since previous experimental analysis in our lab had shown a high endogenous level of *RNASET2* expression in this cell line, in order to study of the effect of human *RNASET2* in macrophages polarization we decided to silence the expression of this gene in these cells by means of RNA

interference. As shown in figure 6, the chosen protocol led to a complete silencing of endogenous RNASET2 expression in THP-1 cells, as evaluated by western blot analysis.

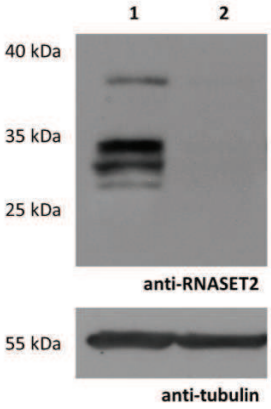

Figure 6. Analysis of RNASET2 protein expression in THP-1 RNASET2-silenced clones.
Western blot analysis for *RNASET2* expression in parental (lane 1) and *RNASET2*-silenced (lane 2) human THP-1 cells. Upper panel: an intracellular lysate was analyzed for RNASET2 expression. Lower panel: the same blot was probed with anti-tubulin polyclonal antibody for normalization.
Image from: Scaldaferri et al. Immunol Lett. 2018.

Thereafter, we proceeded with the differentiation of THP-1 cells into M0 macrophages by culturing them in 5 ng/ml PMA-containing medium, as described in Material and Methods.
The effective differentiation of THP-1 cells in mature macrophages was verified and confirmed by both microscopy analysis and a real-time PCR assay, which showed both the expected shift in cellular behavior from suspension to adherence growth pattern and a marked increase in the expression levels of the CD68 differentiation marker in PMA-treated cells (figure 7).

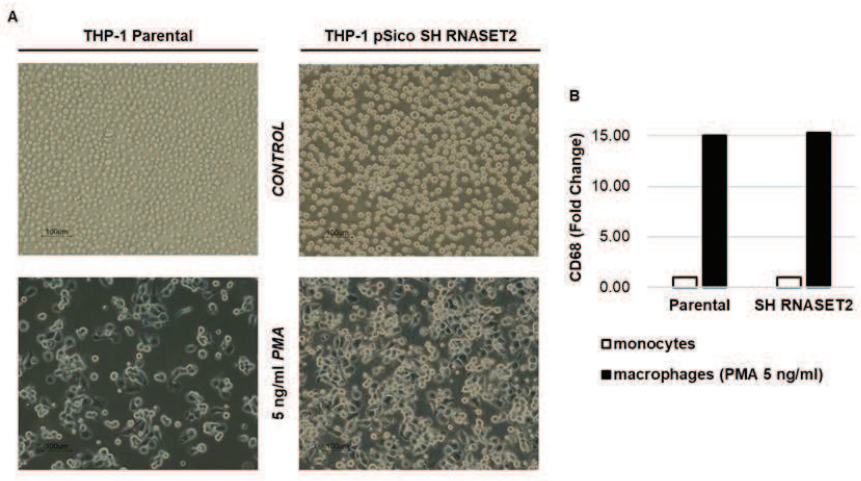

Figure 7. Assessment of PMA–induced macrophage differentiation.
A) Morphological changes associated with PMA–induced THP-1 cells differentiation into macrophages. Representative images are shown for PMA-treated (bottom panel) and vehicle-treated (DMSO, "CONTROL" – upper panel) cells at 20x magnification.
B) Real time PCR analysis was also performed to determine the expression changes of the macrophage differentiation marker CD68. Image from: Scaldaferri et al. Immunol Lett. 2018.

Both parental and *RNASET2*-silenced THP-1-derived M0 cells were then polarized into either M1 or M2 macrophages as described in Material and Methods and the induced polarization pattern was investigated by real time qPCR to evaluate the expression level of known M1 (TNF and CXCL10) and M2 (CCL22 and CCL17) markers.

As expected, we observed a marked increase in the expression of both M1 markers following LPS/IFN treatment, suggesting a proper M1-polarization in parental THP-1 cells (figure 8, black bars in upper panel).

Of note, *RNASET2* silencing turned out to attenuate M1 polarization these cells: indeed, whereas we observed a 50- and 5-fold increase in the expression levels of CXCL10 and TNF markers, respectively, when compared to unpolarized M0 cells in THP1 parental cells, the observed increase in the expression levels of both M1-specific markers was much more weak following RNASET2 knockdown (by 62% for CXCL10 and 28% for TNF, respectively) (figure 8, black bars in upper panel).

These data suggest that the high levels of endogenous RNASET2 expression in THP-1 cells makes them particularly sensitive to M1-polarizing stimuli, since silencing of this gene leads to a weakened M1-response.

By contrast, treatment with IL-4 turned out to be much less effective in driving M2 polarization in parental THP-1 cells, whose expression levels for both CCL22 and CCL17 markers showed a mere 1,5-3-fold increase when compared to unpolarized M0.

By contrast, both CCL22 and CCL17 expression levels turned out to be dramatically increased in *RNASET2*-silenced cells (by 80- and 7-fold, respectively), as shown in figure 8 (white bars, lower panel). These data seem to provide a complementary picture of the M1 polarization pattern, since the high endogenous *RNASET2* expression level in parental THP-1 cells apparently makes them almost unresponsive to M2-polarizing stimuli, and such unresponsiveness is lost following RNASET2 silencing.

Taken together, these data are in keeping with the notion of RNASET2 acting as a macrophage-polarizing molecule, as we previously reported in our *in vivo* xenograft-based assays [19,21].

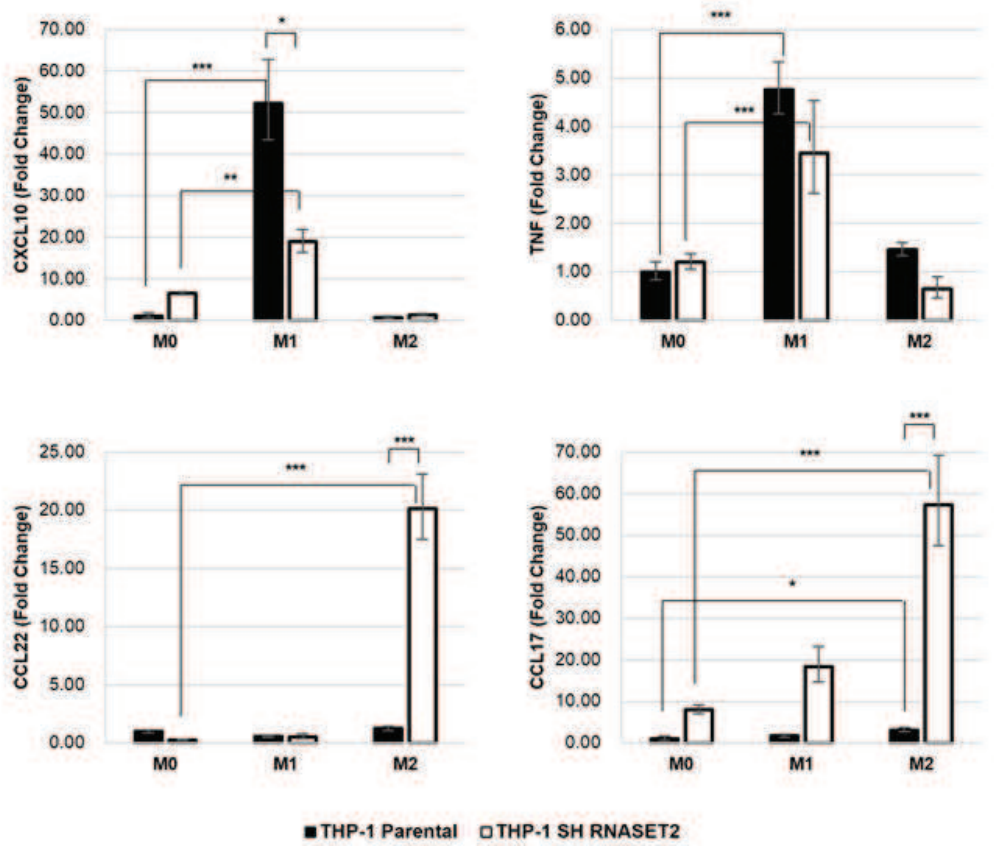

Figure 8. qRT-PCR analysis for M1 and M2 polarization markers in THP-1-derived human macrophages.
Total RNA was isolated from polarized parental (black bars) or *RNASET2*-silenced (white bars) THP-1-derived macrophages from each experimental group. Following reverse transcription, a real time qRT-PCR assay was carried out to analyze the expression changes of 4 different markers of macrophages polarization, using GAPDH as an internal standard.
Gene expression levels are plotted as fold difference in mRNA expression versus THP-1 parental M0 set as 1.0. Error bars represent standard deviation from the mean. Statistical analysis was performed as described in Materials and Methods. *P<0. 05; **P<0. 01; ***P<0. 01. M1 markers: CXCL10 and TNFα; M2 markers: CCL22 and CCL17. Image from: Scaldaferri et al. Immunol Lett. 2018.

The data described above have been obtained following the experimental manipulation of the *endogenous* expression of RNASET2 in THP-1 cells. However, since RNASET2 is described as an extracellular protein, it is difficult to define whether the observed effect of the gene's silencing on the THP-1 cell's polarization pattern was mediated by RNASET2 acting *within* the target cells themselves or rather by means of an autocrine mechanism based on RNASET2 secretion followed by an extracellular signaling.

Indeed, in our *in vivo* xenograft-based assays the observed recruitment of host macrophages was induced by cancer cell-derived extracellular RNASET2, suggesting a non-cell autonomous mechanism of action for this protein.

To better clarify this issue, we decided to test the effect of exogenously added recombinant RNASET2 on the polarization pattern of THP-1 cells.

To this aim, *RNASET2*-silenced THP-1 cells were differentiated into M0 macrophages and subsequently polarized into M1 and M2 subtypes in the presence or absence of *Pichia pastoris*-derived recombinant RNASET2 protein, previously produced in our lab [34]. Since we could not foresee if macrophages exposed to strong M1-inducing cytokines would also be responsive to recombinant RNASET2 treatment, three different M1 polarization regimens (i.e., LPS/IFNγ, LPS alone and IFNγ alone) were applied to THP-1 cells in order to better tune the M1 polarization process. Moreover, a third M1 polarization marker (CCL-19) was included in our qPCR assays, whereas the classical M2-specific MRC1 marker was used in place of CCL17.

As shown in figure 9, most M1-polarizing stimuli led to the expected increase in the expression level of the corresponding M1 markers in the absence of recombinant RNASET2 (white bars, upper lane). As for the M2 polarization, we observed an effective increase of the CCL22 marker, whereas only a weak increase in the expression level of MRC1 was accomplished (white bars, lower lane).

Of note, treatment of *RNASET2*-silenced THP-1 cells with the recombinant protein led to a slight but consistent increase in the expression level of M1-like specific markers under most M1-polarizing regimens (arrow-marked black bars, upper lane). At the same time, treatment with recombinant RNASET2 led to a decrease of both M2 polarization markers, which was particularly evident for MRC1.

Thus, in keeping with the working hypothesis developed from our previous *in vivo* studies, exogenously added recombinant RNASET2 was also able to affect the polarization pattern of THP-1-derived macrophages, although the observed effect was not as marked as the one observed following manipulation of the *endogenous* RNASET2 levels. The putative reasons for such discrepancy are addressed in the Discussion section.

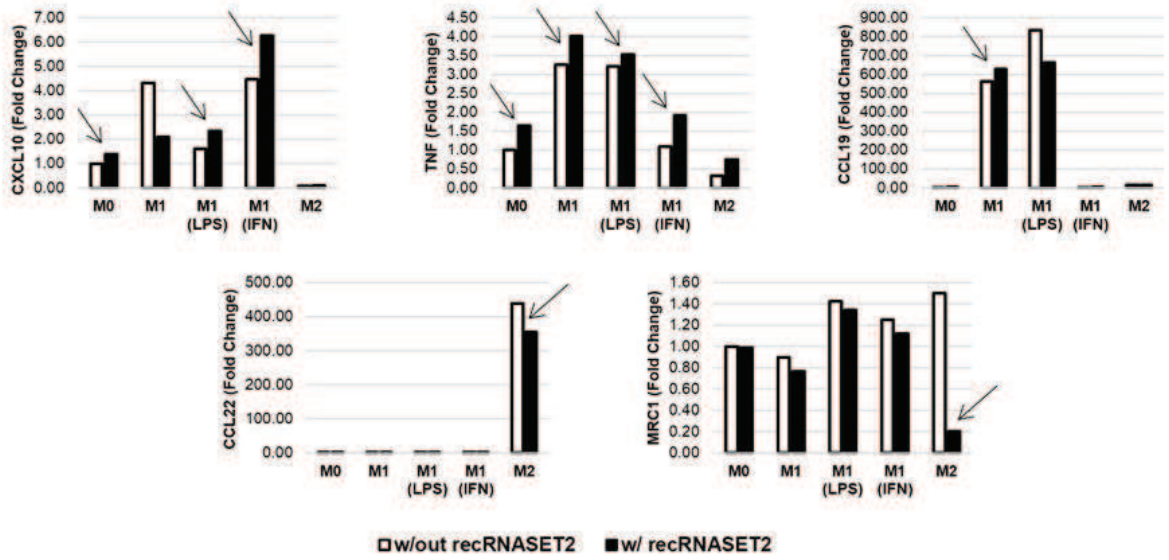

Figure 9. Real time qRT-PCR analysis for M1 and M2 polarization markers in *RNASET2*-silenced THP-1-derived macrophages in the presence or absence of recombinant RNASET2 protein.
Total RNA was isolated from *RNASET2*-silenced, M0, M1- or M2-polarized THP-1 macrophages grown in the presence or absence of recombinant RNASET2. The RNA was reverse transcribed, and a real time qRT-PCR assay was carried out to analyze the expression of 5 different markers of macrophages polarization, using GAPDH as an internal standard. Black arrows point to the experimental groups where treatment with recombinant RNASET2 resulted in an increase of M1 marker expression and a decrease in M2 marker expression. Gene expression levels are plotted as fold difference in mRNA expression versus THP-1 SH RNASET2 M0, not treated with recRNASET2, set as 1.0.M1 markers: CXCL10, TNFα and CCL19; M2 markers: CCL22 and MRC1. Image from: Scaldaferri et al. Immunol Lett. 2018.

Our data with the human THP-1 cell line strongly suggest that *RNASET2* affects the macrophage polarization pattern *in vitro*. To further validate these data, we carried out a preliminary investigation on freshly prepared human PBMC-derived macrophages. PBMC-derived human monocytes were isolated from buffy-coat samples from five independent healthy donors and the homogeneity of the isolated monocyte cell population was assessed by cytofluorimetric analysis, which showed that CD14-positive cells in all preparations represented more than 90% of the total cells (data not shown). The isolated monocytes were then differentiated *in vitro* to macrophages by culturing them in M-CSF-containing medium for 6 days. The effective differentiation of these cells into M0 macrophages was confirmed by both light microscopy and a qPCR assay showing an expected decrease in the expression levels of the CCR2 marker (figure 10).

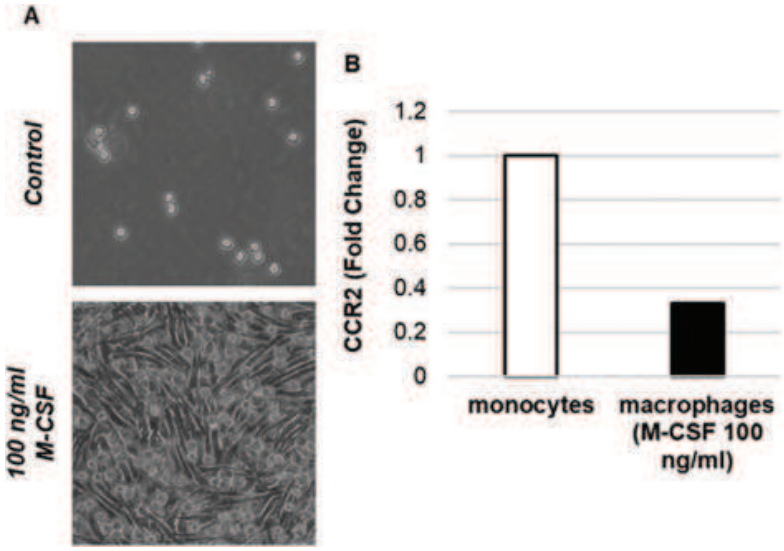

Figure 10. Assessment of the differentiation pattern in PBMC-derived human macrophages.
A) Morphological changes associated with M-CSF–induced monocyte/macrophage differentiation. Representative images are shown for M-CSF treatment (bottom panel) and for control (upper panel) at 20x magnification. B) Real time PCR analysis results showing the expected expression change of the macrophage differentiation marker CCR2 during macrophage differentiation. Image from: Scaldaferri et al. Immunol Lett. 2018.

To evaluate the putative occurrence of significant differences in the endogenous expression level of *RNASET2* among the different samples, a real time qPCR analysis was carried out to compare the expression profile of PBMC-derived monocytes and M0 macrophages from all donors with that of THP-1 cells. The endogenous *RNASET2* expression levels in the monocyte population from five donors turned out to be very similar to that observed in naïve THP-1 cells (figure 11).

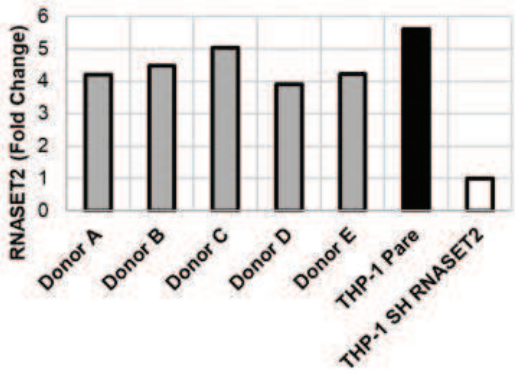

Figure 11. Real time qRT-PCR analysis for RNASET2 expression in THP-1- and PBMC-derived monocyte.
Total RNA was isolated from both parental/RNASET2-silenced THP-1 and PBMC-derived monocytes. The RNA was reverse transcribed and a real time qRT-PCR assay was carried out to analyze the expression of the RNASET2 gene, using GAPDH as an internal standard. Image from: Scaldaferri et al. Immunol Lett. 2018.

Therefore, the monocyte population from these five donors was selected for a preliminary evaluation of the effect of recombinant RNASET2 on their polarization pattern.

M0 macrophages were induced to polarize into M1 or M2 macrophages following treatment with IFN-γ/LPS or IL4, respectively, in the presence or absence of human recombinant RNASET2, as described in Materials and methods.

To evaluate the efficacy of the applied polarization procedure and the effect of RNASET2 treatment on cell polarization pattern, a real time PCR analysis was performed to evaluate the expression of two M1-specific (CXCL10 and TNF-α) and two M2-specific (CCL17 and MRC1) markers.

Unfortunately, in the macrophage populations derived from all five donors the expected pattern of M1 polarization following IFN-γ/LPS treatment was observed for one donor only (figure 12). These results are possibly attributable to the well-known issue of donor-to-donor variability inherent to human PBMC-derived monocytes.

Of note, when the effect of human recombinant RNASET2 administration was evaluated in IFN-γ/LPS treated monocytes, one of the five donors showed a marked increase of the CXCL10 marker expression following treatment with recombinant RNASET2 (figure 12). Moreover, when the expression of the same M1 polarization markers was evaluated in M2-polarized macrophages, a clear trend supporting an effect of recombinant RNASET2 was observed. Indeed, in all five donors both M1-specific markers (CXCL10 and TNF-α) showed a consistent trend toward an increase in their expression level in M2 macrophages following RNASET2 treatment (figure 12).

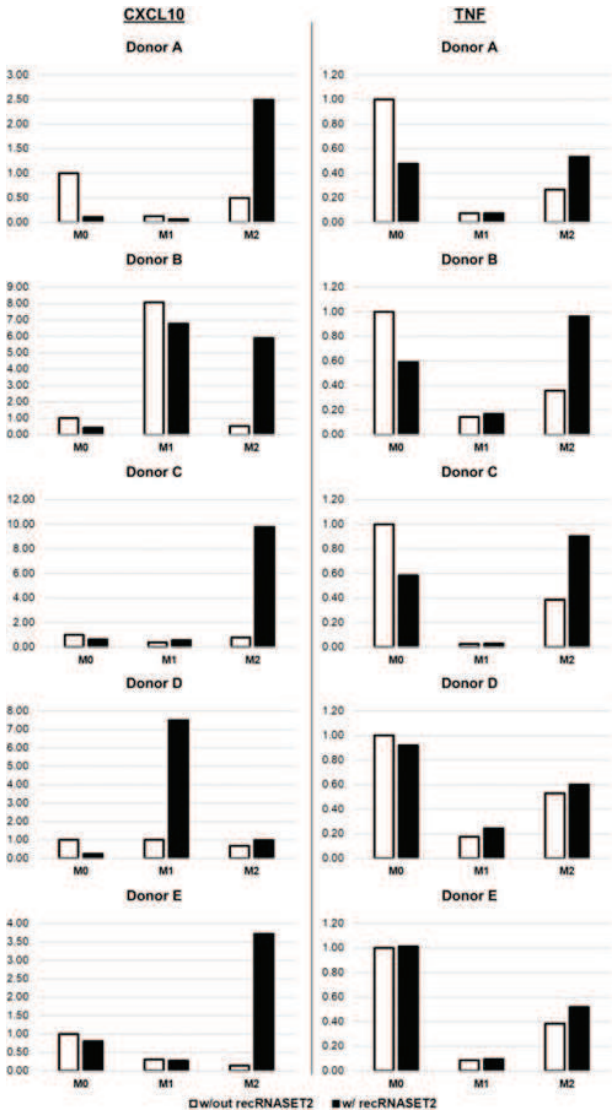

Figure 12. Real time qRT-PCR analysis for M1 polarization marker expression in PBMC-derived macrophages in the presence or absence of recombinant RNASET2 protein.
Total RNA was isolated from M0, M1- and M2-polarized macrophages from five independent donors, reverse transcribed as described in Materials and Methods and analyzed by real-time qRT-PCR assay to evaluate the expression of two different markers of M1 macrophages polarization (CXCL-10 and TNF-α) under each experimental condition, using GAPDH as an internal standard. Gene expression levels are plotted as fold-change differences in mRNA expression using macrophages M0 without recRNASET2 as a reference set as 1. Image from: Scaldaferri et al. Immunol Lett. 2018.

Thus, even though PBMC-derived macrophages did not show a proper M1 polarization response following IFN-γ/LPS treatment, recombinant RNASET2 was nevertheless associated with a significant increase of M1 specific markers in M2-polarized macrophages (figure 13).

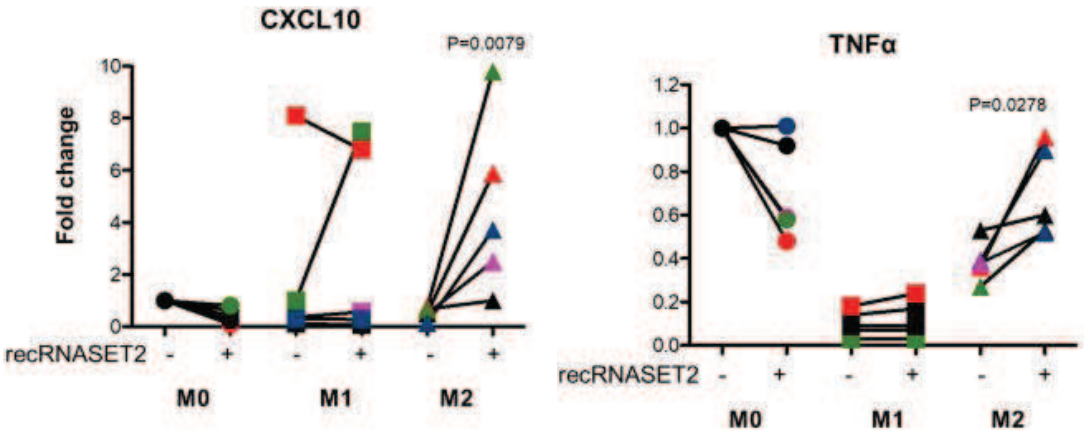

Figure 13. Increase in M1 markers in PBMC-derived M2 macrophages with recombinant RNASET2
Data in figure 5 plotted as donors with and without recombinant RNASET2, with a Mann-Whitney two tailed test was used to compare the data. A statistically significant increase in M1 markers in PBMC-derived M2 macrophages was apparent. Image from: Scaldaferri et al. Immunol Lett. 2018.

When we turned our attention on the expression profile of M2 polarization markers in PBMC-derived macrophages, although IL-4 treatment was highly effective in triggering a M2-polaritazion response in all samples, the effect of recombinant RNASET2 was very difficult to define. Indeed, MRC1 expression turned out to be largely unaffected in M2 macrophages, whereas CCL17 expression showed a marked donor-dependent trend for either an increase or a decrease in its expression level (data not shown).

These very preliminary experiments in PBMC-derived macrophages were therefore quite inconclusive and did not provide clear-cut results comparable to those observed in *RNASET2*-silenced THP-1 cells, likely due to the well-known phenomenon of donor-to-donor variability when using PBMC-derived cells. However, notwithstanding the intrinsic limitations of this approach, we were still able to observe a trend for an RNASET2 effect on PBMC-derived macrophages as well, prompting us to repeat the investigations on this *in vitro* polarization model following some improvements of the experimental plan, as described in the discussion section.

Part II: Interplay of RNASET2 with the tumor microenvironment in an immunocompetent mouse

So far, the *in vivo* experiments aimed at characterizing the oncosuppressive activity of *RNASET2* have been carried out by injecting immunocompromised mice with human ovarian cancer cells.
Xenograft-based assays represent a widely used experimental model in cancer research, given their potential to faithfully recapitulate several features of the carcinogenic process *in vivo*.
On the other hand, these models suffer from the key limitation of making use of animals whose immune system is inactivated to different extents.
Given the prominent role of both innate and adaptive immune systems in modulating the growth rate of cancer [6] and the results from our previous experiments, pointing to a functional crosstalk between RNASET2 and cellular components of the immune system, we decided to further investigate the oncosuppressive role of RNASET2 *in vivo* in the context of a totally immunocompetent experimental model.
This would represent a key task to expand our knowledge on the physiological mechanisms underlying *RNASET2*-mediated tumor suppression and at the same time to shed light on its putative interaction with all component of the host immune system.
Based on these premises, we therefore sought to investigate the role of *RNASET2* in a syngeneic mouse model.

ANALYSIS OF RNASET2 ENDOGENOUS EXPRESSION IN DIFFERENT MOUSE CELL LINES

To this end, we first assessed the endogenous expression levels of the orthologous Rnaset2 gene in different murine cell lines, by comparing them with multiple human cell lines routinely used in our lab (figure 14).

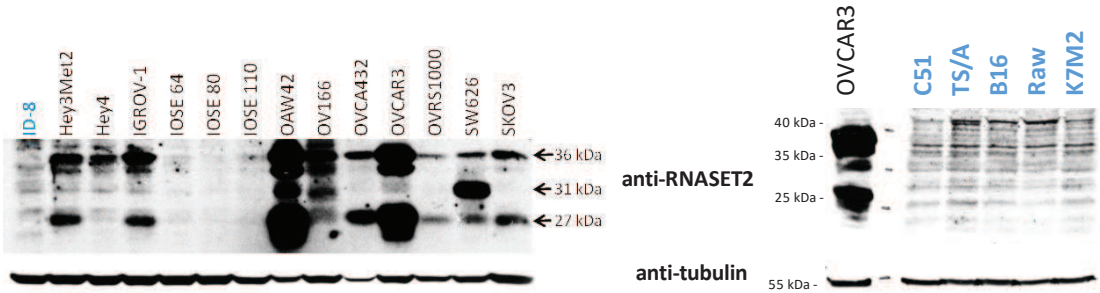

Figure 14. Immunoblot probed with polyclonal antibody anti-RNASET2 and anti-tubulin
Upper panel: intracellular lysates of different murine cell lines were analysed for RNASET2 expression. A very low level of endogenous expression was observed when compared with other cell lines commonly used in our lab. Lower panel: the same blot was probed with anti-tubulin antibody for normalization.

Quite unexpectedly, the expression of RNASET2 turned out to be quite low in all the murine cell lines tested.

Based on our previous experience with *RNASET2*-negative human cancer cell lines [16], we therefore decided to carry out transiently transfection assays to overexpress both human and murine RNASET2 proteins in three different murine cell lines (C51, ID8, TS/A) and one human cell line (Hey3Met2), using a plasmid bearing a constitutive promoter as described in Materials and Methods. To our surprise, whereas the human RNASET2 protein was efficiently overexpressed in all cell lines tested, the murine protein was never expressed (figure 15).

The putative inefficacy of our rabbit polyclonal anti-RNASET2 antibody (which had been raised against the *human* protein) to recognize the murine protein was ruled out, since we were able to easily detect murine RNASET2 by western blot analysis on protein extracts from two different adult murine tissues (heart and kidney, arrow-marked bands in figure 15, right panel).

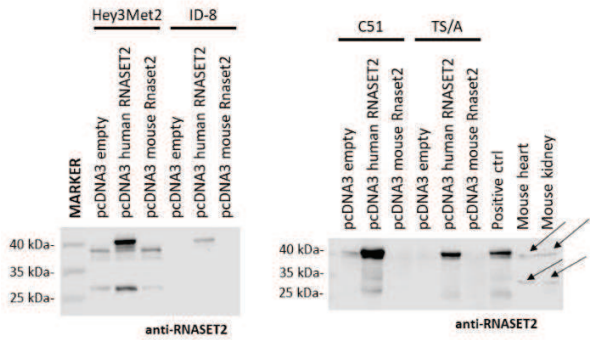

Figure 15. Immunoblot probed with polyclonal antibody anti-RNASET2
Total protein extracts from transiently transfected cells were analyzed for RNASET2 expression. In all four cell lines tested, the human RNASET2 protein was expressed whereas the mouse Rnaset2 was never detected. As a control, protein extracts from two murine tissues were loaded and the protein was efficiently detected (right panel).

Therefore, we hypothesized that, unlike human RNASET2, constitutive expression of the murine orthologous gene might result in a toxic or growth-suppressing effect on the cells.

EXPRESSION OF MOUSE RNASET2 USING AN INDUCIBLE EXPRESSION VECTOR.

We therefore decided to turn to an inducible expression vector bearing either the human or the murine cDNA. We bought a customized commercial vector bearing the mouse Rnaset2 cDNA and the genetic elements needed for inducible transgene expression with the Tet-on system. Moreover, we added a 3xFLAG tag-coding sequence at the 3' end of the Rnaset2 cDNA in order to detect the corresponding protein with a different antibody. Finally, we chose this vector to be a bicistronic construct, allowing co-expression of the reporter GFP protein with Rnaset2. This was meant to allow us to understand if the Rnaset2 transcript itself was expressed. As a control, we cloned the human RNASET2 cDNA in the same vector. A schematic representation of the vector is shown in Figure 4 of materials and methods.

The murine TS/A cell line was chosen for inducible RNASET2 expression assays, so we first transiently transfected the inducible plasmids into these cells. 24 hours after the transfection, RNASET2 expression was induced by doxycycline treatment for 24 hours. By checking expression of the GFP reporter using fluorescence microscopy, we observed only a few positive cells for both constructs (Figure 16a). Moreover, when we tested protein extracts from these cells by western blot, we could again observe the overexpression of the human protein only (Figure 16b).

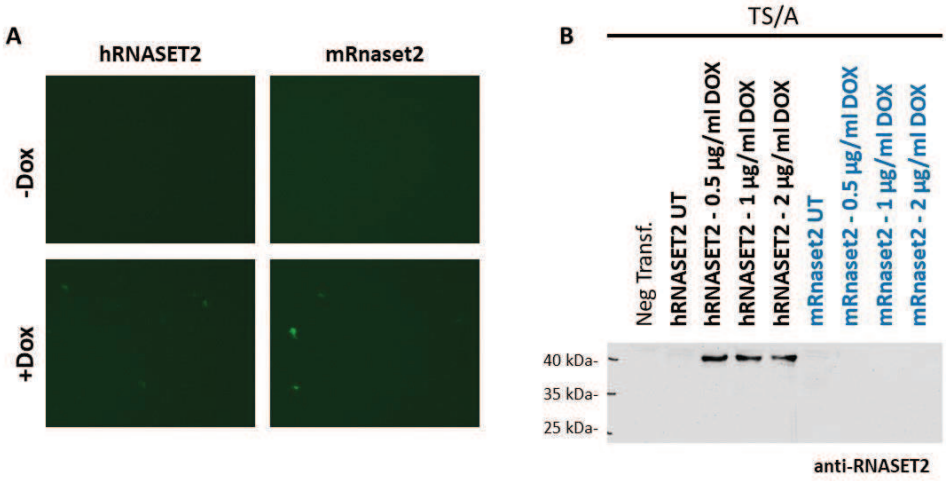

Figure 16. Analysis of TS/A cell line transiently transfected with inducible vectors bearing human or mouse RNASET2

The murine TS/A cell line was transfected with both the inducible vector for the expression for human RNASET2 and the same vector for the expression of the murine protein. After 24 hours, the expression was induced with doxycycline at three different concentrations. Cell were analyzed at the fluorescence microscopy and then protein extracts were produced and analyzed by immunoblot analysis. A) Representative images of positive GFP cells in the lower panel. B) Total protein extracts were analyzed for RNASET2 expression: cells transfected with the plasmid bearing the human cDNA expressed the protein at all the concentrations, whereas no expression was found when transfected with the mouse-bearing one.

We nevertheless went on to stably transfect the cells, in order to enrich for cellular pools bearing the inducible plasmids and then induce them.

Since the vectors carry a neomycin resistance gene as a selectable marker for eukaryotic cells, we tested the toxicity of the selective antibiotic G418 on TS/A cells before transfection. Once the optimal G418 concentration to be used for clone selection was estimated to be 300 µg/ml, we proceeded with stable transfection. The transfected cells were subsequently detached from the 6-wells plate, re-suspended in fresh medium supplemented with the selective agent G418 and seeded. Following a 14-days period of selection, we obtained a pool of G418-resistant clones that were induced for 48 hours with Doxycycline at 1 µg/ml of concentration.

Although the observation of cells under fluorescence microscopy confirmed the functionality of the inducible system for both recombinant constructs, a phenomenon of "leakiness" emerged in

untreated cells, which showed a weak expression of GFP even in absence of the inducer (figure 17).

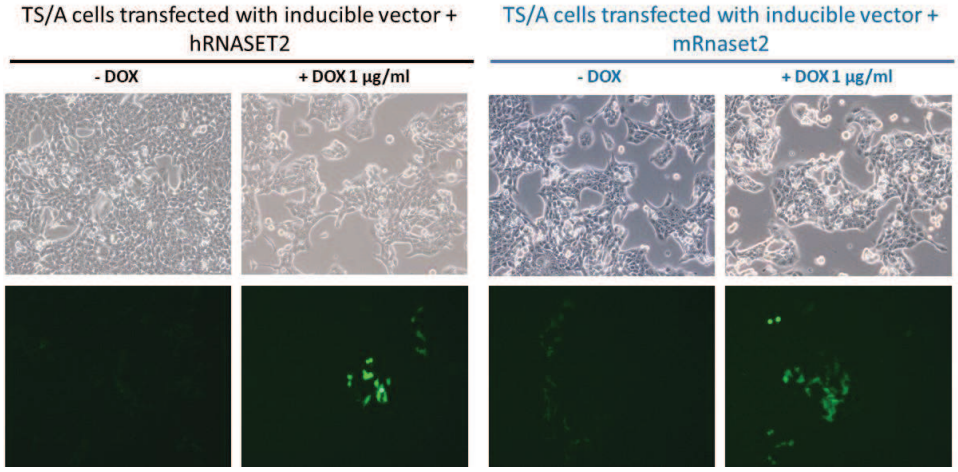

Figure 17. Analysis of GFP expression in TS/A cell line stably transfected with inducible vectors
The murine TS/A cell line transfected with the inducible vectors bearing the human or mouse RNASET2 cDNA were induced with doxycycline for 48 h. Cell were analyzed at the fluorescence microscopy: GFP positive cells are present in both the pool of clones, but a weak leaking expression is also visible in untreated ("-DOX") cells.

This phenomenon, which was not observed following transient transfection, was confirmed by western blot analysis using both an anti-RNASET2 and anti-FLAG antibody (figure 18) and by FACS analysis (figure 19).

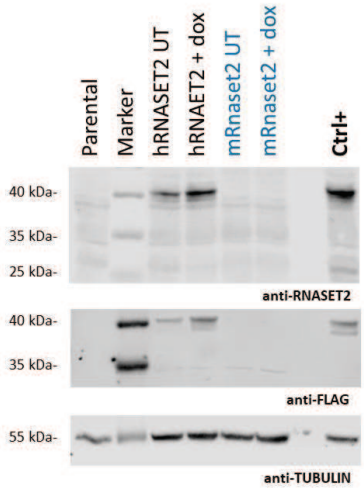

Figure 18. Immunoblot analysis of RNASET2 expression in pool of clones of the TS/A cell line stably transfected with inducible vectors
Total protein extracts from transfected clones were analyzed for the expression of RNASET2. Again, only cells transfected for the human RNASET2 protein were positive.
An anti-FLAG antibody was also used and the result is the same: the mouse protein isn't expressed. Unfortunately, a minimal expression of RNASET2 is also present in untreated cells. Specific signals were normalized using α-tubulin.

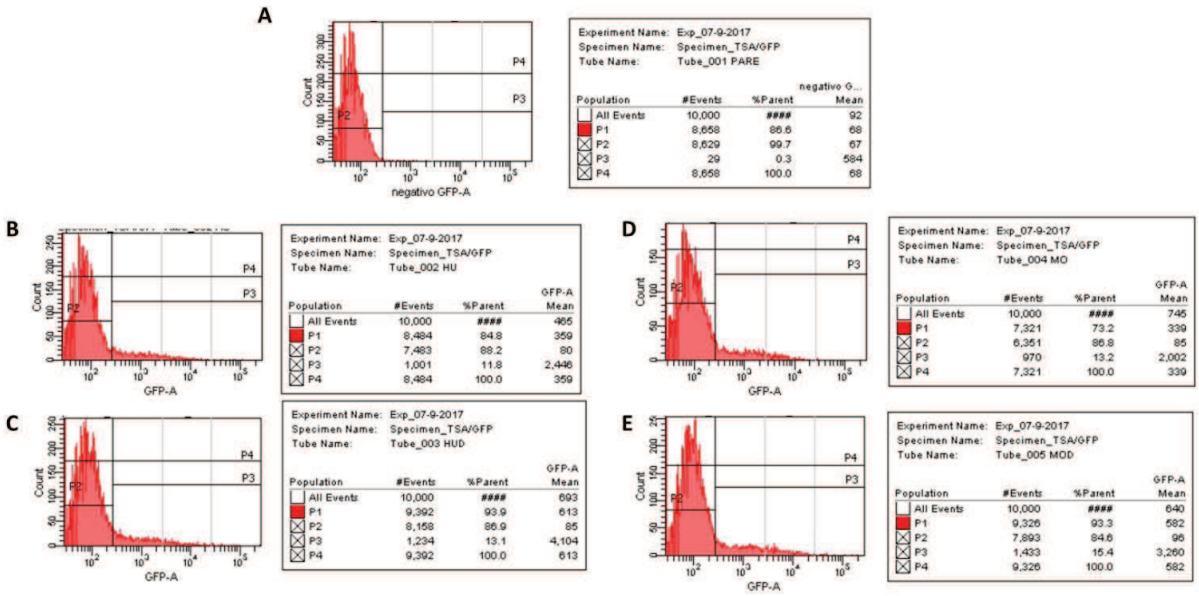

Figure 19. Cytofluorimetric analysis of GFP positive cells in TS/A parental cells and pool of clones
Pool of clones were analyzed for percentage of GFP positive cells. Differences between untreated and treated cells were very small, probably due to the leaking expression phenomenon. A) TS/A parental; B) TS/A inducible vector with hRNASET2 untreated; C) TS/A inducible vector with hRNASET2 treated with doxyxicline; D) TS/A inducible vector with mRnaset2 untreated; C) TS/A inducible vector with mRnaset2 treated with doxyxicline.

Most importantly, besides the vector leakiness problem, the expression of mouse Rnaset2 was still not detectable by western blot analysis using both anti-RNASET2 and anti-FLAG antibodies (with the latter specifically used to recognize with the same efficiency the human and mouse proteins).

On the basis of these results, we decided to move our expression analysis at the transcription level. Total RNA from stably transfected pools of clones was extracted, reverse transcribed into cDNA and used for a qPCR analysis with primers designed on the 3xFLAG coding sequence.

As shown in figure 20A, we observed a 2-fold expression increase in cells treated with doxycicline at 1 µg/ml for 48 h. Interestingly, this increase, though weak, was observed in cells transfected with both the human and murine RNASET2 expression vectors. We considered this as an indication that the murine transcript was properly produced with this system, although we were not able to detect its expression at the protein level.

As a further evidence that the RNASET2 transcripts were expressed and intact, we carried out a classical RT-PCR on the same cDNAs with primers crossing the IRES region, with the forward primer designed on the RNASET2 3xFLAG sequence and the reverse primer on the GFP sequence. In keeping with our previous RT-PCR data, the bicistronic transcript was observed in all the samples (figure 20b).

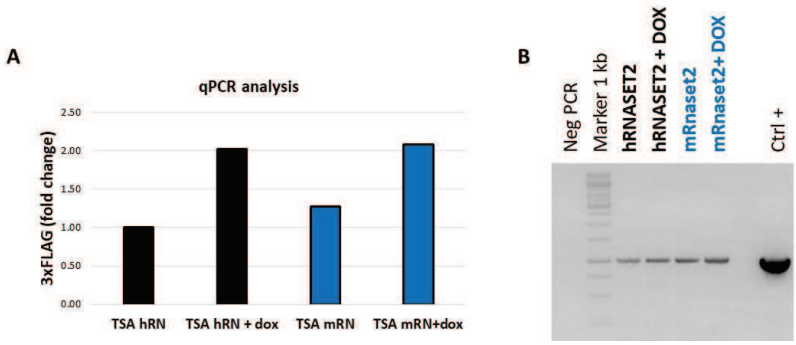

Figure 20. qRT-PCR analysis of the expression levels of the transcripts and classical PCR with primers spanning the IRES region

Total RNA was isolated from TS/A clones untreated or treated with doxycycline, reverse transcribed and used for both a quantitative PCR with primers designed on the 3xFLAG coding sequence and a classical PCR to verify the integrity of the transcript cDNA-IRES-GFP. A) Gene expression levels are plotted as fold difference in mRNA expression versus untreated TS/A transfected with the inducible vectors bearing the human cDNA set as 1.0. The addition of doxycycline increased the expression of the transcript, even though of only 2-fold, in cells transfected both for the human or mouse protein. B) Further evidence supporting the presence and integrity of the transcript were provided by a classical PCR using a forward primer on the region of the transcript before the IRES sequence and a reverse primer on the region after that sequence: the expected PCR amplicon was detected in all samples.

Taken together, these data seemed to suggest the occurrence of a functional difference of murine *versus* human RNASET2, at least in the context of *in vitro* transfection assays.

To exclude a cell-line specific effect for this unexpected observation, a second murine cell line (C51) was transiently transfected with both plasmids but the results were still consistent with overexpression of the human protein only (data not shown).

Overall, these data confirmed the difficulties in expressing the murine Rnaset2 protein in these experimental models and, at the same time, suggested that the underlying problem could reside at the protein rather than at the transcript level.

PROTEIN SWAP APPROACH

In the light of the observed discrepancy in the expression efficiency between the human and mouse T2 RNase protein, we turned to a "protein domain swap" approach in the attempt to map the putative region from murine Rnaset2 responsible for this biological difference.

For this purpose, we aligned the primary sequence of the human RNASET2 protein with the sequences of the two mouse Rnaset2 (A and B) isoforms (the latter derive from two different genes whose coding sequence are identical). The human and mouse proteins show a 67% identity and 79% similarity. However, a putative TRAF-binding motif (PKQE), which has been reported to be potentially involved in RNASET2-mediated cell apoptosis [37], was not detected in the orthologue mouse protein (figure 21).

We therefore decided to break the human protein at the 219 aa residue, therefore excluding the TRAF2 binding domain that is absent in the mouse protein. Reciprocally, we interrupted the mouse protein at the 223 aa, as described in figure 21.

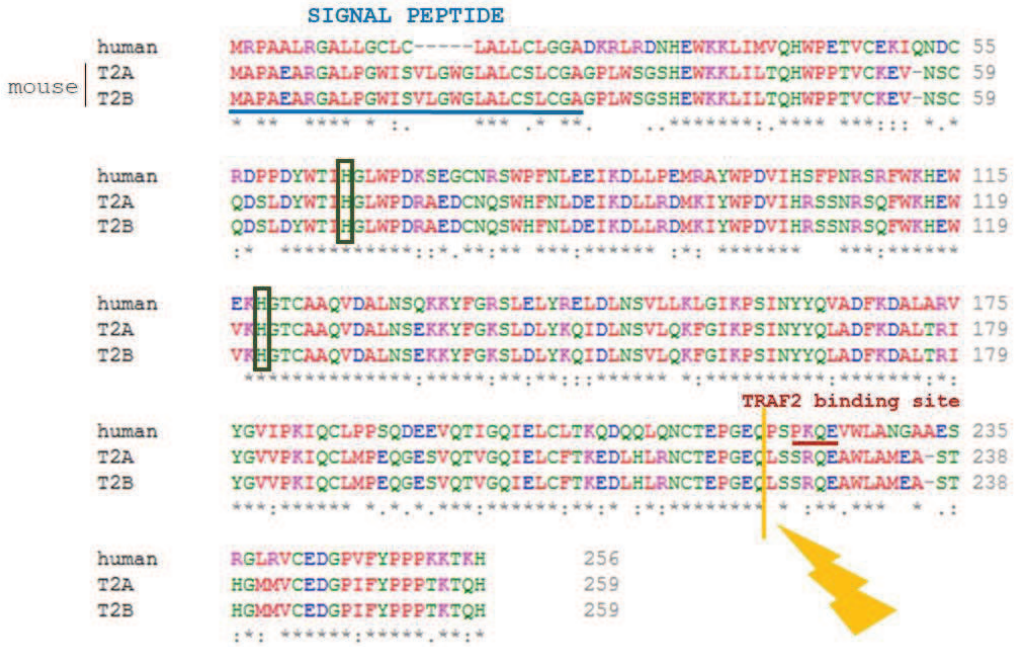

Figure 21. Primary sequences alignment of the human RNASET2 with the mouse Rnaset2A and Rnaset2B
The three sequences were aligned using the ClustalOmega program. Signal peptides, CAS sites (represented by the two histidines in the green boxes) and the TRAF2 binding site are indicated. The thunderbolt shows the chosent breakpoint.

Besides the issue concerning the TRAF-binding site, the decision to interrupt both proteins in that point was based on their tertiary structure as well. Indeed, although bioinformatics prediction tools suggest that the mouse protein should have a quite similar structure to the human one, the C-terminal region, which include the putative TRAF2 binding site, appear to be the most unstructured and disordered and it should accordingly withstand a domain swap manipulation (figure 22) [38].

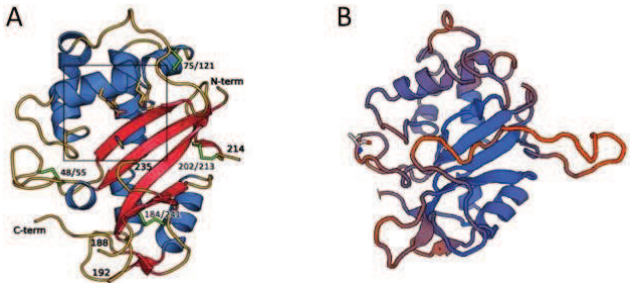

Figure 22. 3D structures of the human RNASET2 and the mouse Rnaset2 proteins
The 3D structure of the human RNASET2 protein (A) has been determined by X-ray cristallography [38 – Image from Nucleic Acids Res. 2012 Sep; 40(17): 8733–8742], whereas the mouse protein structure (B) has been predicted by the SWISS model bioinformatic tool.

Since we could not successfully address the problem of absent murine RNASET2 expression using an inducible expression vector, we turned back to the constitutive pcDNA3 expression vector, which has been successfully used in our lab for most RNASET2-overexpression assays in the past. Following the addition of a 3xFLAG coding sequence in the vector's backbone (see Materials and Methods) we cloned into this vector five independent RNASET2 coding sequences:

(1) a full-length human RNASET2 cDNA

(2) a full-length mouse Rnaset2 cDNA

(3) a truncated version of mouse Rnaset2 coding region, spanning aa 1-223

(4) a chimeric version with the *human* RNASET2 sequence including aa 1-219 followed by the *murine* protein sequence spanning aa 224-259 ("*N-ter human/C-ter mouse*")

(5) a complementary chimeric version, with the *murine* RnaseT2 sequence spanning aa 1-223 followed by the human protein sequence spanning aa 220-256 ("*N-ter mouse/C-ter human*").

A schematic representation of these five expression plasmids, assembled following the protocol described in materials and methods, is shown in figure 5.

All these recombinant constructs were transiently transfected into the TS/A cell line. After 24 hours, total protein extracts were prepared, quantified and analyzed by western blot. Using both the anti-RNASET2 and anti-FLAG antibody, we detected a strong signal for the human full-length and a weaker signal for the chimeric *N-ter human/C-ter mouse* proteins, respectively (figure 23). This preliminary result seemed to point to the mouse N-terminal region as the one responsible for the previously observed lack of expression.

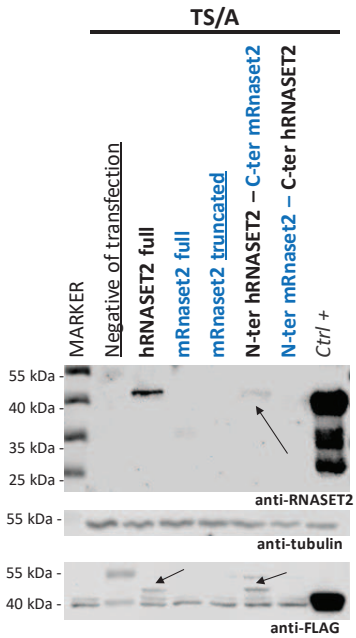

Figure 23. Western Blot analysis of TS/A transiently transfected
Total protein extracts were analysed for RNASET2 expression. The anti-FLAG antibody was used as further analysis. In both cases, we observed positivity only for the cells transfected with the vector bearing the full-length human RNASET2 and with the vector bearing the chimeric region N-ter human/C-ter mouse. A Ponceau S staining is reported to show the correct balance and transfer of the proteins.

On the basis of these data, we decided to produce stably transfected pools from both C51 and TS/A cell lines and to subsequently analyze RNASET2 expression on both protein extracts and supernatants.

As show in figure 24, switching from transient to stable expression assays substantially confirmed the previous data, since a clear expression signal was mainly observed for both the human full-length protein and the *N-ter human/C-ter mouse* chimeric protein in TS/A cells. However, a faint band corresponding to the *N-ter mouse/C-ter human* was observed in both cell extracts and supernatants as well. Interestingly, a weak signal was also observed in cells transfected with the vector bearing the mouse truncated cDNA (arrow mark).

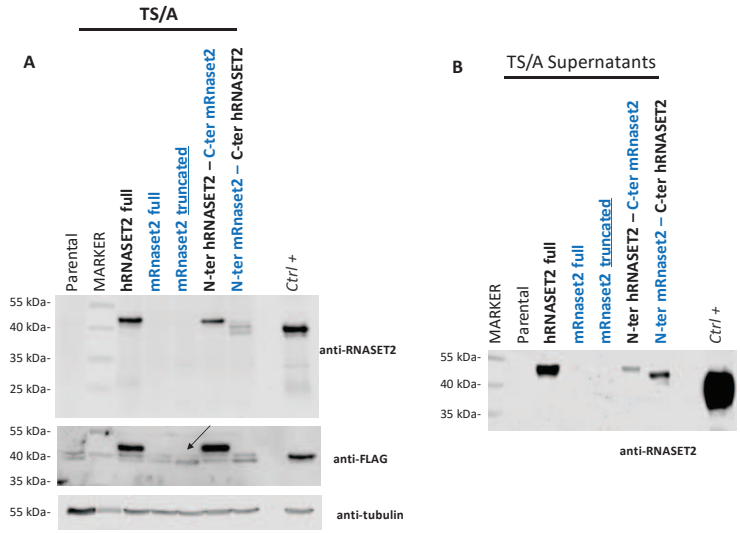

Figure 24. Western Blot analysis of TS/A stably transfected pools
(A) Total protein extracts were analysed with anti-RNASET2 and anti-FLAG antibodies. In both cases, we observed clear positivity for cells transfected with the vector bearing the full-length human RNASET2 and with the vector bearing the chimeric region N-ter human/C-ter mouse. Moreover, a weaker band was observed for cells transfected with N-ter mouse/C-ter human cDNA. (B) The analysis of the supernatants with the anti-RNASET2 antibody confirmed the results.

When the same vector series was used to transfect the C51 cell line the results were quite comparable, except for the detection with the anti-FLAG antibody of a band corresponding to truncated version of the mouse Rnaset2 (figure 25, arrow).

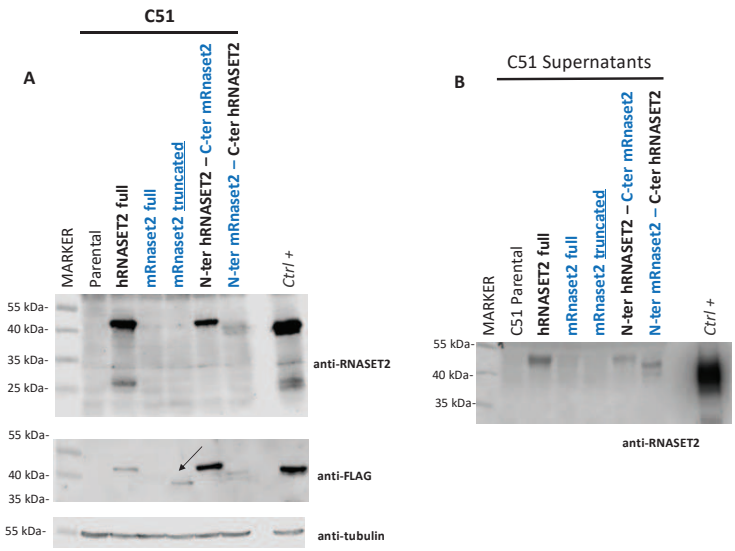

Figure 25. Western Blot analysis of C51 stably transfected pools

(A) Total protein extracts were analysed with anti-RNASET2 and anti-FLAG antibodies. In both cases, we observed clear positivity for cells transfected with the vector bearing the full-length human RNASET2 and with the vector bearing the chimeric region N-ter human/C-ter mouse. Moreover, a weaker band was observed for cells transfected with N-ter mouse/C-ter human cDNA. Interestingly, the anti-FLAG antibody revealed a weak band also for cells transfected with the truncated version of the mouse Rnaset2. (B) The analysis of the supernatants with the anti-RNASET2 antibody were consistent with that of TS/A cell line pools.

A quantitative PCR analysis carried out on all transfectants showed a slight decrease of mouse full-length and truncated transcripts (figure 26), but this result was apparently not sufficient to explain the marked difference observed by western blot analysis, once again suggesting the occurrence of a post-transcriptional event to explain the absent or reduced expression of the murine protein.

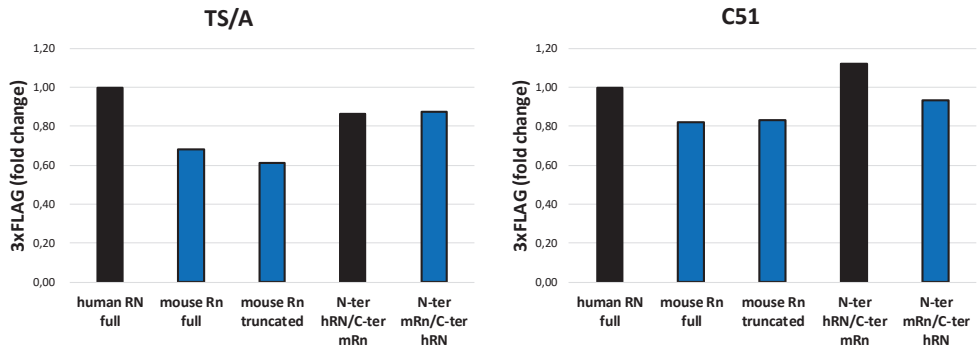

Figure 26. qRT-PCR analysis of the expression levels of the transcripts in TS/A and C51 pools

Total RNA was isolated from TS/A and C51 pools of clones, reverse transcribed and used for both a quantitative PCR with primers designed on the 3xFLAG coding sequence. Gene expression levels are plotted as fold difference in mRNA expression versus cells transfected with the human cDNA set as 1.0.

Taken together, the data from our transfection series confirmed the extreme difficulty to express a full-length Rnaset2 protein in murine cells. Unfortunately, we were not able to unambiguously

assign such phenomenon to a particular region of the murine protein, since we could observe expression of both the *N-ter human/C-ter mouse* and *N-ter mouse/C-ter human* chimeric proteins, although at a quite weak level when compared to the human full-length protein.

However, the observation of a weak expression of the mouse truncated protein suggested that some cells could express even the full-length murine Rnaset2, although to a weaker level compared to human RNASET2, and that by using pool of transfected cells such putative weak expression could pass unnoticed.

We therefore decided to generate single murine Rnaset2-transfected stable clones in both murine cell lines and to screen a very high number of clones in the hope to obtain a few murine Rnaset2-expressing clones.

Indeed, as shown in figures 27 and 28, following an intensive screening of stably transfected C51 and TS/A clones (70 and 60 clones), we were finally able to select a few clones expressing low to moderate levels of both truncated and full-length murine Rnaset2.

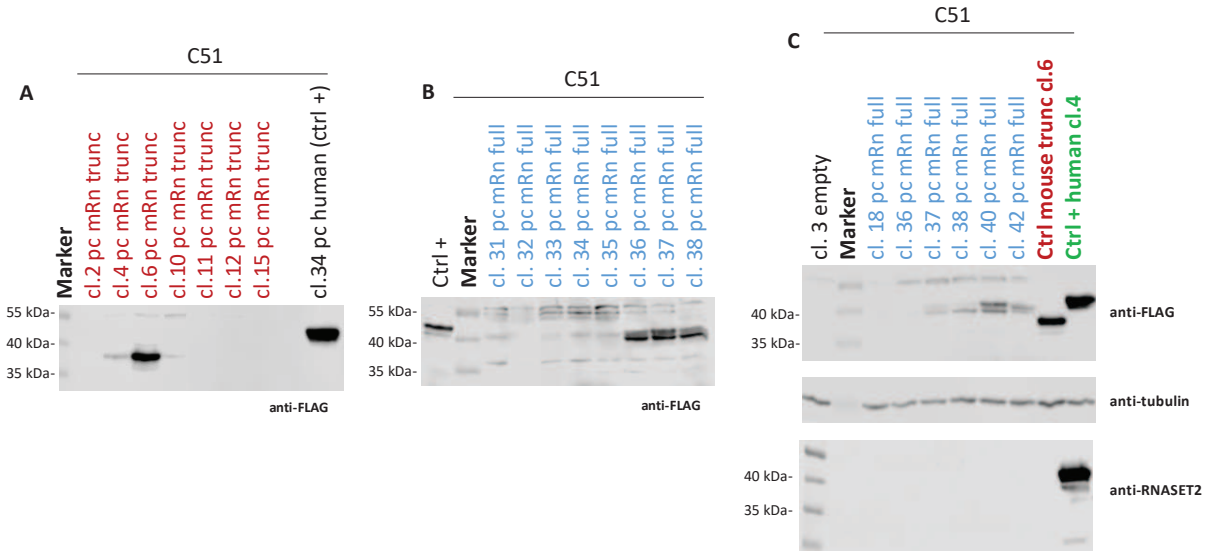

Figure 27. Western blot analysis of C51 single stable clones
Total protein extracts were analysed with anti-FLAG antibodies. Representative images for screening of clones overexpressing the mouse truncated (A) or full-length (B) protein. When some clones were positively selected, the anti-humanRNASET2 antibody was also used for analyzing the same clones but we observed an inability of the antibody to detect the mouse protein.

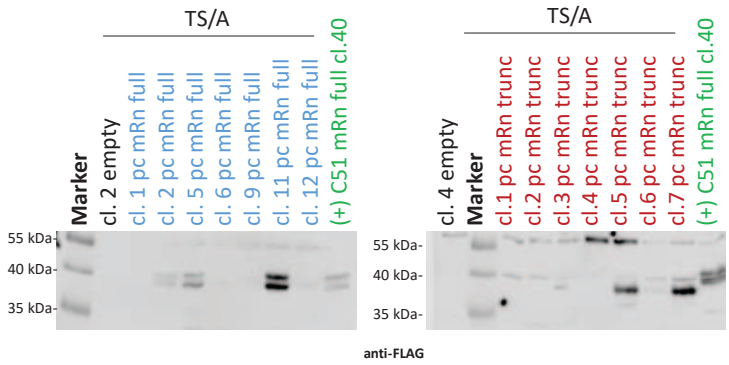

Figure 28. Western blot analysis of TS/A single stable clones
Total protein extracts were analysed with anti-FLAG antibodies. Representative images for screening of clones overexpressing the mouse full-length (left panel) or truncated (right panel) protein.

The overexpressing clones were also analysed with the anti-RNASET2 antibody, but unfortunately, this antibody has not the same efficiency to detect the mouse orthologue protein as to detect the human one (figure 27c).

We next decided to test murine Rnaset2-expressing clones (both truncated or full) for their *in vitro* cell proliferation rates. As shown in Figure 29 (left panel), a significant decrease in cell growth was observed for C51 cells overexpressing the full-length murine protein when compared to the control cells. Interestingly, the decrease in cell growth was slighter, and not statistically significant, in C51 cell clones expressing the truncated protein.

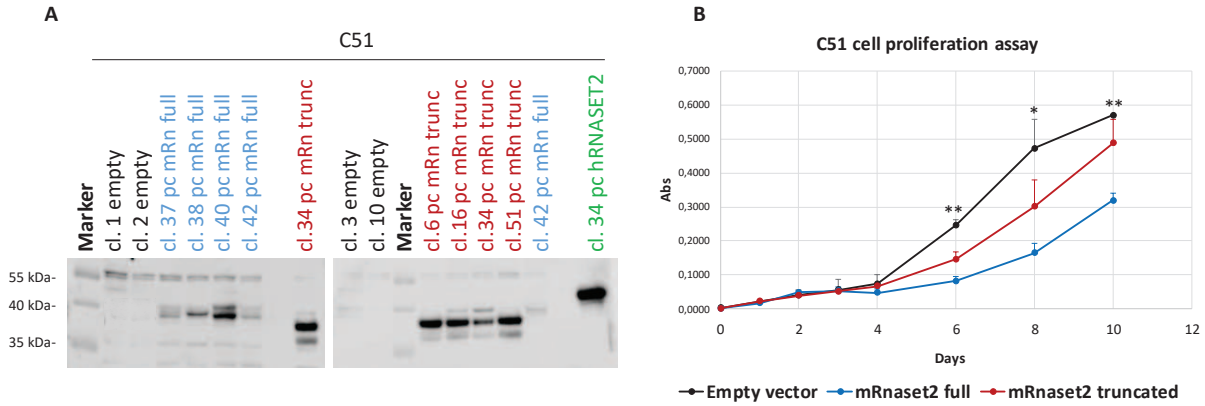

Figure 29. Analysis of cell proliferation assay and western blot analysis of C51 single stable clones
(A) Immunoblot analysis of mouse Rnaset2 overexpressing C51 cell clones with anti-FLAG antibody.
(B) MTT assay: four clones for each experimental group were analyzed. An ANOVA statistical analysis was performed assuming $p<0.05$ as a threshold value.

When the same analysis was performed with TS/A clones, the same trend was observed, with mRnaset2 full length protein overexpression correlating with a modest decrease in proliferation rate, even though the observed difference was not statistically significant (figure 30).

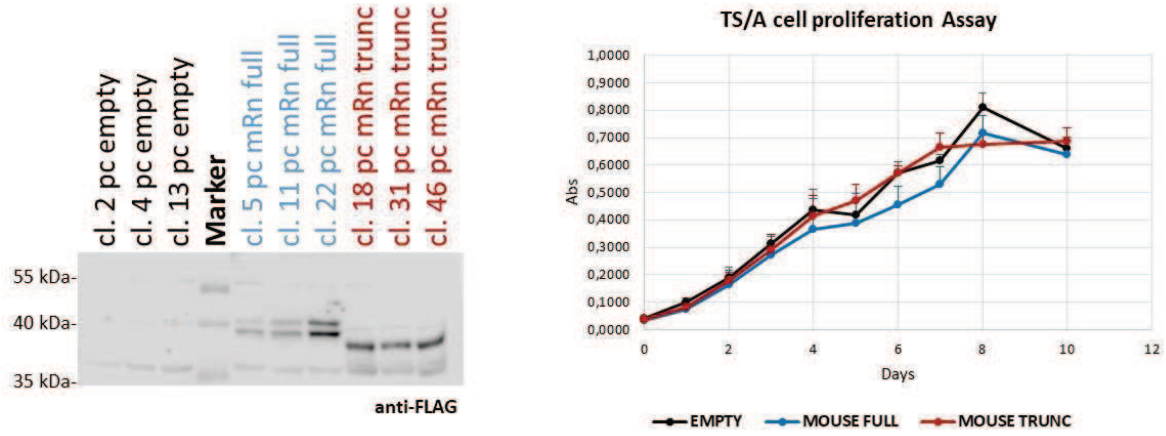

Figure 30. Analysis of cell proliferation assay and western blot analysis of TS/A single stable clones
(A) Immunoblot analysis of mouse Rnaset2 overexpressing TS/A cell clones with anti-FLAG antibody.
(B) MTT assay: three clones for each experimental group were analyzed.

Finally, to further investigate the role of murine RNASET2, we performed a preliminary *in vivo* assay. Balb/c mice were injected subcutaneously with Rnaset2-expressing C51 clones previously investigated *in vitro* (one clone for each experimental group) and the tumor growth rate was followed for up to 18 days. Interestingly, large tumours developed in mice inoculated with empty vector-transfected cells or with vector expressing the truncated mRnaset2 form, whereas cells expressing the wild-type full-length form of mRnaset2 were clearly suppressed in their tumorigenic potential (figure 31).

Though preliminary, these data represent the first experimental evidence supporting the *in vivo* tumor suppressive role for the RNASET2 protein in the context of a perfectly competent immune system.

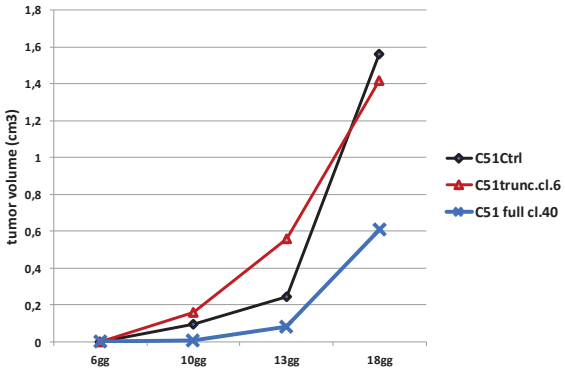

Figure 31. Analysis of *in vivo* tumor growth rates of C51 cell clones
One clone for each experimental group was inoculated s.c. in nude mice as described in Materials and Methods. Tumor growth was monitored every 2 d until day 40. At least three mice were inoculated for each tested clone.

Part III: RNASET2 in the context of mammary morphogenesis and tumorigenesis

So far, the oncosuppressive activity of the RNASET2 gene (both *non-cell autonomous* and *cell-autonomous*) has been mainly investigated in ovarian carcinoma models. However, since the *RNASET2* gene maps to the 6q27 chromosomal region, which is reported to be frequently deleted or rearranged in several solid and hematological cancers, an interesting issue for future investigations concerns the role of *RNASET2* in other cellular models of cancer, such as the mammary epithelium. Of note, a preliminary analysis of breast cancer relapse-free survival (RFS) data (Kaplan-Meyer plots) from the TCGA breast cancer patients' collection showed a trend for a positive association between RNASET2 expression levels and higher RFS for both the total breast cancer sample and some cancer subtypes (figure 32), suggesting that RNASET2 expression might be involved in breast cancer as well.

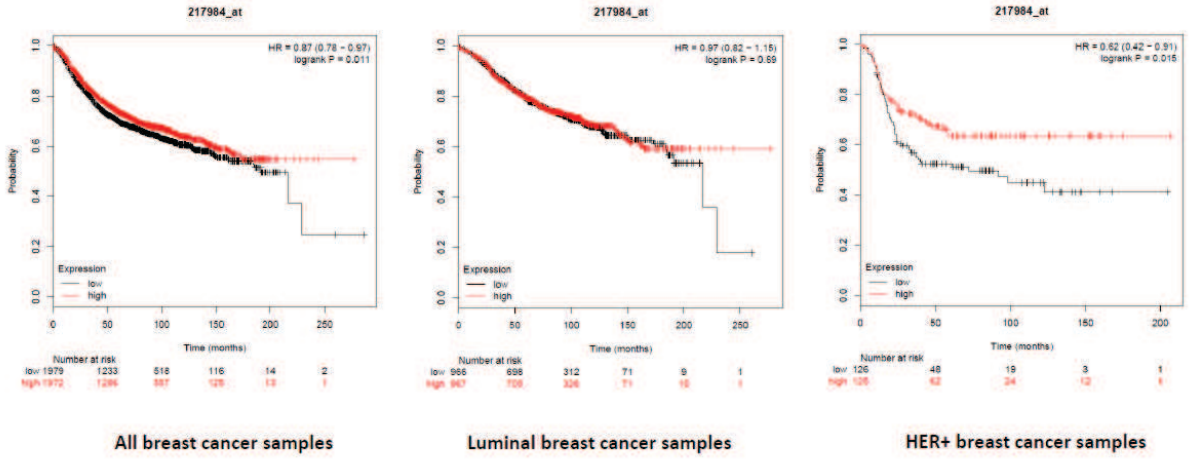

Figure 32. Kaplan-Meyer plots for Relapse-Free Survival (RFS) in breast cancer patients according for RNASET2 expression level

The three curves show the RFS plots from the TCGA database for total breast cancer sample (left panel), Luminal-A breast cancer patients (middle panel) and HER+ cancer patients (right panel).

Moreover, given the widely acknowledged pleiotropic roles reported for members of the T2 Rnase family, investigations on RNASET2's tumor suppressive role in an independent cancer model might shed light on previously unrecognized mechanisms by which this versatile class of proteins operate in cancer cells.

Within this frame, the human breast cancer cell line MCF7 was therefore chosen as an experimental model. Interestingly, analysis of RNASET2 expression in this cell line by western blot analysis showed that, compared to other cell lines previously used in our laboratory, MCF7 cells display very low levels of RNASET2 endogenous expression (figure 33). These findings prompted us to generate human RNASET2-overexpressing MCF7 clones, which were later used for further investigations.

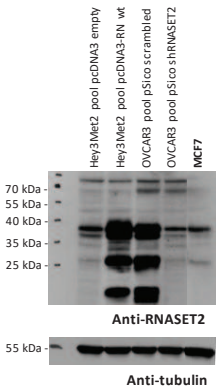

Figure 33. Immunoblot probed with polyclonal antibody anti-RNASET2
Upper panel: intracellular lysate of MCF7 cell line was checked for RNASET2 expression. A very low level of endogenous expression is shown compared with other cell lines. Lower panel: the same blot was probed with anti-a-tubulin polyclonal antibody for normalization.

2D *IN VITRO* ASSAYS

We therefore proceeded to stably transfect MCF7 cells with our constitutive human RNASET2 expression vectors. The transfected cells were subsequently put in medium with a selective agent. Following a 2-weeks period of selection, single clones were picked-up, expanded and analyzed by western blot with an anti-RNASET2 antibody. On the basis of the observed expression levels, three clones from each experimental group were chosen for further investigations (figure 34).

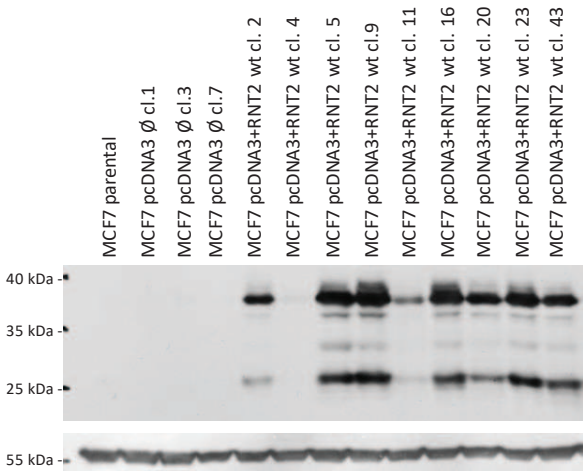

Figure 34. Immunoblot probed with polyclonal antibody anti-RNASET2
Total protein extracts of MCF7 clones were checked for RNASET2 expression. Specific RNASET2 signals were normalized using a-tubulin.

Once RNASET2-overexpressing clones were isolated, we first carried out a panel of standard cancer-related *in vitro* assays, such as cell proliferation and colony formation.

By comparing the *in vitro* cell proliferation rates of RNASET2-overexpressing clones to that of control clones transfected with the empty vector (MCF7 pcDNA3 empty) a slight decrease in the proliferation rate of RNASET2-overexpresing clones was observed, with no statistical difference (figure 35a).

This result was not completely unexpected, being in keeping with experimental data previously observed in our lab with some human ovarian cancer-derived cell lines.

We then assessed the ability of the same clones to form colonies in culture dishes. Strikingly, this parameter was significantly decreased in RNASET2-overexpressing clones with respect to control clones, as shown in figure 35b.

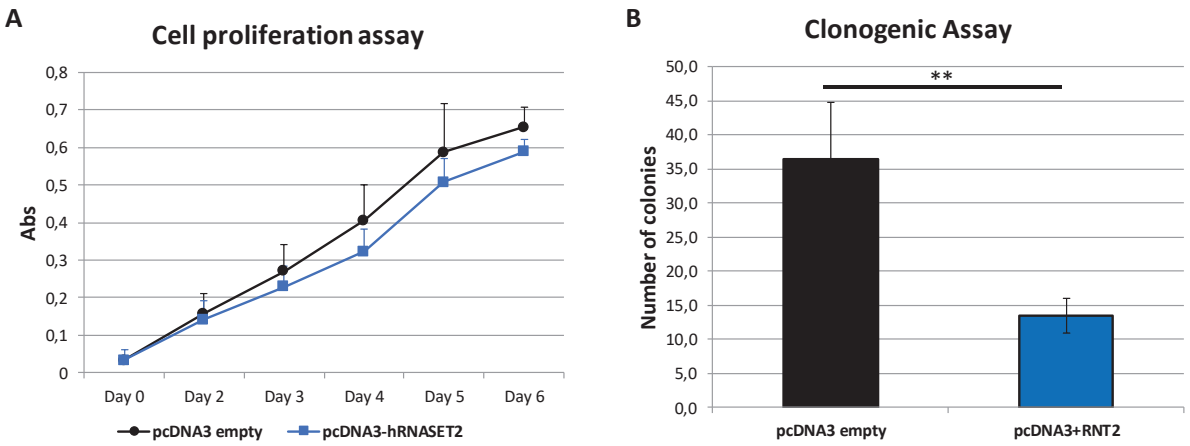

Figure 35. Analysis of cell proliferation rates and colony formation ability

(A) A cell proliferation assay was performed in RNASET2-overexpressing clones and control cell clones using an MTT assay as described in *Materials and Methods*. Triplicate experiments were performed with three clones of each experimental group. Mean values of the three clones of pcDNA3 empty clones and pcDNA3-RNASET2 overexpressing clones. A slight variability in their proliferation rate was observed, but there was no statistical difference.

(B) RNASET2-overexpressing MCF7 cell clones generated a smaller number of colonies than control cell clones in colony formation assay. Experiments were carried out on three independent clones for each experimental group and then mean value were considered. Mean value of control clones significantly differs from both RNASET2-overexpressing clones.

We next decided to test whether RNASET2 overexpression could also affect apoptosis in MCF7 cells. As shown in figure 36, in the absence of any particular apoptotic induction, RNASET2-overexpressing cells showed a slight increase in the number of apoptotic cells when compared

to control cells, although the fraction of apoptotic cells was very small in both samples. Again, these data are in keeping with those previously obtained in the ovarian cancer-derived OVCAR3 cell line [24]. To better investigate the role of RNASET2 in the induction of apoptosis, we then challenged cell clones with 200 µM $CoCl_2$: strikingly, an increased apoptotic rate was observed in RNASET2-overexpressing cell clones, although the effect was quite weak. Moreover, when cells were challenged with a non-physiological apoptogenic stimulus (100 nM cis-platinum) the percentage of apoptotic cells increased in both control and RNASET2-overexpressing cells, but the trend for an increased apoptotic rate in the latter cells was still detected.

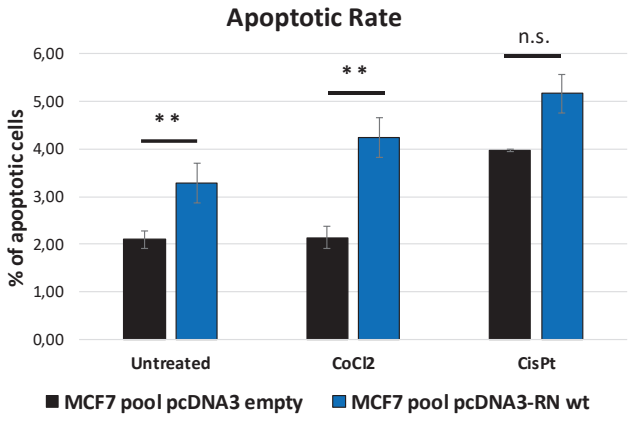

Figure 36. Analysis of cell-death rate in response to pro-apoptotic stimuli.
Flow cytometry analysis was performed in RNASET2-overexpressing MCF7 cell clones with respect to control clones stained with propidium iodide, following a 24-hours treatment with either cobalt chloride or cis-platinum. In all of the three experimental conditions, RNASET2-overexpressing clones showed a percentage of apoptotic cells significantly higher than control clones. Three technical replicates were performed. Statistical analysis was performed using t-test student (assuming $p<0.05$ as a threshold value to discard the null hypothesis.

RNASET2 CYTOPLASMIC LOCALIZATION AND PHALLOIDIN STAINING

In a previous work carried out in ovarian cancer cells, a RNASET2-dependent cytoskeleton reorganization was reported [24]. Of note, the effect of RNASET2 on the cell cytoskeleton seem to represent an evolutionary conserved feature, since it has been reported for human RNASET2 and for its two orthologues Omega-1 and ACTIBIND as well [20,25]. We thus investigated the cytoskeletal organization in our panel of MCF7 clones.

The observed actin staining profile was indicative of a complex network of actin filaments in clones overexpressing wild-type RNASET2, with several long actin-filament bundles crossing the whole cell length. Strikingly, this pattern was significantly altered in control clones, which showed a mainly peripheral actin filament bundle pattern instead (Figure 37). These data are totally in keeping with our recent findings in OVCAR3 cells and further suggest a prominent cell-autonomous role for RNASET2 in the regulation of the cell cytoskeleton organization.

Taken together, these data provide a first evidence that the cell-autonomous oncosuppressive role of RNASET2 that we previously reported in human ovarian cancer-derived experimental models can be faithfully recapitulated in a breast cancer cell model as well.

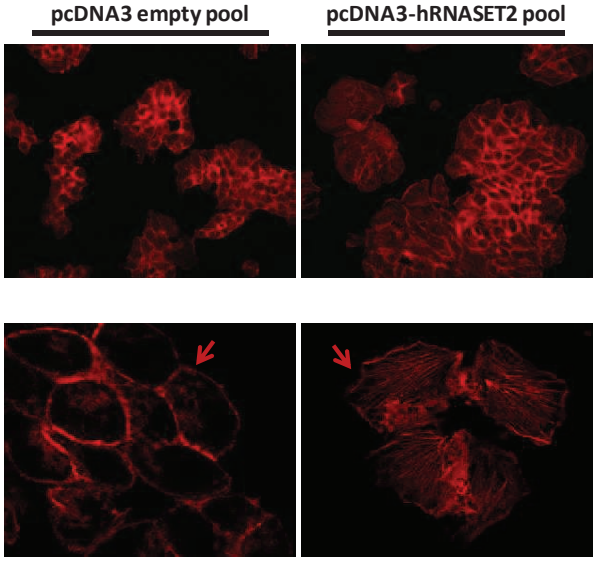

Figure 37. Structural organization of the actin cytoskeleton
MCF7 cell clones were seeded on coverslips and stained with TRITC-conjugated Phalloidin. A complex network of actin filaments is evident in RNASET2-overexpressing cells, while the actin cytoskeleton appears different in control clones. Details on Ph-TRITC stained cell (Confocal microscopy images).

EVALUATION OF MAMMOSPHERE FORMATION IN 3D CULTURE CONDITIONS

On the basis of the promising results obtained by investigating the role of RNASET2 on breast cancer cells cultured in 2D culture conditions, we decided to turn our attention to a more physiological 3D experimental model.

Thus, as a preliminary assay for 3D culture, some of the previously selected MCF7 clones were also tested for their ability to form mammosphere in culture. The mammosphere assay originally developed by Dontu et al. [39] represents a well-established *in vitro* culture system commonly used for propagating healthy human mammary epithelial stem cells, based on the assumption that only undifferentiated cells will be able to survive and proliferate in suspension culture. The ability to form such structures is therefore related to the self-renewal ability.

Of note, it is now widely accepted that most advanced tumors contain a sub-population of cells with stem cell properties that are considered responsible for the onset and progression of tumors [40]. In fact, cancer stem cells (CSCs) are uniquely able to reestablish the tumor when transplanted into xenograft models, they exhibit enhanced resistance to therapy and they can drive tumor recurrence and metastasis [40]. For all these reasons, CSCs represent critical therapeutic targets [41].

In light of the previously reported pleiotropic roles of RNASET2 in tumor suppression, we reasoned that mammosphere growth assays could be used to determine whether *RNASET2* can affect the number or growth pattern of mammosphere and/or on the expression of stem cell markers in the cells forming these structures.

We therefore evaluated the mammosphere-forming ability of *RNASET2*-overespressing MCF7 clones compared to control clones (MCF7 cells transfected with pcDNA3 empty vector).
Interestingly, as shown in Figure 38 (upper panel), control clones formed a higher number of mammosphere compared to RNASET2-expressing clones, allowing us to hypothesize that RNASET2 might inhibit stem cell properties. Of note, this result was observed when considering two different size of mammosphere (50 or 100 μm).
To further define the effects of RNASET2 on CSCs, we analyzed the expression pattern on known markers of stemness in this experimental system. Breast CSCs are generally identified as $CD24^{-/low}/CD44^{+}$ cells and also display high expression of ALDH1.
Two independent realtime qPCR expression assays were carried out in our model system for different epithelial and stemness markers. Significantly, clones overexpressing RNASET2 showed a higher expression of CD24 and a lower expression of ALDH1, supporting the hypothesis that

RNASET2 could be involved a signaling pathway controlling the cellular differentiation state (Figure 38 – lower panel).

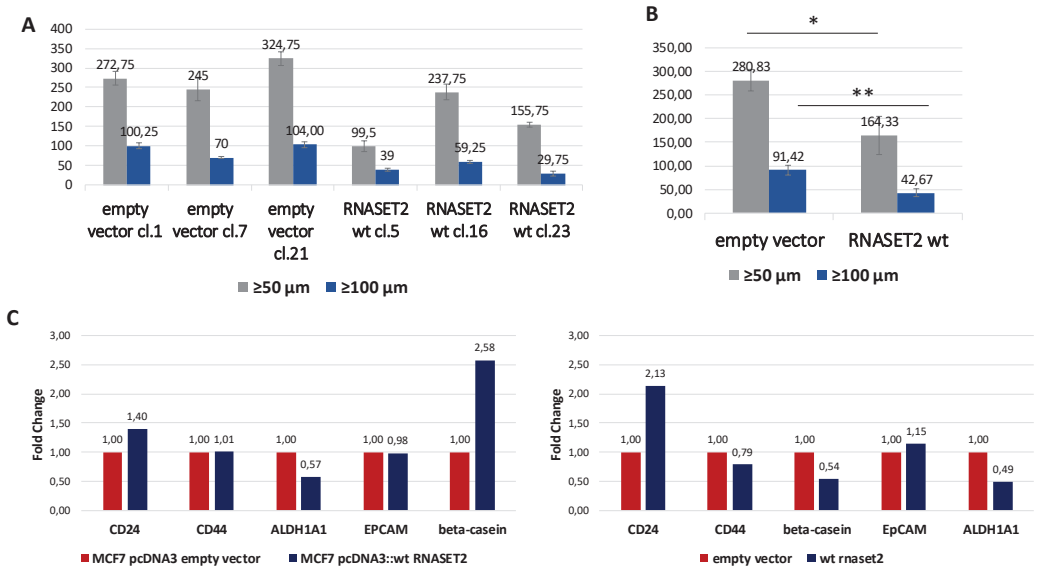

Figure 38. Difference in mammosphere-forming ability between MCF7 RNASET2-overexpressing clones and control clones and gene expression analysis

(A) Three clones overexpressing RNASET2 were compared to control clones for their efficiency in forming mammospheres and the first ones showed a decrease in the number of these structures. Every sample was seeded in quadruplicates; results are shown as mean ± SEM. (B) The histograms show the mean for each of the experimental group of the same experiment. Statistical analysis was performed using one-tailed Student's *t*-test. *p<0.05. (C) Total RNA was isolated from three (left panel) or six (right panel) MCF7 control/RNASET2-overexpressing clones, reverse transcribed and a qRT-PCR was carried out to analyze the expression of 5 different markers, with GAPDH as an internal standard. The fold change in gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and considering the mean of control clones with a level of expression equal to one.

RNASET2 EXPRESSION IN HUMAN MAMMARY GLAND CELL POPULATIONS

To further analyze the role of human RNASET2 in the mammary gland physiology, we analyzed its expression levels in different populations of healthy mammary gland tissue.

Strikingly, when four different cell populations belonging to the healthy mammary gland (namely, luminal terminally differentiated cells, myoepithelial basal-like cells, stem cells and cells undergoing Epithelial-Mesenchymal Transition - EMT cells) were sorted by cytofluorimetry and subsequently analyzed for gene expression, the *RNASET2* gene showed a cell type-dependent gradient of expression, with the highest expression levels observed in the luminal population

(which represents the more differentiated one) and, conversely, a lower expression level in the stem cell and EMT populations (figures 39a and 39b).

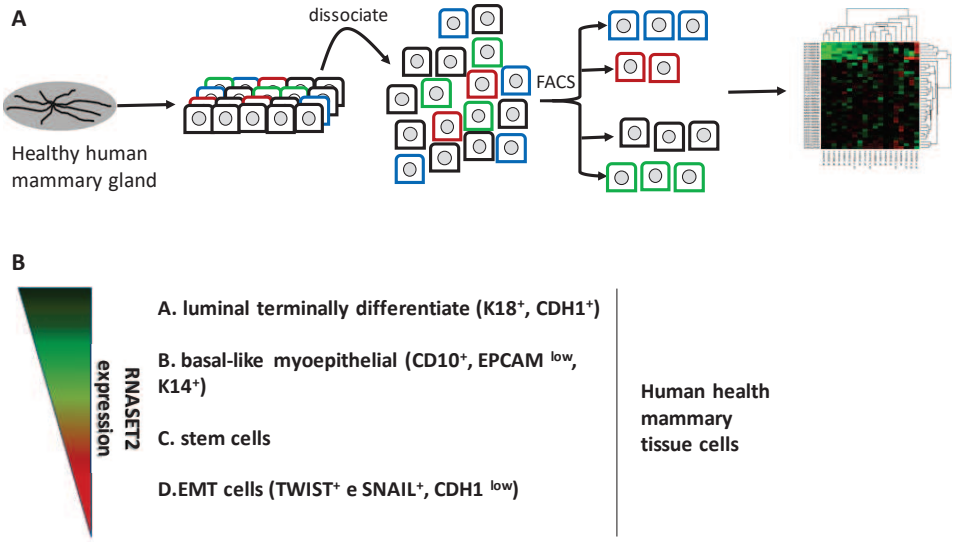

Figure 39. RNASET2 role in normal breast morphogenesis
(A) Cells derived from mammary gland tissue were sorted by lineage markers and a microarray analysis was performed. (B) RNASET2 has a higher expression in the more differentiated population, the luminal cells.

These results suggest a putative role for RNASET2 in stemness control in the mammary gland. Of note, our preliminary analysis on relapse-free survival data (figure 32) indicated that high RNASET2 expression levels were associated with a higher survival in luminal-A breast cancer patients.

To better address this issue, luminal cells derived from normal breast samples were transduced with a lentiviral system in which a short hairpin RNA (shRNA) for RNASET2 gene silencing was constitutively expressed with the GFP as reporter gene.

When these cells were cultured under conditions that promote 3D organoid formation, downregulation of *RNASET2* resulted in a significantly altered morphogenetic pattern leading to abnormal 3D structures formation, whose main feature was a loose dissociated morphology (figure 40b). Indeed, these structures were not comparable with the development of branching tubules and cauliflower-like structures reminiscent of multi-branched lobular clusters that were

instead observed by culturing scrambles shRNA-expressing control luminal cells in 3D (figure 40a).

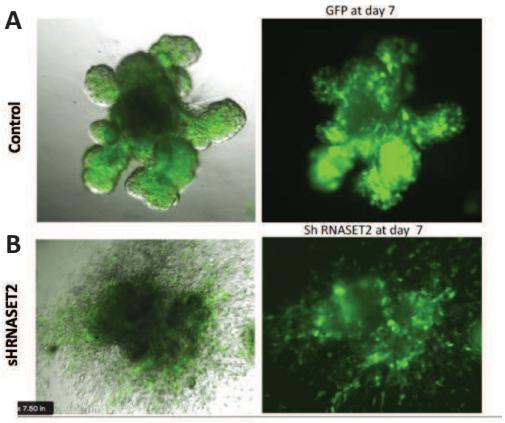

Figure 40: RNASET2 role in mammary organoid morphogenesis from donor sample BR20-11
(A) 3D organoids structures from cells transduced with control vector. (B) 3D disordered structures from cells transduced with a vector for silencing RNASET2 expression.

The proper downregulation of the *RNASET2* gene in the luminal cell population was verified by real-time qPCR analysis, which confirmed a dramatic reduction of this gene's expression in the luminal cell population derived from sample BR20-11 (figure 41).

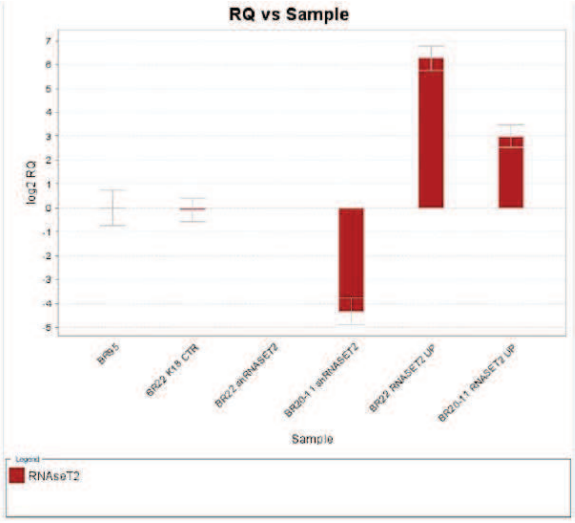

Figure 41: Evaluation of RNASET2 expression in mammary organoids generated from samples BR20-11 and BR22 following shRNA-mediated downregulation of RNASET2
Quantitative PCR analysis was carried out in scrambled ("RNASET2 UP") vs. *RNASET2* shRNAs-expressing ("shRNASET2") luminal cells from two independent healthy donors (BR20-11 and BR 22).

To define putative *RNASET2*-driven molecular alterations associated with the observed structural disruption of the organoids, the latter were analyzed by immunofluorescence assays. In keeping with the notion that *RNASET2*-silenced 3D structures were not representative of normal 3D mammary organoids, epithelial cell-to-cell contacts were found to be significantly disrupted in these structures (data not shown). Moreover, a significant remodeling on the actin cytoskeleton, coupled to a marked downregulation of proteins associated with the differentiated state of luminal cells (cytokeratins K14 and K18) was observed in *RNASET2*-silenced structures compared to control organoids. (figure 42).

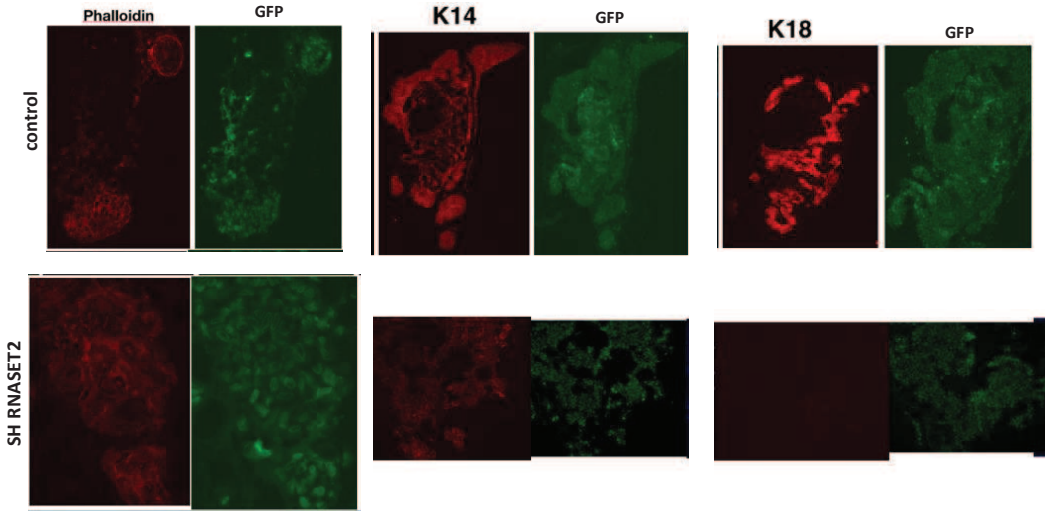

Figure 42: Immunofluorescence assays on mammary organoids
Cells derived from mammary gland tissue and transduced with control vector or SH RNASET2 vector were analysed for the actin cytoskeleton pattern and the expression of two cytokeratins marker (K14 and K18).

The loss of normal 3D organization in the mammary parenchyma represents one of the hallmarks of breast tumor's morphology and is characterized by the appearance of de-differentiated (due to loss of epithelial cell morphology) and invasive-like parenchyma luminal cells. In agreement, downregulation of *RNASET2* in the 3D structures was associated with a decrease in beta-catenin epithelial marker expression (figure 43), coupled to an increase in expression of the canonical EMT markers alpha-SMA (data not shown).

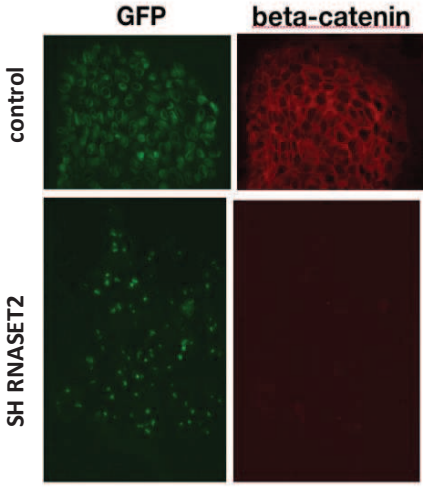

Figure 43: Immunofluorescence assays on mammary organoids cryosections
Analysis by immunofluorescence of the beta-catenin epithelial marker. SH RNASET2 cells unable to form an ordered 3D structure show a marked downregulation of this protein.

By repeating the experiment to analyze samples at different time points (7 or 14 days), we discovered that *RNASET2* silencing prevented the proper development of the organoid (figure 44, compare upper and lower pictures). Indeed, as shown in figure 44, control cells are able to develop a proper structure with a clear actin cytoskeleton pattern, whereas RNASET2-silenced cells eventually die, cells contacts are lost and actin fibers are disrupted.

Of note, since lentiviral transduction efficiency was probably lower than 100%, the luminal cell population was likely composed of both infected, RNASET2-silenced and uninfected RNASET2-expressing cells at the beginning of the experiment. Indeed, the percentage of GFP-positive cells in control organoids at 7 days was ranging from 10 to 50% (figure 44, upper panel).

In this regard, it is worth noting that during organoid formation $GFP^+/RNASET2^-$ cells were apparently lost with time, as demonstrated by the very low number of $GFP^+/RNASET2^-$ cells at day 7 following organoid culture, coupled to the concomitant occurrence of $GFP^-/RNASET2^+$ cells (figure 44, right lower panel). Significantly, $GFP^+/RNASET2^-$ cells were completely missing at 14 days.

We therefore reckon that, in the first days of 3D culturing of luminal cells, the likely predominance of RNASET2-silenced cells prevents the proper formation of the organoid, since

the few RNASET2-expressing cells present at early stages are not able to compensate for the lack of this protein in most cells, which impairs organoid formation.

We therefore hypothesize that the lack of RNASET2 in luminal cells, which initially outnumber other cells, is a key factor in the first steps for the organoid formation process.

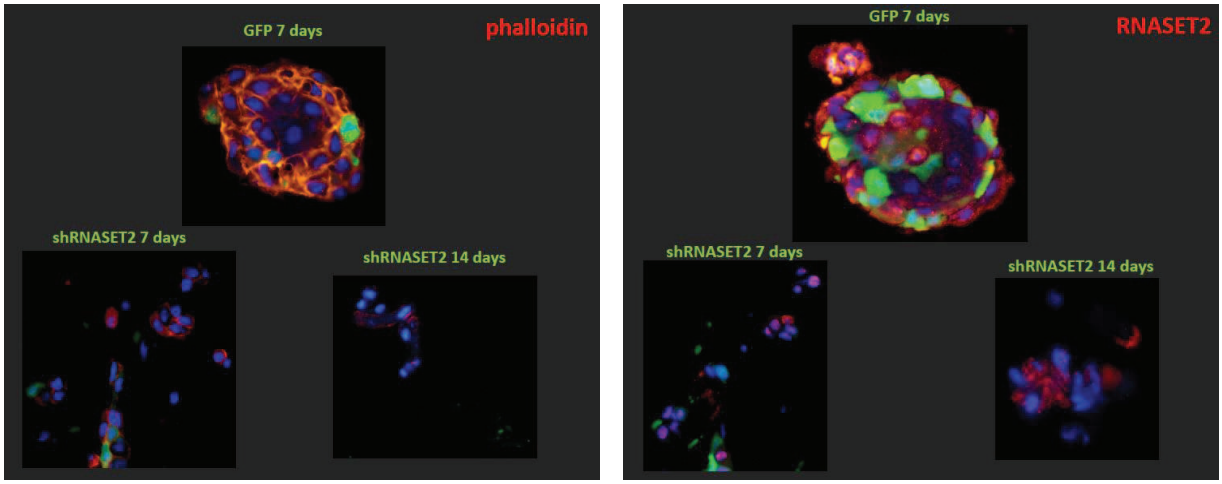

Figure 44: Immunofluorescence assays on mammary organoids cryosections
Analysis by immunofluorescence of actin cytoskeleton (Phalloidin) and RNASET2 expression. SH RNASET2 cells unable to form an ordered 3D structure are lost over time and no actin fibers material is visible already at 7 days.

Taken together, the above described data, obtained in several independent experimental breast cancer models, are of key relevance. In fact, whereas on one hand they provide an independent support to the notion of the *RNASET2* gene as a tumor suppressor acting on a wide spectrum of human cancers, on the other hand they shed new light to an unprecedented potential mechanism by which this highly pleiotropic gene carries out its anti-tumoral activity, i.e. by controlling the differentiation state of mammary epithelial cells, which in turn is tightly linked to their tumorigenic potential.

DISCUSSION

Ribonucleases represent a very ancient class of enzymes, whose role in degrading or processing RNA molecules has been exploited by nature to carry out an impressive range of biological functions. Indeed, the key role assigned to RNases in living organisms is testified beyond any reasonable doubt by the impressive number of RNase-encoding genes that have been discovered in many species, particularly in higher eukaryotes. For instance, the human genome is known to encode at least thirteen members just for the vertebrate-restricted RNAse A superfamily [42], the most widely investigated so far. However, several ribonuclease genes belonging to other subfamilies have been reported as well in higher organisms in the last decades.

Of note, notwithstanding the disparate biological processes regulated by this large class of enzymes, a common theme which has soon emerged from the functional analysis of several RNases is their role in host defense. In this context, a key role in displaying anti-viral and anti-bacterial activities has been reported for an impressive number of ribonucleases. Of note, such host defense role, which most often relies on the catalytic activity of these enzymes, entails a strong cytotoxic activity, which in turn has attracted a growing attention in order to exploit ribonucleases as potential anticancer molecules [43]. Moreover, one relevant biological process by which several ribonucleases are engaged in host defense is immune modulation, in particular for those RNases that are secreted in the extracellular space [12]. For instance, an impressive number of human RNAses belonging to the A superfamily are known to be secreted by several cell types in order to carry out a range of activities involved in host defense, inflammation and tissue remodeling by means of immune modulation [22].

More recently, the role in cancer growth control coupled to immune modulation has been extended to some members of the Rh/T2/S family of extracellular ribonucleases, the most ancient and evolutionary conserved [22]. The ubiquitous distribution of these enzymes among taxa, coupled to the fact that, unlike RNase A members, T2 ribonucleases often carry out their biological functions independently from their catalytic activity, suggest that these enzymes represent highly pleiotropic molecules involved in very ancient biological processes.

Unlike RNase A family members, the human genome is known to encode a single gene encoding a T2 RNase member. This gene, called *RNASET2*, has been shown by our group to

behave as a powerful tumor suppressor. In particular, in recent years we reported that *RNASET2* overexpression in human ovarian cancer cells is associated with a strong suppression of their tumorigenicity *in vivo* [16]. Noteworthy, the observed control of tumor growth apparently relied on the establishment of a cross-talk between RNASET2-overexpressing cancer cells and the tumor microenvironment, with the monocyte/macrophage cell lineage representing the most likely target of *RNASET2* activity [19].

Interestingly, our first experimental data, reported with the Hey3Met2 cell line, seemed to suggest an asymmetric tumor-suppressive activity for *RNASET2*, which was apparently carried out *in vivo* but not *in vitro* [19]. These biological properties led us to rank *RNASET2* as a novel member of the growing family of "tumor antagonizing/malignancy suppressor genes" [44], whose main feature is their ability to carry out a non-cell autonomous, microenvironment-mediated control of tumorigenesis [45]. Of note, the catalytic activity of RNASET2 turned out to be dispensable for its oncosuppressive role [16].

With further investigations in other ovarian cancer cell line model, however, independent *cell-autonomous* oncosuppressive roles were attributed to this gene as well, which might further enhance its tumor suppressive activity. In particular, *RNASET2* was reported to behave as a stress response gene endowed with a marked effect on the actin cytoskeleton organization and with the ability to affect several cancer-related parameters *in vitro* [24]. Moreover, the tumor suppressive role of *RNASET2* has been reported for other human cancer types in recent years, such as colorectal cancer and malignant melanoma [17].

Altogether, the experimental data gathered on different human cancer models thus suggest a highly pleiotropic role for *RNASET2* in tumor suppression, whereby different cellular parameters related to cancer growth are affected by changes in the expression levels on this protein, but a marked perturbation of the cancer microenvironment is also carried out by this gene at the same time.

Within this frame, the general aim of my Ph.D work was to further characterize the oncosuppressive role of the human *RNASET2* gene at the molecular and functional level.

As mentioned above, since T2 RNases represent the most ancient and evolutionarily widespread ribonuclease family, they probably share some very ancient and critical functions. One of these biological properties is likely the modulation of the innate immune system. Indeed, the T2 ribonuclease orthologous gene encoded by the trematode *Schistosoma mansonii* encodes for a protein (Omega-1) that has been reported to prime the host's dendritic cell toward a Th2-polarized response [20]. Moreover, a role for *RNASET2* in the activation of the innate immune system in the invertebrate model *Hirudo medicinalis* has also recently described in our lab [46] and at least two other members of the T2 ribonuclease protein family have been reported to modulate the mammalian innate immune system [20,47].

These experimental evidences, coupled to our previous *in vivo* experiments and immunohistochemistry analysis on human ovarian cancer cell-derived xenograft tumours, prompted us to investigate a potential effect of RNASET2 on macrophage polarization pattern *in vitro*.

Using the human promyelocytic THP-1 cell line, a widely used and established cellular model of macrophage differentiation and polarization, we found that, following the silencing of endogenous *RNASET2* expression in these cells, a marked effect on their polarization pattern was observed, consisting in both a dramatic reinforcement of the M2 polarization pattern upon IL-4 stimulation, coupled with a moderately weakened M1 response under IFNγ/LPS stimulation were observed in these cells.

This was the first evidence supporting a role for *RNASET2* in the regulation of macrophages polarization pattern *in vitro,* and these data lead us to further wonder if exogenous administration of RNASET2 protein could mimic the effect observed by experimentally manipulating the endogenous protein expression levels.

Of note, injection of human recombinant RNASET2 in *Hirudo medicinalis* was recently shown by our group to induce both a massive migration of cells belonging to the macrophages lineage to the injection site and an increase in the expression level of the endogenous T2 RNase protein in the same innate immune cells [46].

A rescue assay in *RNASET2*-silenced THP-1 cells was therefore carried out using recombinant RNASET2 expressed in the yeast system *Pichia pastoris*. Indeed, treatment of

RNASET2-silenced THP-1 cells with recombinant RNASET2 could rescue the polarization pattern seen in parental THP-1 cells, although the observed reversion of the polarized phenotype was only partial. We reckon that such partial effect might be attributable to the expression system used to produce the recombinant protein. Indeed, a recent work on the T2 Rnase Omega-1 from *Schistosoma mansonii* reported a key role for the mannose-receptor in Omega-1 uptake by mammalian dendritic cells [48], which is required for Th-2 polarization of these cells. Since the glycosylation pattern of proteins produced in *P. pastoris* is known to substantially differ from that observed in mammalian cells (specifically concerning mannose residues), should the mannose receptor be involved in *human* RNASET2 binding by macrophages as well, the *Pichia pastoris*-derived recombinant RNASET2 protein might not have completely rescued the polarization phenotype of THP-1 cells due do some critical differences in its glycosylation pattern when compared to the human *endogenous* protein that was silenced by RNA interference.
To address this issue, the production of human recombinant RNASET2 in mammalian CHO cells is undergoing in our lab.

Of note, since the mannose receptor has long been described as an M2-specific macrophage marker, the report from the Omega-1 binding pattern suggests that the human target cells for RNASET2-mediated polarization might be represented by M2 macrophages themselves. Under this scenario, RNASET2 might operate by promoting an M2 to M1 shift *in vivo*, which in turn might trigger a transition from a pro- to an anti-tumoral microenvironment.

Therefore, one task of key relevance for our future investigations will be to assess the role of the mannose receptor in RNASET2-mediated macrophage polarization, by means of RNAi assays coupled to the use of specific agonists of the mannose receptor.

To further validate the results obtained in the THP-1 experimental model, we started a preliminary polarization assay using PBMC-derived human monocytes as a target cell population.

The result form our first attempts were quite disappointing, since the macrophage polarization regimen applied was only partially effective, thus precluding a reliable evaluation of the *RNASET2* role in these cells. Moreover, the problem of donor-to-donor variability, which has long been recognized in experiments using PBMC-derived cells, has probably confounded the interpretation of the experimental data obtained.

However, even in this imperfect experimental system we were able to observe a trend for an RNASET2-mediated effect on these cell's polarization pattern. Therefore, further efforts are ongoing in our lab in order to both improve the polarization protocol for PBMC-derived monocytes (particularly for M1 macrophages) and at the same time change our experimental plan by pooling PBMC-derived monocytes from multiple independent donors rather than using single-donors-derived independent cell populations.

Moreover, in order to get a more comprehensive picture of the role played by RNASET2 in macrophage polarization, we plan to significantly increase the number of polarization markers to be analyzed in both THP-1 and PBMC-derived-monocytes, by using customized qPCR arrays including more than one hundred genes involved in innate immunity.

Human cancer cell lines whose RNASET2 expression level has been experimentally manipulated will also be co-cultured with both THP-1 and PBMC-derived macrophages, in order to reproduce *in vitro* and better investigate the role of macrophages in *RNASET2*-mediated tumor suppression.

Finally, several collaborations with medical oncology research labs have been recently launched by our group in order to start a detailed survey of a panel of human cancer types, aimed at both evaluating the correlation of RNASET2 expression with tumor grade or stage and the putative involvement of stromal macrophage infiltrates.

Taken together, the experimental data presented in this first part of my PhD work are in keeping with our previously reported *in vivo* xenograft-based data, where *RNASET2* overexpression and secretion by cancer cells resulted in the suppression of their tumorigenic potential, coupled to a massive recruitment of M1-polarized macrophages in the tumour mass, likely polarized by RNASET2 at the expense of M2 macrophages.

Of note, RNASET2 has been recently described as a stress-response protein, whose expression and secretion is markedly increased following induction of a wide range of cellular stresses [24]. Among these are included oxidative stress and hypoxia, which represent two stress conditions typically experienced by cancer cells. On the basis of this observations, RNASET2 has been proposed to act as a cancer-related alarmin molecule, whose role is to send a danger signal

to alert microenvironmental components (such as cells of the innate immune system) of the occurrence of a pre-neoplastic state in order to mount a proper host defense response.

In this context, it is worth noting that several current therapeutic approaches in cancer research are focused on reprogramming the immune cell populations within the tumor microenvironment towards an anti-cancer response. For instance, one largely explored strategy entails Tumor-Associated Macrophages (TAMs) reprogramming towards an M1-polarized, anti-tumor role [49]. Thus, our results potentially suggest a translational application of RNASET2 as a powerful tool in this area of anti-cancer research.

In light of the experimental evidences supporting a role of the immune system in RNASET2-mediated tumor suppression, to further address the potential therapeutic applications of this protein we next asked whether the tumor suppressive role observed for *RNASET2* in xenograft-based assays carried out in immunocompromised experimental models could be confirmed in the context of a perfectly efficient immune system. We therefore sought to develop a syngeneic murine model to get further insights into the non cell-autonomous tumor suppressive role of RNASET2 *in vivo*. This model could provide us with key information on the interplay of RNASET2 with the tumor microenvironment in a more physiologic context and therefore allow us to perform a deeper investigation on the extent of the RNASET2-mediated cross-talk between cancer cells and immune cells.

One of my PhD work's aims was therefore to define a suitable experimental model consisting of a mouse cell line for manipulating RNASET2 expression and producing cell clones to be subsequently used in *in vivo* experiments.

Several cell lines representative of a range of murine cancers were chosen to this aim and their endogenous RNASET2 expression levels was first analyzed at both the transcript and protein level. Since we observed a very low endogenous expression of mouse Rnaset2 in all murine cell lines, we choose a gene over-expression approach for our purposes.

To this end, we set up transient transfection assays in at least three different cell lines with recombinant expression vectors encoding either mouse Rrnaset2 and human RNASET2 cDNAs.

Quite unexpectedly, in all cell lines tested only the human protein was effectively over-expressed, while the murine protein was either expressed at very low levels or undetectable.

Since a protein alignment approach revealed some potentially relevant differences between the human and mouse RNASET2 proteins, we speculated that the observed data might be attributable to some potentially toxic or anti-proliferative activity of the murine protein with respect to its human counterpart. To address this issue, we turned to the development of an inducible expression system.

Unfortunately, we were once again unable to get the murine protein expressed following doxycycline-induced expression in any of the cell lines tested, although we could demonstrate that the murine transgene was properly induced at the RNA level.

These data provided further support to the notion that the mouse Rnaset2 protein is somehow incompatible with cell viability and/or proliferation. In this regard, it is worth noting that although our anti-RNASET2 antibody was raised against the human protein, it could easily detect murine Rnaset2 following western blot analysis on murine adult *post-mitotic* tissues.

This observation seems to rule out any problem of antibody sensitivity as a possible reason for the lack of murine protein detection in our transfection assays, and at the same time points to an antiproliferative rather than cytotoxic effect of this protein.

Drawing from these experimental results, we next decided to investigate whether some mouse protein portion could be responsible for the marked human *vs.* murine biological difference. To this end, we carried out a protein swap domain approach by assembling different constitutive expression vectors bearing the human and mouse full-length cDNA, a C-terminal deleted version of the mRnaset2 and two chimeric human-murine versions of RNASET2.

At a first sight, transient transfection assays seemed to point to the N-terminal murine portion as the one responsible for the observed lack of protein expression, but when we turned to stable transfections in TS/A and C51 cell lines, both chimeric versions turned out to be expressed in cell pools, although at a very low level. Moreover, the mouse truncated version was expressed as well. On one hand, these data were disappointing since we could not unambiguously define a portion within the murine Rnaset2 protein that might be responsible for

its lack of expression. However, the observation of a weak expression signal from some murine expression vectors prompted us to make a large-scale effort in order to isolate stably transfected single clones expressing the murine protein.

The achievement of this task turned out to be quite expensive and time-consuming, since a very high number of transfected clones had to be screened in order to obtain a few murine Rnaset2-expressing cell clones, in line with the previous difficulty experienced with pools of transfected clones. Of note, by comparing the expression levels in the few T2 RNase-expressing single clones, the mouse full-length Rnaset2 protein turned out to be always expressed less efficiently that the murine truncated version, which was in turn expressed at lower levels when compared to human full-length RNASET2. Thus, our results confirmed the occurrence of a kind of functional difference between the human and murine proteins, whose nature is still to be defined.

Despite this difference, the successful isolation of a handful of murine Rnaset2-expressing clones paved the way for studies aimed at defining the functional features of this protein. We started to address this issue by analyzing the proliferation rate *in vitro.* Interestingly, the full length mRnaset2 protein displayed a marked antiproliferative ability in C51 cells, which was much more attenuated for cell expressing the truncated protein. The same trend was observed in TS/A cells, although the observed differences were not statistically significant.

These data are in keeping with our hypothesis of an antiproliferative rather than toxic effect of murine RNASET2, since cell viability was apparently unaffected by mouse Rnaset2 expression by visual inspection (data not shown). Moreover, the observed difference between the full-length and truncated versions of the protein pointed at the C-terminal portion of murine Rnaset2 as the likely determinant of a key functional property.

Besides these *in vitro* studies, the availability of murine Rnaset2-expressing clones allowed us to start developing a syngeneic experimental model for further investigations.
To this end, we used one clone for each experimental group from the C51 cell line to carry out a pilot experiment on immunocompetent Balb/c mice. Strikingly, we observed a marked suppression of the *in vivo* tumor growth rate in cells overexpressing the full length mRnaset2. Of note, cells overexpressing the truncated protein gave rise to tumors whose growth rate was very

similar to control clones, again pointing at some crucial functional element at the C-terminal end of the protein.

To our knowledge, this is the first evidence supporting the *in vivo* oncosuppressive role of a T2 ribonuclease in a fully immunocompetent experimental model. In this context, this result paves the way for a new line of research, aimed at both defining the putative occurrence of a cross-talk between cancer-cell derived RNASET2 and the components of the adaptive immune system and at the same time to develop preclinical model aimed at investigating the potential of RNASET2 as a potentially useful agent in cancer therapy.

Our laboratory will be particularly involved in the first task, whereby murine Rnaset2-expressing clones will be deeply investigated to assess a wide panel of cancer-related parameters *in vitro* assays and to compare them with those associated with human RNASET2 overexpression.

At the same time, further *in vivo* studies will be carried out in order to achieve for the first time a thorough characterization of an immunocompetent tumor microenvironment in mouse Rnaset2-expressing tumors, by means of both IHC assays (to define the putative involvement of cellular component of the innate and adaptive immune system in Rnaset2-mediated tumor suppression) and functional *in vitro* co-culture experiments with Rnaset2-expressing cancer cells and several cellular component of the tumor microenvironment.

Collectively, the experimental data presented in the first part of this PhD work provide a strong evidence in support of both a marked and evolutionary conserved activity of RNASET2 as a tumor suppressor and at the same time the role played by the immune system in RNASET2-mediated tumor suppression.

However, T2 ribonucleases are widely known as pleiotropic proteins involved in several biological processes [22]. For instance, in recent years several research groups (including ours) reported a cell-autonomous oncosuppressive role for several members of the T2 family [23,24,25]. According to the chosen experimental model and the human T2 Rnase under investigation, such cell-autonomous role entails a wide range of cancer-related biological processes, such as induction of apoptosis, remodeling of the cytoskeleton, prevention of angiogenesis, control of autophagy and decrease of anchorage-independent growth [21]. These

observations, coupled to the broad spectrum of tumors associated with genetic alteration in the genomic region in which the RNASET2 gene maps, prompted us to confirm (and possibly extend) the current knowledge about the oncosuppressive roles of human RNASET2 in an independent cancer model. In this context, since one of the cancer types in which the 6q27 region is frequently deleted is breast cancer, we decided to investigate the putative involvement of RNASET2 in this cancer type. The human MCF7 cell line was chosen for this purpose, since it represents a valuable model to investigate the role played by a gene of interest not only in 2D *in vitro* assays but also in three-dimensional cultures, due to its ability to grow in suspension under proper culture conditions to form 3D structures (called mammospheres) that better reproduce the architectural features of the human mammary gland *in vitro*, thus providing a more physiological experimental system [32;50-53].

A preliminary analysis of the RNASET2 expression level in this cell line showed that MCF7 cells has low levels of endogenous expression compared to other cell lines previously used in our laboratory. These findings prompted us to generate human *RNASET2*-overexpressing MCF7 clones for further investigations. A panel of such clones were therefore generated and few of them were selected for preliminary functional assays.

We first evaluated the effect of *RNASET2* overexpression on some cancer-related parameters and observed a slight variability on cell proliferation rate between *RNASET2*-overexpressing clones and control clones. By contrast, a colony-formation assay showed that control clones produced a significant greater number of colonies with respect to *RNASET2*-overexpressing clones. Since colony formation is an *in vitro* cell survival assay that tests the ability of single cells to undergo "unlimited" proliferation, the results are in keeping with the notion of a cell-autonomous tumor suppressive role of RNASET2 in an experimental model different from ovarian cancer.

Furthermore, since apoptosis represents a biological process deeply involved in cancer development and progression, we decided to assess whether *RNASET2* could affect this biological process as well in MCF7 cells, both at basal condition and following apoptogenic treatment. Again, we found a clear effect of *RNASET2* on this parameter, since clones overexpressing wild-type RNASET2 showed an increased apoptotic rate in all tested conditions (untreated, treated

with CoCl2 or *cis*-platinum) with respect to control clones. This result is once again in keeping with our previous data in the ovarian cancer model.

A similar conclusion could be drawn by turning our attention to RNASET2's effects on the cell cytoskeleton. Malignant cancer cells utilize their intrinsic migratory ability to invade adjacent tissues and cytoskeletal rearrangements are known to be of paramount importance in this process. In agreement with our previous data in ovarian cancer models, a distinct *RNASET2*-mediated re-organization of the cell cytoskeleton was observed in MCF7 cells as well, since the actin structural network was consistently rearranged to a complex network with several long actin-filament bundles crossing the cell length following *RNASET2* overexpression.

The observed cytoskeletal dynamics was in keeping not only with our previous data on an ovarian cancer-derived experimental model, but also with the previously reported role of *Schistosoma mansoni* Omega-1 protein in cytoskeleton rearrangements [20;24], demonstrating once again the evolutionary conservations of a biological process mediated by T2 ribonucleases.

Taken together, the results from these *in vitro* assays lend further credit to the notion of a strong *cell-autonomous* oncosuppressive role for RNASET2 in a human breast cancer cell model. If the previously reported data from human malignant melanoma are also taken into account [17], the emerging picture points at *RNASET2* as an oncosuppressor gene endowed with a common role in most cancer types showing chromosomal rearrangements in the 6q27 region that have been investigated so far.

However, the well-established pleiotropic roles assigned to T2 ribonucleases, coupled to the intrinsic complexity of the tumorigenic process, suggest a putative scenario by which *RNASET2* might act as a tumor suppressor in different ways for different cancer types. Of note, the MCF7 cell model is particularly attractive to address this issue, due to the previously mentioned ability of this cell line to grow in both 2D and 3D culture condition. The latter feature is of key relevance in this regard, since it allows to address a critical issue related to cancer growth, which is the role of cell differentiation and tumor heterogeneity.

Intra-tumor heterogeneity represents a well-documented feature of cancer and posits great challenge to the successful development of antineoplastic therapies. Indeed, it is now widely accepted that tumors contain a sub-population of cells endowed with stem cell properties that

are considered responsible for the onset and progression of tumors. Of note, cancer stem cells (CSCs) are uniquely able to reform the tumor when transplanted into *xenograft* models, exhibit enhanced resistance to therapy and can drive tumor recurrence and metastasis. For all these reasons CSCs represent key current therapeutic targets [41]. We therefore decided to exploit the experimental versatility of the MCF7 cell model to further investigate the putative effect of RNASET2 in breast cancer development, with a particular attention to its putative role in CSCs biology.

To this end, we decided to take advantage of the mammosphere assay [39], a 3D *in vitro* culture system commonly used for propagating human mammary epithelial stem cells and based on the assumption that only undifferentiated cells such as CSCs will be able to survive and proliferate in suspension culture. The ability to form such structures is therefore related to self-renewal ability.

We therefore carried out this assay to determine the ability of *RNASET2* to affect both mammospheres formation and/or the expression level of stem cell markers in the cells forming these 3D structures, under the assumption that breast CSCs are generally identified as a $CD24^{-/low}/CD44^{+}$ population and have high expression of ALDH1 [54].

Strikingly, our experimental data supported the hypothesis that RNASET2 could be involved in the regulation of MCF7 differentiated state. In fact, a clear negative correlation was found between RNASET2 expression and the number of mammospheres formed. Additionally, mammospheres derived from RNASET2-overexpressing clones showed a higher expression of CD24 and a lower expression of ALDH1, supporting the hypothesis of a role for *RNASET2* in breast cell differentiation.

Recent data gathered on a collaborative basis with our group further support this hypothesis. In fact, to get a deeper insight into the role of human *RNASET2* in the mammary gland, we proceeded to analyse its expression levels in different populations of the healthy mammary gland. Strikingly, the *RNASET2* gene showed the highest expression levels in the more differentiated breast cell population (i.e. the luminal one) whereas less differentiated cells (such as CSCs and cells undergoing EMT) showed a drastically decreased RNASET2 expression level.

To better investigate this issue, we evaluated the effect of *RNASET2* downregulation in human organoids generated from healthy breast luminal cells.

Of note, silencing RNASET2 expression in the luminal population resulted in the formation of abnormal 3D structures with a concomitant decrease on the expression of some epithelial markers.

Though preliminary, we reckon that these data are of key relevance since they further support the extensive pleiotropy of the *RNASET2* gene by unveiling a previously unknown mechanism by which this gene act to suppress tumor growth. In particular, the apparent role of RNASET2 in controlling the cell differentiation state in MCF7 cells might be put in relation with the previously established ability of the RNASET2 protein to affect the actin cytoskeleton [24].

Indeed, the cell polarization pattern of epithelial cells (which is in part controlled by specific cytoskeletal organization patterns) has long been recognized to control their differentiation pattern. Therefore, the previously reported ability of T2 ribonucleases to affect the actin cytoskeleton might provide a functional link to explain the ability to control the cell differentiation pattern that we observed in the MCF7 experimental model.

Although many details still need to be further investigated to draw a comprehensive picture on the molecular and cellular mechanisms underlying *RNASET2*-mediated tumor suppression, our results laid the bases for future studies aiming at dissecting, at the widest possible range, the involvement of *RNASET2* in tumor suppression by means of several mechanisms, such as innate immune cells recruitment and polarization, cell-autonomous antiproliferative and pro-apoptotic activity, and cell cytoskeleton remodeling, which might in turn endow T2 RNases with the ability to control the cell differentiation state.

All these disparate functional features of the *RNASET2* gene are going to be addressed in our laboratory in the near future, by exploiting the above-mentioned experimental models in order to shed more light on the molecular and cellular effectors involved in the several biological processes in which this very ancient and highly pleiotropic gene is involved.

As long as our breast cancer model is concerned, we plan to carry out a detailed survey of a wide panel of cell differentiation and stem cell markers in control *vs.* RNASET2-silenced

organoids. At the same time, a zebrafish-based model is going to be developed on a collaborative basis in order to investigate the role of *RNASET2* in cell differentiation and organ morphogenesis in a well-established and easily available animal model.

On the long-term, we reckon that these investigations might be exploited in medical translation approaches aimed at exploiting the RNASET2 protein in anticancer therapy. Indeed, ribonucleases have already been considered as potential anticancer drugs and some RNase A family members have already proven to represent promising therapeutic tools for cancer treatment [55,56].

For example, *Onconase*, an amphibian ribonuclease belonging to the RNase A family, has been included in clinical trials for the treatment of mesothelioma, a rare tumor with no effective treatments to date. We reckon that human RNASET2 might soon be included in the list of therapeutically useful anti-cancer ribonucleases.

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