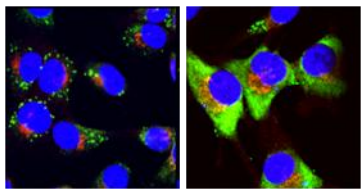
SAPIENZA

UNIVERSITA' DI ROMA

DOTTORATO DI RICERCA IN MEDICINA SPERIMENTALE
XXIX CICLO

"Fatty acids role in cancer: Stearoyl-CoA desaturase 5 and its product oleic acid reduce melanoma dissemination by impairing tumor microenvironment"



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A chi ha asciugato le mie lacrime

ed accarezzato i miei sorrisi

A Voi, la mia bussola, il mio porto sicuro dove posso sempre approdare

A Te, il mio vento in poppa, il mio compagno di Viaggio

Ed alla mia stella che mi indica sempre il cammino

Grazie

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ABSTRACT

Breakthroughs in the understanding of the basic biology of melanoma have yielded continue progresses on a variety of fronts. In the past decade, several specific inhibitors have been utilized against melanoma. However, after a short period of remission, their clinical use inevitably evidenced drug resistance and disease exacerbation. Finding new arms against the advanced phase of melanoma is one of the major challenges in the struggle against this cancer. New insight highlighted the rewiring of cellular signaling and the reprogramming of metabolic pathways in cancer. In normal cells a precise balance between saturated and unsaturated Fatty Acid (FA) synthesis is required for maintaining cell homeostasis. Conversely the anabolic pathways responsible for constructing these molecules appear altered during tumorigenesis. It is now appreciated the importance of fatty acid regulation in malignant disease and the opportunities to target these pathways, required not only for cell growth and division, but also for tumor dissemination.

The role of the stearoyl-CoA desaturase 5 (SCD5) was investigated in melanoma, being its pathophysiological function virtually unknown. This enzyme, converting saturated into monounsaturated FAs, is downregulated during progression of melanoma by epigenetic and miR221/-222-dependent mechanisms. SCD5 restored expression significantly reduced melanoma malignancy, in human A375M melanoma and in murine 4T1 mammary carcinoma cell lines, mainly by inducing oleic acid. In both cell systems we

also evidenced SCD5 effects on tumor microenvironment, through reduced secretion of protumoral protein such as the Secreted Protein Acidic and Rich in Cysteine (SPARC), Collagen IV and Cathepsin B. The net effect of SCD5 action was evidenced by the intracellular acidification (pHe > pHi) and, in turn, by the inhibition of the vesicular trafficking across plasma membranes. This acidification also depends on SCD5-induced reduction of the C2 subunit of the proton pump vacuolar H+-ATPase. Supplementation of oleic acid was per se able to mimic SCD5 enforced expression by reducing the protumoral matrix protein secretion.

Our data support a role for SCD5 and its enzymatic product, oleic acid, in protection against malignancy, offering an explanation for the beneficial Mediterranean diet. Furthermore, SCD5 appears to functionally connect tumor cells and the surrounding stroma, with consequences on tumor spread and eventually resistance to treatment.

INTRODUCTION

1. MELANOMA

Melanocytic neoplasms range from benign lesions, termed melanocytic naevi, to malignant ones, termed melanomas. All originate from melanocytes, neural crest-derived cells, that during development colonize the skin, eye and a board range of other tissues throughout the body (Mort RL., 2015). The incidence of melanoma has more than doubled in the white population over the last 30 years, and melanoma currently is the sixth most common cancer in the United States (American Cancer Society, 2016). and it still continues increasing (Fig. 1). At present melanoma represents the tenth most common cancer in men and the seventh in women (Rapporto AIOM-AIRTUM 2015).

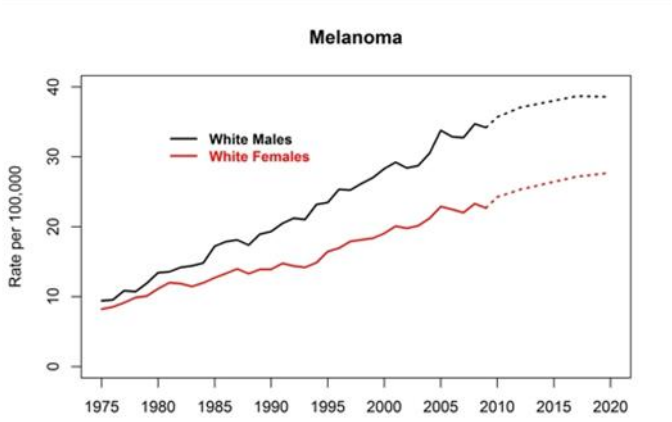


Fig 1: Expected incidence rate of melanoma, United States 1975-2020
www.cdc.gov/cancer

While melanoma accounts for roughly 4% of all skin cancers, it is responsible for nearly 75% of skin cancer deaths. Treatment of melanoma in its early stages provides the best opportunity for cure. The most common types of melanoma in Caucasians are found on sun-exposed skin. This cutaneous melanomas can be broadly categorized by their origins from skin that is or not chronically sun damaged (CSD) and non-CSD melanomas, respectively.

CSD and non-CSD melanomas differ in their anatomical sites of origin, degree of cumulative exposure to ultraviolet (UV) radiation, host age, mutation burden and types of oncogenic alteration (Shain AH., 2016). CSD melanomas arise on skin that shows macroscopic and microscopic signs of long-term exposure to UV radiation, specifically marked solar elastosis. Thus CSD melanomas typically originate from the head, the neck and the dorsal surfaces of the distal extremities of older individuals (>55 years of age). They have a high mutation burden and are associated with neurofibromin 1 (NF1), Neuroblastoma RAS Viral Oncogene Homolog (NRAS), B-Raf Proto-Oncogene, Serine/Threonine Kinase (BRAF) nonV600E or KIT Proto-Oncogene Receptor Tyrosine Kinase (KIT) mutations.

In contrast, non-CSD melanomas typically affect the more intermittently sun-exposed areas such as the trunk and proximal extremities of younger individuals (<55 years of age) that do not show marked solar elastosis. They are associated with a moderate mutation burden and a predominance of *BRAF* V600E mutations (Bastian BC., 2014). Primary melanomas are often found in

association with different types of precursor lesions, ranging from benign naevi and dysplastic naevi to melanoma *in situ*. Idealized progression models often imply a single path of evolution from naevus, to dysplastic naevus, to melanoma *in situ*, to invasive melanoma (Fig. 2).

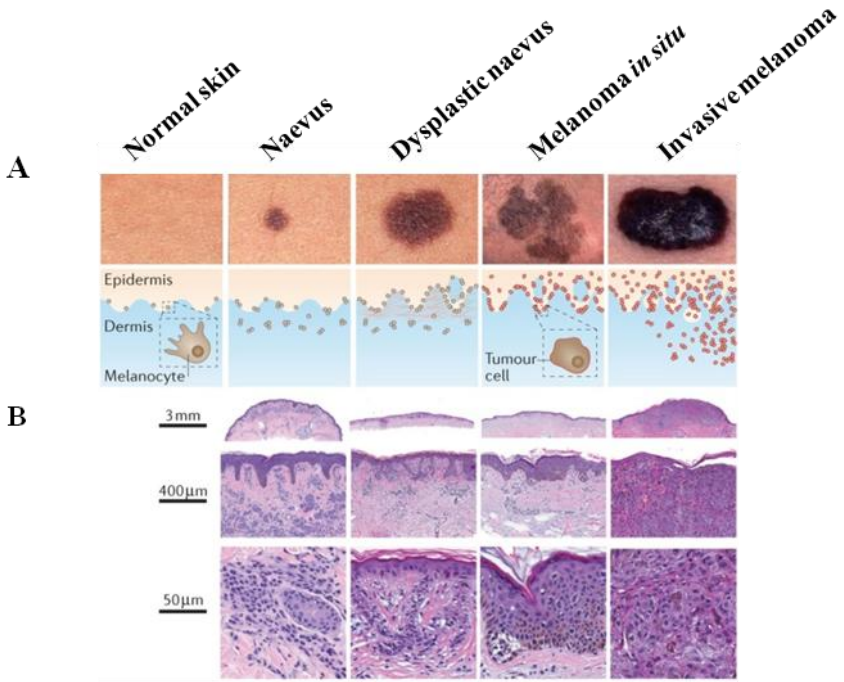


Fig 2: The morphological spectrum of melanocytic neoplasms.
(A) Clinical images showing a free-standing naevus, a dysplastic naevus, melanoma in situ and invasive melanoma, with schematics illustrating the architectural features for each type of lesion and (B) histopathological features. (Adapted from Shain AH, Nat Rev Cancer. 2016)

1.2. RISK FACTORS

There is a complex interactions between exogenous environmental and endogenous factors (Cohen C., 2002; Maldonato JL., 2003). The most relevant environmental factor is intermittent sun exposure, while intrinsic factors are represented by individual's family history and inherited genotypes.

Although up to 65% of malignant melanomas may be related to sun exposure, the role of chronic sun exposure is still controversial in view of host factors such as skin and hair color (see Fitzpatrick skin classification, (Table 1).

Skin phototype	Typical features	Sun exposure risk
Type I	Tends to have freckles, red or fair hairs and blue or green eyes	Often burns, rarely tans
Type II	Tends to have light hairs and blue or brown eyes	Usually burns, sometimes tans
Type III	Tends to have brown hairs and eyes	Sometimes burns, usually tans
Type IV	Tends to have dark brown eyes and hairs	Rarely burns, often tans
Type V	Naturally black-brown skin. Often has dark brown hairs and eyes	
Type VI	Naturally black-brown skin. Usually has dark brown hairs and eyes	

Table 1: Fitzpatrick Skin phototypes classification, features and sun exposure risk

Moreover the development of melanoma among albinos is a very rare occurrence (Perry PK. 2001), which further questions the role of UV light in melanoma genesis. A multifactorial model of cancerogenesis with complex interactions between genes and environment is likely to be the explanation for these apparently conflicting evidences.

Other risk factors in developing melanoma include:

-dysplastic naevi which are histologically characterized by the presence of an architectural disorder with some fusion of rete ridges, lymphocytic infiltrate in the upper dermis and nuclear atypia of the melanocytes in the nest (Hussein MR., 2005, Naeyaert JM., 2003). Naevi number is a very useful intermediate phenotype for melanoma as it is to date the highest risk factor for this tumor and this trait can easily be documented in all Caucasian populations whilst melanoma is rare.

-race: as melanoma is much more frequent in whites, being the proportion between Caucasians and Asian/Black population about 20:1 (Balch CM., 2003). In non-whites melanoma is rare and mostly confined to non pigmented sites such as the subungual regions, the palms of the hand and the soles of the feet. Although the incidence is lower, the mortality rate in non-white patients is higher, which might be due to late diagnosis.

-phototype: malignant melanoma risk is more than doubled in people with skin phototype I compared with skin phototype IV (Olsen CM., 2010) (for skin phototype features see Table 1).

1.2.1. SPORADIC MELANOMA

Over the past decade, it has become evident that subsets of melanoma can be defined at molecular level by recurrent "driver" mutations that occur in multiple oncogenes, mainly BRAF, NRAS, Mitogen-Activated Protein Kinase Kinase 1 (MEK1) and KIT (Table 2). Such driver mutations lead to constitutive activation of mutant signaling proteins that induce specific pathways sustaining tumorigenesis (My cancer genome, 2016).

Gene symbol	Mutation frequency in melanoma	Refs
BRAF	37-50% (>85% in benign naevi)	Hodis, 2012
NRAS	13-25%	Curtin, 2005
MEK1	6-7%	Nikolaev, 2012
KIT	2-8%	Handolias, 2010

Table 2: Main melanoma mutated genes
(Adapted from Lovly, C., J. Sosman, W. Pao. 2015. My Cancer Genome)

The activation of Mitogen-Activated Protein Kinases (MAPK) pathway through mutations of BRAF appears to be the most common. Although the activation of the MAPK signaling (Fig. 3) has been recognized as a critical event, Khavari and colleagues have demonstrated that activation of BRAF–MAPK requires concomitant PI3K–AKT pathway alteration to affect melanoma development

(ChudnovskyY., 2005). Consistent with these data, over 60% of human melanomas exhibit activated AKT (Dhawan P., 2002) and mutational inactivation and/or deletion of the PI3K negative regulator PTEN occurring in 30–40% of melanoma cell lines (Sherr CJ., 2001).

- **BRAF**

The most prevalent BRAF mutations detected in melanoma are missense that introduce an amino acid substitution at valine 600. Approximately 80–90% of V600 BRAF mutations are V600E (valine to glutamic acid), while 5-12% are V600K (valine to lysine) and 5% or less are V600R (valine to arginine) or V600D (valine to aspartic acid) (Lovly CM., 2012). The result of these mutations is to enhance BRAF kinase activity increasing phosphorylation of downstream targets, particularly MEK (Wan PT., 2004**)**. In the vast majority of cases, BRAF mutations are non-overlapping with other oncogenic mutations found in melanoma (e.g., NRAS mutations, KIT mutations, etc.). Clinically, BRAF inhibitors have been investigated in combination with MEK inhibitors in subsets of patients with BRAF V600E mutation-positive melanoma previously resistant to BRAF inhibitors (Johnson DB., 2014; Ribas A., 2014).

- **NRAS**

In the majority of cases these mutations are missense mutations which introduce an amino acid substitution at positions 12, 13, or 61. The result of these mutations is constitutive activation of NRAS signaling pathways. NRAS mutations are found in all melanoma

subtypes, but may be slightly more common in melanomas derived from chronic sun-damaged skin (Ball NJ., 1994).

- **MEK1**

The prevalence of MEK1 mutations in different melanoma subtypes is not yet known. However, most of the reported MEK1 mutations involve C>T and G>A nucleotide changes, which frequently result from exposure to UV radiation (Nikolaev SI., 2012).

- **KIT**

Somatic point mutations in melanoma tumor specimens have been detected predominantly in the juxtamembrane domain, but also in the kinase domain of KIT. They can induce ligand-independent receptor dimerization, constitutive kinase activity and in turn transformation (Growney JD., 2005).

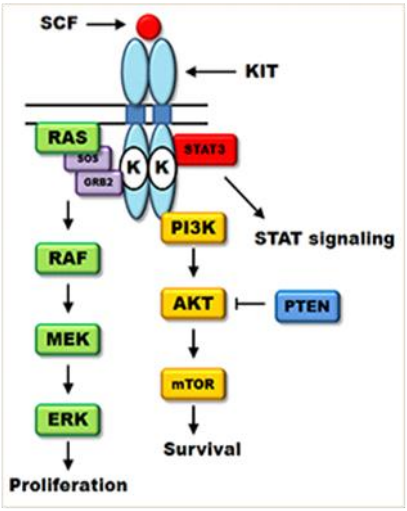


Fig 3: MAPK pathway
(Lovly et al., 2015. My Cancer Genome)

1.2.2. FAMILIAR MELANOMA

Intrinsic risk factors belong to the familiar melanoma, which include mutations or total loss of selected Tumor Suppressor Genes (TSGs). Familial melanoma patients tend to have an earlier age at first melanoma diagnosis, thinner tumors and higher frequencies of multiple primary melanomas (MPMs) respect to sporadic melanoma patients (Hansson J., 2008). A family history of melanoma greatly increases a person's risk because of mutations in three high-penetrance and one low-penetrance melanoma predisposing genes. The best understood melanoma susceptibility gene is cyclin-dependent kinase inhibitor 2A (CDKN2A) on chromosome 9. Hereditary mutations in this gene underlie susceptibility to melanoma in 40% of families with 3 or more cases of melanoma (Goldstein AM., 2007). The CDKN2A locus codes for two alternatively spliced products: p16 and p14ARF. P16 binds to CDK4 and is a cell cycle inhibitor (Kamb A., 1994) playing a role on cellular senescence, specifically inducing melanocyte senescence (Sviderskaya EV., 2003). P14ARF is a tumor suppressor gene too. Mutations at the CDKN2A locus may lead to melanoma susceptibility if they impact on p16 alone, p14ARF alone (Randerson-Moor JA., 2001; Rizos H., 2001), or on both p16 and p14ARF. Very rare families exist (2% of families with detectable mutations) who have hereditary mutations in the CDK4 gene which code for the p16 binding site (Goldstein AM., 2000; Zuo L., 1996). The lifetime risk (i.e. penetrance) of melanoma in CDKN2A

mutation carriers is high, but variable, ranging from 58% in Europe to 91% in Australia by the age of 80 years (Bishop DT., 2002), thus underscoring the importance of the environment (geographical location) even in familial melanoma.

Mutations in these gene products are inherited in an autosomal dominant mechanism and confer 'extremely high risk' (Pho L., 2006). Besides high-penetrance genes we can also find low-penetrance genes, as the melanocortin receptor gene (MC1R), located on 16q24.3, which is established as a determinant of susceptible phenotypes such as red hair (Raimondi S., 2008), sun sensitivity in the absence of red hair (Healy E., 2000) and freckles (Bastiaens M., 2001). MC1R gene encodes the G-protein coupled receptor for α-melanocyte stimulating hormone (α-MSH) (Hayward NK., 2003). Binding of α-MSH to the receptor increases the expression of enzymes involved in the production of melanin in melanocytes. Other pigmentary genes as tyrosinase (TYR), TYR related protein 1 (TYRP1) agouti signalling protein, (ASIP) have been also recently identified as melanoma susceptibility genes (Gudbjartsson DF., 2008).

1.3. DIAGNOSIS AND HYSTOLOGICAL CLASSIFICATION

It is worth to mention the mnemonic of melanoma risk factors "*ABCDE"*. It is a simple but extremely important "must to know" that every health care giver should be familiar with since it may prevent a potentially curable atypical melanocytic proliferation from

progressing to malignant melanoma. These *ABCDE* are abbreviation for: ***A****SYMMETRY*, ***B****ORDER* irregularity (map like configuration), ***C****OLOR* variegation (more than one color: light-dark brown color is usually due to presence of melanin in epidermis, blue and black colors due to melanin in dermis; red color due to inflammation and white color due to fibrosis in regression), ***D****IAMETER* more than 0.6 cm and ***E****VOLUTION* (Healsmith MF., 1994).

For the diagnosis of malignant melanoma several other factors must be evaluated:

•Type of melanoma

•Thickness

•Ulceration

•Infiltration of vessels

•Resection margins

•Mitotic rate, or Clark`s level if mitotic rate cannot be determined reliably.

•In addition, possible lymphocyte invasion, presence of necrosis, regression and neurotropism (tumor growth along nerves) should be considered.

In 1967 malignant melanomas were originally classified by Dr. Wallace Clark and co-workers into several subtypes including. Superficial Spreading Melanoma (SSM), Nodular Malignant Melanoma (NMM) and Lentigo Malignant Melanoma (LMM). Later in 1976 Dr. Richard Reed added another subtype; i.e., Acral

Lentiginous Malignant Melanoma (ALMM). Histological criteria for diagnosing malignant melanoma subtypes is basically is related to the location of the melanocytes (lentiginous, Pagetoid, etc), organization pattern of melanocytes (nested or single cells), cytological features, location of malignant melanoma (for instance acral or subungual region) and other morphologic features such as lymphocytic host response.

The major malignant melanoma subtypes are as follow:

1) **Superficial spreading melanoma (70%)** is by far the most common type, accounting for about 70 percent of all cases. This is the one most often seen in young people. As the name suggests, this melanoma grows along the top layer of the skin for a fairly long time before penetrating more deeply. The first sign is the appearance of a flat or slightly raised discolored patch that has irregular borders and is somewhat asymmetrical in form. The color varies, and you may see areas of tan, brown, black, red, blue or white. This type of melanoma can occur in a previously benign mole. Melanomas can be found almost anywhere on the body, but is most likely to occur on the trunk in men, the legs in women, and the upper back in both.

2) **Nodular melanoma (15%)** is usually invasive at the time it is first diagnosed. The malignancy is recognized when it becomes a bump. It is usually black, but occasionally is blue, gray, white, brown, tan, red or skin tone. The most frequent locations are the trunk, legs and arms, mainly of elderly people, as well as the scalp

in men. This is the most aggressive form of the melanomas.

3) **Lentigo maligna (10%)** is similar to the superficial spreading type, as it also remains close to the skin surface for quite a while, and usually appears as a flat or mildly elevated mottled tan, brown or dark brown discoloration. This type of in situ melanoma is found most often in the elderly, arising on chronically sun-exposed, damaged skin on the face, ears, arms and upper trunk. Lentigo maligna is the most common form of melanoma in Hawaii. When this cancer becomes invasive, it is referred to as lentigo maligna melanoma.

4) **Acral lentiginous melanoma** (**5%**) also spreads superficially before penetrating more deeply. It is quite different from the others, though, as it usually appears as a black or brown discoloration under the nails or on the soles of the feet or palms of the hands. This type of melanoma is sometimes found on dark-skinned people, and can often advance more quickly than superficial spreading melanoma and lentigo maligna. It is the most common melanoma in African-Americans and Asians, and the least common among Caucasians. (Skin Cancer Foundation).

1.4. PROGNOSTIC FACTORS

Malignant melanoma prognostic factors can be classified in three groups:

A) Morphological (histology) factors

B) Clinical Factors

C) Other Factors

In the first group we include lots of different aspects, including Tumor thickness (T), Lymph node involvement (N), Distant metastasis (M), Ulceration, Regression, Mitotic rate, Lymphovascular invasion, Age, Histological type, Tumor infiltrating lymphocytes, Molecular markers. Tumor thickness is one of the most important prognostic available factors, together with nodal involvement and distant metastasis (see pTNM section) (Balch CM., 2001; Balch CM., 2004). In this field we can include 1) the Breslow's system, which is based upon the measurement of the maximum thickness of melanoma from the granular layer of the epidermis to the deepest point of invasion into the dermis; 2) the Clark's levels describing melanoma depth according to the invasion of the four skin layers (level I confined to epidermis, level II with partial infiltration of the papillary dermis by single cells or small nests, level III characterized by the contact between tumor cells and papillary-reticular dermal interface, level IV with reticular dermis infiltration and level V characterized by subcutaneous fat infiltration). The Clark's level is connected with the concept of Radial Growth Phase (RGP) and Vertical Growth Phase (VGP) which was introduce by Dr. Clark itself and his colleagues. Excluding some aggressive variants of nodular malignant melanoma, in general the pathology begins with an intra-epidermal proliferation of transformed melanocytes (RGP). This type of melanoma is always curable with surgical excision and can remains

confined for decades. When an aggressive infiltration of reticular dermis occurs we talk about VGP, that is defined as "dermal nests or expansive dermal nodule(s) composed of fully transformed malignant cells which exceeds the size of any junctional nest(s)". The importance of the Vertical Growth Phase is connected with the fact that melanoma acquires the capability to metastasize, linking the prognosis with the depth of invasion.

In particular the pTNM stages is able to identify four different groups with different pathological outcome.

-**Stage I**: characterized by a good overall survival (OS) rates (5-year OS ≥90%), underlying the importance of early diagnosis;

-**Stage II:** associated with a very variable outcome, the 5-year OS ranging from 45% (stage IIC) to 78% (stage IIA). The balance between this survival range is connected with the presence of ulcerations, adverse prognostic factors that shift patients with the same tumor thickness from stage IB to IIA (T2), from IIA to IIB (T3), and from IIB to IIC (T4). The impact on survival is evident as T4 melanoma (thickness >4 mm) is associated with a 67% 5-year OS rate when ulceration is absent (T4a, stage IIB), but the survival rate drops to 45% when ulceration is present (T4b, stage IIC).

-**Stage III:** survival rates in part overlap with that of stage II (5-year OS rates range from 24% to 69%). With stage III disease, only 49% of all patients with nodal metastases survive 5 years (37% at 10 years), but the range of melanoma-specific survival is large, going from 13% at 5 years for patients with the combination of the worst

risk factors (ulceration of the primary tumor, high regional lymph node burden) to 69% at 5 years for the most favorable prognostic factors.

-**Stage IV:** characterized by a miserable prognosis, although some variability is found according to the site of metastasis and the lactate dehydrogenase (LDH) plasma levels (5-year OS rates range from 9% to 18%).

Of note, there is a significant variability in overall survival rates within each single TNM stage and substage (Fig. 4).

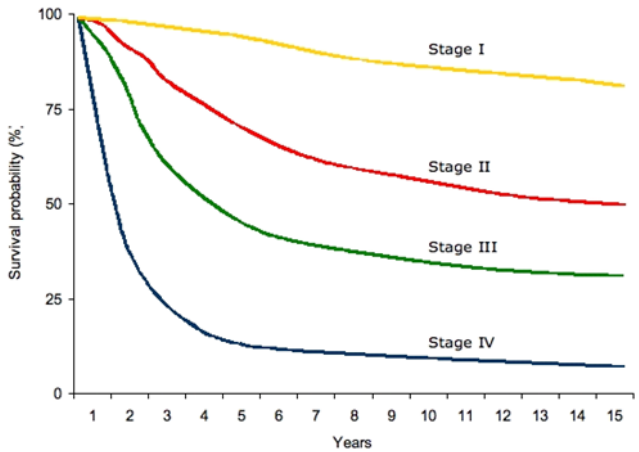


Fig 4: American Joint Commitee on Cancer TNM stages and overall survival of patients with cutaneous melanoma (Molecula Melanoma Map Project)

1.5. TREATMENT OPTIONS

Different type of treatment are available for melanoma patients:

1.5.1. SURGERY

Surgery removal of the tumor is the primary treatment of melanoma at all stages. A wide local excision is used to remove the tumor lesion and some of the normal tissue around it. If the tumor is removed very early, no other treatment is usually needed.

1.5.2. TARGET THERAPY

Targeted therapy is a form of treatment in which drugs are developed with the goal of destroying cancer cells while leaving normal cells intact. These drugs are designed to interfere with the specific molecules that are driving the growth and spread of the tumor. While BRAF inhibitor therapy is associated with clinical benefit in the majority of patients with BRAF V600E-mutated melanomas, resistance to treatment and tumor progression occurs in nearly all patients, usually in the first year (Sosman JA., 2012). A variety of mechanisms have been implicated in primary and acquired resistance to BRAF inhibitors, primarily through reactivation of the MAPK and other cell signaling pathways.

- **Vemurafenib (Zelboraf®)**

Was approved by the FDA in 2011 for the treatment of BRAF V600E mutant melanoma that cannot be removed by surgery. This drug is only approved for those patients who have tested positive

for the BRAF mutation.

- **Vemurafenib (Zelboraf®) + Cobimetinib (Cotellic®)**

Was approved by the FDA in November 2015 for the treatment of BRAF V600 mutant melanoma (https://clinicaltrials.gov/show/NCT02427893). Vemurafenib was previously approved in 2011 but in research studies, this combination showed improved progression-free and overall survival compared to Vemurafenib alone, (Larkin J., 2014)

- **Dabrafenib (Tafinlar®)**

was approved by the FDA in 2013 for the treatment of BRAF V600 mutant melanoma that cannot be removed by surgery. This drug is only approved for those patients who have tested positive for the BRAF mutation. It is not indicated for the treatment of patients with wild-type BRAF mutation.

- **Trametinib (Mekinist®)**

was approved by the FDA in 2013 for the treatment of BRAF V600E or V600K mutations. It is a first-in-class MEK inhibitor approved for the treatment of unresectable or metastatic melanoma. It is not indicated for the treatment of patients who have received a prior BRAF inhibitor therapy.

- **Dabrafenib (Tafinlar®) + Trametinib (Mekinist®)**

received accelerated approval by the FDA in 2014 for demonstrating durable responses in patients with unresectable or metastatic melanomas that carry the BRAF V600E or V600K mutation. Randomized trials are in progress to assess the ability of

the combination to improve time to progression and overall survival compared with dabrafenib alone (https://clinicaltrials.gov/show/NCT02196181) (Robert C., 2015).

1.5.3. IMMUNOTHERAPY

Advanced melanomas that recur after surgery are difficult to treat with radiation and chemotherapy. New treatments, such as immunotherapies, offer benefits for these more advanced cancers. Immunotherapies stimulate the body's immune system to attack cancer cells in various ways. Some forms of immunotherapy are already approved and routinely used to treat some melanomas, while others are still experimental (**Seattle Cancer Care Alliance**).

- **Interferon**

After surgery to remove melanoma, interferon is sometimes recommended to reduce the chance of melanoma returning. Another form of immune-stimulating treatment, but less powerful than ipilimumab, interferon is being tested in patients after surgery. While interferon has been a standard FDA-approved treatment for nonmetastatic melanoma following surgery, its ability to prevent relapse is very weak, and it causes many side effects (**Seattle Cancer Care Alliance**)

- **Interleukin-2**

A synthetic version of interleukin-2 (IL-2), a natural cytokine (protein that can stimulate the immune system), may be used to control advanced melanomas. Sometimes IL-2 is given along with

chemotherapy (Ridolfi R., 2002). It has been used for several decades and is sometimes used to treat patients whose melanoma has spread. The potential benefit is that some patients on this therapy may achieve long-term complete remission, but due to the potential for severe side effects IL-2 use is limited.

- **Tumor-Infiltrating Lymphocytes**

Tumor-infiltrating lymphocyte (TIL) therapy is another promising form of immunotherapy being investigated in people with stage IV melanoma. TILs are unique because they are immune system cells that naturally exist in people and that infiltrate melanoma tumors.

TILs can recognize and attack melanoma cells, but melanoma responds by suppressing TILs and placing them in a sleeping state. Doctors can remove some suppressed TILs from the patient, reactivate them in the laboratory, grow them into billions, and then reinfuse them into the patient. The idea is that the high number of activated TILs will be able to attack the melanoma more effectively.

Prior to the infusion of TILs, patients receive one week of lymphodepleting chemotherapy, which prepares their body by clearing out immune cells suppressed by their cancer and making space for the incoming TILs. After the TIL infusion, patients receive high-dose IL-2, which "feeds" the freshly infused TILs so they survive longer and attack cancer cells more effectively. The hospital stay for chemotherapy, TIL infusion, and high-dose IL-2 is around 10 to14 days (**Seattle Cancer Care Alliance**).

1.5.4. NEW IMMUNOTHERAPIES

- **Drugs anti CTLA-4**

In 2011, the U.S. Food and Drug Administration (FDA) approved ipilimumab (**Yervoy®**) to treat metastatic melanoma. It was the first new drug approved to treat this disease in over 14 years.

Ipilimumab is a monoclonal antibody that sticks to an important protein on blood cells T-lymphocytes, the most important immune-system cells in controlling tumor growth. Ipilimumab blocks a signal on T-cells that suppresses their activation after a normal, vigorous response to infection or inflammation. This suppression protects our tissues from being attacked by our own overactivated T-cells. But it can also prevent our T-cells from attacking tumor cells. Blocking the signal with ipilimumab allows T-cells to recover their activity and kill tumor cells (Fig. 5A). While ipilimumab can provide long-term remission (20% of the cases) (Schadendorf D., 2015) for a fraction of patients, it occasionally causes dangerous side effects resulting from an autoimmune reaction in which the patient's overstimulated immune system attacks not only cancer cells but the patient's healthy tissues.

- **Drugs that Block PD-L1 or PD-1**

In 2015, the European Medicines Agency (EMA) approved **OPDIVO®** (Nivolumab) and **KEYTRUDA®** (Pembrolizumab) as drugs that block PD-L1 or PD-1 and tumor-infiltrating lymphocyte therapies. Melanoma cells often have a protein on their surface called PD-L1 that helps them evade the immune system (Fig. 5B).

The PD-L1 on tumor cells links to a receptor called PD-1 on T-cells, causing the activation of their programmed cell death. This lethal phenomenon can be prevented by blocking the interaction between PD-L1 and PD-1. Blocking either PD-L1 or PD-1 can allow the T-cells to attack the tumor effectively. Antibodies that block PD-L1 or PD-1 may have higher antitumor activities than Ipilimumab (Weber JS., 2015). They also appear to be associated with milder and less frequent autoimmune complications. Combinations of ipilimumab and PD-1-blocking antibodies are being studied because their effects are slightly different, so together they may stimulate the immune system even more effectively than either drug alone.

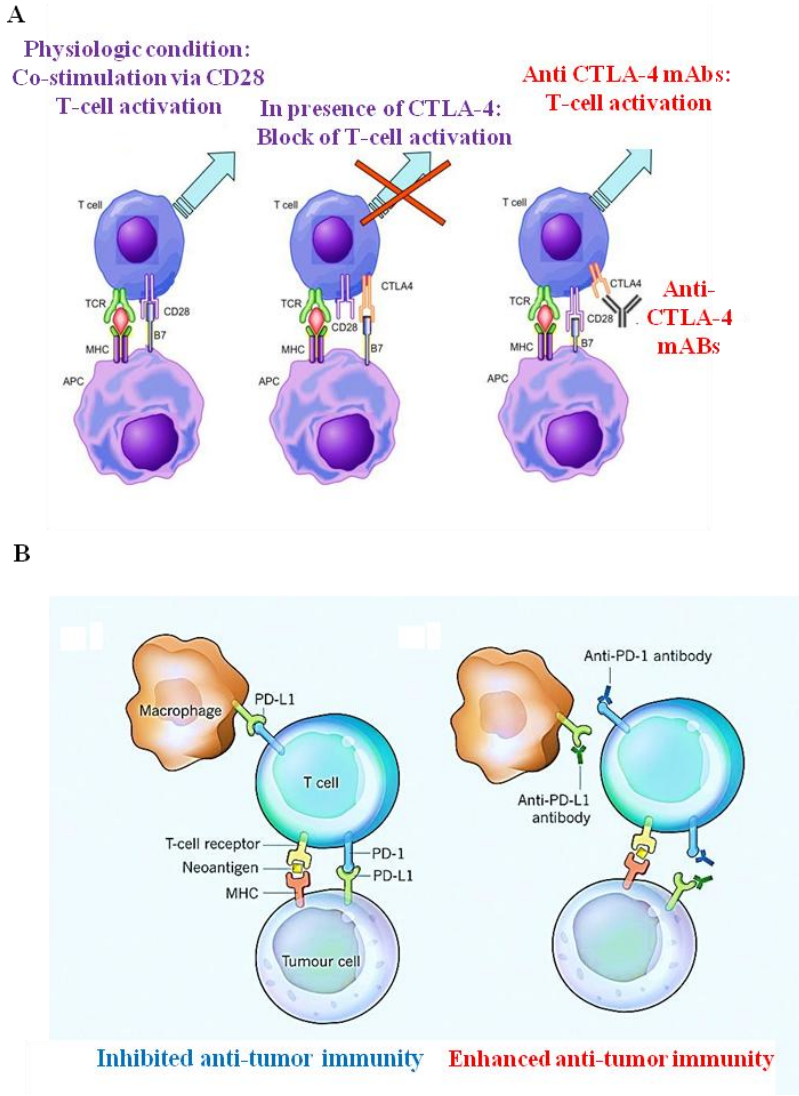


Fig 5: Anti-PD1 and –CTLA-4 mechanisms of action
(A) Adapted from Lesbo et al., ESMO 2008**.** (B) Adapted from http://www.nature.com/nature/journal/v515/n7528/images/515496a-f1.jpg
Antigen-presenting cell (APC), Cytotoxic T-lymphocytes antigen-4 (CTLA-4), Major histocompatibility complex (MHC), T-cell receptor (TCR)

2. THE TUMOR MICROENVIRONMENT

An important regulator of normal cell behavior and tissue homeostasis is the surrounding extracellular matrix (ECM). The ECM has many functions, including acting as a physical scaffold, thus facilitating interactions between different cell types and providing survival and differentiation signals. Maintaining organ homeostasis can prevent neoplastic transformation in normal tissues by ensuring stable tissue structure, mediated by tight junction proteins and cell adhesion molecules such as β1 integrins and epithelial (E)-cadherin (Nelson CM., 2006). However, the presence of transformed tumor cells initiates crucial changes that can convert this environment into one that supports cancer progression (Junttila MR., 2013). In fact, cancers are not just masses of malignant cells, but complex "organs" where many other cells are recruited to be corrupted by transformed cells. Interactions between malignant and non-transformed cells create the tumor microenvironment (TME) (Balkwill FR., 2012). The orchestration of these changes involves recruitment of fibroblasts, migration of immune cells, matrix remodeling and eventually development of vascular networks. Regional differences into the same tumor mass, such as hypoxia, acidity and the presence of growth factors, actively shape its development. A unique environment emerges in the course of tumor progression as a result of interactions between tumor and host. It is created and at all times shaped and dominated by the tumor, which orchestrates molecular and cellular events taking place in

surrounding tissues (Whiteside TL., 2008). The evolution, structure and activities of the cells in the TME have many parallels with the processes of wound healing and inflammation (Grivennikov SI., 2010; Hanahan D., 2011; Mantovani A., 2008).

Although the expansion of neoplastic cells generates the initial insult that instigates the creation of the tumour niche, non-transformed cell types (Junttila MR., 2013) and protein compartment of the ECM (Balkwill FR., 2012) co-evolve with the tumor cells, so that both continuously participate in the process of tumorigenesis (Joyce JA., 2009).

2.1. TUMOR MICROENVIRONMENT ARRANGEMENT

Different stromal cell types have been recruited to the primary tumor site as endothelial cells, pericytes, fibroblasts and various bone marrow derived cells (BMDCs), including macrophages, neutrophils, mast cells, myeloid cell-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs).

- **VASCULATURE**

Vascular networks are derived through formation of new vessels (angiogenesis), co-option and modification of existing vessels within tissues, or recruitment and differentiation of endothelial precursors from bone marrow (vasculogenesis), all of which contribute to vascular heterogeneity in and among tumors. Vessel formation involves degradation and reincorporation of existing vascular basement membranes that vary in a tissue-specific manner

(Kalluri R., 2003). Many soluble factors present in the TME, such as Vascular Endothelial Growth factors (VEGFs), Fibroblast Growth factors (FGFs), platelet-derived growth factors (PDGFs) and chemokines stimulate endothelial cells and their associated pericytes during the neovascularization (Carmeliet P., 2011). The organization of tumor vascularization is abnormal in almost every aspect of its organization and function (Jain RK., 2005): blood vessels are characterized by chaotic branching structures with irregular and permeable vessel lumen. The leakiness of the vessels raises the interstitial fluid pressure causing unevenness of blood flow, oxygenation, nutrient and drug distribution, thus increasing hypoxia and facilitating metastasis.

Microvessel high density has been reported to be a significant prognostic factor for poor outcome in various carcinoma types as non-small-cell lung cancer (NSCLC) (Meert AP., 2002) colorectal (Des Guetz G., 2006) and breast cancers (Uzzan B., 2004). Moreover, elevated expression of the predominant pro-angiogenic ligand VEGF-A has been shown to be associated with a worse prognosis compared with cancer with low expression of VEGF-A in metastatic colorectal, lung and renal cell cancers (Hegde PS., 2013).

- **IMMUNE CELLS**

The immune system collectively functions to recognize and protect tissues from infections and damage. Both the innate and adaptive immune systems have been implicated in promoting and preventing tumor growth (Junttila MR., 2013). Tumor masses are generally

characterized by high heterogeneity of the tumor immune context, being influenced by various additional factors, as the extent and permeability of the vasculature, and by the actions of tumor cells themselves. Even within the same lesion, the distribution of immune cell infiltration is not uniform. In fact the presence of immune cells on the leading edge of the lesion has a different prognostic significance than in central areas, thus indicating the crucial nature of intratumoral localization (Fridman WH., 2012).

- **EXTRACELLULAR VESICLES**

In order to achieve these defining features of the tumor microenvironment, cancer and non cancer cells have to exchange continuously information. One way to sustain this "informational-trafficking" is packaging different molecules into membrane-enclosed vesicles widely known as extracellular vesicles (EVs). They contain cargos such as lipids, proteins, various RNAs and DNA fragments as well as metabolic products. These vesicles, with different subcellular origin, can shuttle these molecules between tumor cells and surrounding stroma inducing signaling pathway or directly altering their phenotype. One kind of EVs, so called exosomes (50-150 nm), origins in secretory multi-vesicular bodies that fuse with the cellular membrane releasing intraluminal vesicles (Wendler F., 2016; Felicetti F., 2016). They have been recently implicated as direct mediators of the response of solid tumors to cytotoxic chemotherapy and as putative "real-time" circulating biomarkers; finally circulating EVs can also transport important

molecules to remote destinations in order to lead metastatic properties in other healthy tissues (Felicetti F., 2016) (Fig. 6).

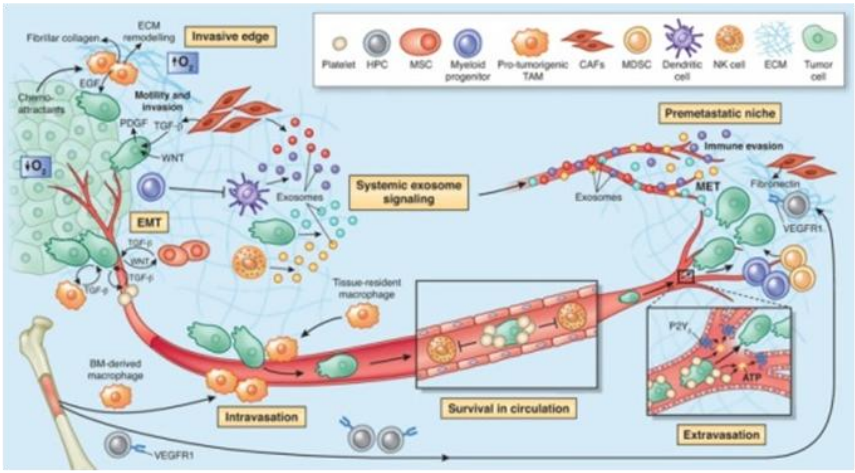


Fig 6: Tumor microenvironment cells component
(Daniela F Quail and Johanna A Joyce, Microenvironmental regulation of tumor progression and metastasis. Nat. Med. 2013).

2.2. *ECM*

The ECM provides not only a physical scaffold for all cells in the TME, but also has a dynamic role in the evolution and spread of cancers, especially considering that cell adhesion to the ECM is a key step for its movement out of and into the TME. The ECM also contains fundamental growth factors, such as angiogenic factors and chemokines, which interact with cell surface receptors giving to each tissue its tensile and compressive strength and elasticity (Frantz C., 2010). Tumors are typically stiffer than the surrounding

normal tissues owing to an increased ECM deposition by CAFs (cancer–associated fibroblasts) (Weigelt B., 2008). Collagen and elastin fibers are reorientated and cross-linked by lysyl oxidase (LOX) and transglutaminase present in the TME, resulting in larger, more rigid fibrils (Levental KR., 2009). Matrix metalloproteases (MMPs), that degrade ECM proteins, are secreted and activated by malignant cells, Tumor-associated macrophages (TAMs) and CAFs. MMPs further remodel the ECM, thereby releasing chemokines, growth and angiogenic factors. Other proteases that are upregulated in the cells of the TME include a large family of cysteine proteases, the cathepsins. Cathepsin L, for instance, processes and activates heparanase, thereby aiding metastasis, angiogenesis and inflammation (Edovitsky E., 2004; Lerner I., 2011). In addition diverse group of matricellular proteins includes the glycoproteins secreted protein acidic and rich in cysteine (SPARC/osteonectin/BM40), thrombospondin (TSP), tenascin C (TNC), osteopontin (OPN) and periostin (POSTN) (Bornstein P., 2002).

2.2.1. SPARC IN CANCER: A FOCUS ON MELANOMA MODEL

SPARC is an extracellular matrix-associated glycoprotein first identified by Termine and coworkers as a major non-collagenous constituent of bovine bone (Termine JD., 1981). SPARC is a single-copy gene with a high degree of evolutionary conservation. The

human gene has been localized to chromosome 5q31-33, whereas the murine homolog is in the central region of chromosome 11. The vertebrate SPARC gene encodes proteins of 298-304 amino acids, whose initial 17 aa constitute a signal sequence that is removed prior to secretion of the protein. SPARC protein structure is organized in three different domains: the NH2-acidic region, the follistatin-like domain and the COOH-extracellular Ca^{2+} region.

The role of SPARC in tissue homeostasis is exemplified by the phenotypes of SPARC-deficient mice characterized by cataract formation and osteopenia, decreased size and tensile strength of dermal collagen fibers, and increased deposition of adipose tissue (Chavey C., 2006; Nie J., 2009). Reported phenotypes were related to defects of fibroblast differentiation and plasticity and increased leukocyte recruitment (Bradshaw AD., 2009; Chiodoni C., 2010, Rentz TJ., 2007; Sangaletti S., 2011). SPARC role in carcinogenesis is particular interestingly as it plays multi-faceted contextual roles depending on the tumor type and on its origin, being either produced by cancer or surrounding stromal cells (Said N., 2005; Said N., 2013; Said N., 2009)

In melanoma, SPARC expression has been reported to increase with tumor progression, and its expression shown to be a marker of poor prognosis (Girotti MR., 2011). Accordingly SPARC knockdown in melanoma cells lead to the complete loss of their *in vivo* tumorigenic growth in nude mice (Ledda MF., 1997) through a mechanism involving the activation of polymorphonuclear cell anti-

tumor activity. Importantly, SPARC expression in melanoma cells has been associated with the acquisition of mesenchymal characteristics with reduced E-cadherin expression (Robert G., 2006). However, the *in vivo* tumorigenicity and invasiveness of melanoma cells injected in nude mice were dependent on SPARC released by tumor cell, but not by fibroblasts (Alvarez MJ., 2005; Prada F., 2007).

2.3. LIPID ROLE IN TUMOR FORMATION AND PROGRESSION

Hallmarks of cancer comprise six biological capabilities acquired during the multistep development of human tumors. These hallmarks constitute an organizing principle for rationalizing the complexities of neoplastic disease. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. The origins of these hallmarks are genome instability, which generates the genetic diversity that expedites the acquisition of these properties, and inflammation, which fosters multiple hallmark functions. Recent data in the last decade has evidenced two emerging hallmarks of tumor formation and progression, such as reprogramming of energy metabolism and evading immune system activation (Fig. 7) (Hanahan D., 2011).

Fig 7: Hallmarks of Cancer: The Next Generation
(Adapted from Hanahan D and Weinberg RA, Cell 2011)

Today the central role played by bioactive lipids and fatty acids (FAs) as mediators of the crosstalk between cancer cells and stroma has been clearly recognized (Beloribi-Djefaflia S., 2016).

The required changes in metabolic activity are driven by oncogenic signals and by the particular organization of the surrounding stroma, which is characterized by poor oxygenation and nutrient scarcity. As a result tumor cells are forced to modify their metabolic balance to overcome this hostile microenvironment. One of the first events in tumor cells metabolism is an alteration on glucose metabolism, known as "Warburg effect" (Warburg O., 1956). Besides these

events, cancer cells show a strong lipid and cholesterol avidity in order to sustain their highly proliferative rate (Zaidi N., 2013).

Lipid synthesis describes the processes that convert nutrient derived carbons into FAs. The first step involved in FA and cholesterol biosynthesis is the production of two-carbon units in the form of acetyl-CoA. Acetyl-CoA is generated from citrate by the enzyme ATP-citrate lyase (ACLY) and then converted to malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC). Acetyl-CoA and malonyl-CoA are then coupled to the acyl-carrier protein domain of the multifunctional enzyme fatty acid synthase (FASN). Repeated condensations of acetyl groups generate a basic 16-carbon saturated FA, named palmitic acid (C16:0). Palmitic acid is further elongated and desaturated to generate the diverse spectrum of saturated and unsaturated FAs synthesized by mammalian cells, as stearic acid (C18:0). One of the main desaturases in mammalian cells are the stearoyl CoA desaturases (SCDs), integral endoplasmic reticulum proteins that catalyse the introduction of the first double bond in the *cis*-Δ9-position to convert saturated FAs (SFAs), palmitic and stearic acid, into mono-unsaturated FAs (MUFAs), palmitoleic acid (C16:1) and oleic acid (C18:1) respectively (Ntambi JM., 2004).

There are two isoforms in humans, SCD1 (also named SCD) (Whang J., 2005), which is highly expressed in adipose tissues, as well as in brain, liver, heart and lung, and SCD5, which is expressed in few normal tissues (brain, pancreas), playing physiological and pathological functions virtually unknown (Igal RA., 2010). In

contrast, the murine genome encodes four SCD (SCD1-4) enzymes with partially overlapping functions. SCD1 was found to be overexpressed in tumors from several cancer types, including prostate (Fritz V., 2010), liver (Huang GM., 2015), kidney (Von Roemeling CA., 2013) and breast cancer, where it correlates with reduced survival (Holder AM., 2013). Antisense-mediated downregulation of SCD expression was found to inhibit proliferation and tumor formation in human lung cancer cell lines (Scaglia N., 2008), thus leading to cell cycle arrest and apoptosis (Hess D., 2010). Further evidence that lipid desaturation is an important metabolic process in tumors is provided by investigating the composition of membrane lipids in human breast cancer samples. Tumor tissues showed an overall increased amount of membrane phospholipids, sphingomyelins and ceramides, while triacylglycerides were unchanged. Interestingly, the most prominent changes were seen in triple negative breast cancers, the most aggressive form of the disease. In contrast to normal breast tissue, lipids from the tumor samples contained a higher proportion of saturated and mono-unsaturated acyl chains, which are indicative of increased reliance on de novo synthesis rather than uptake of dietary lipids, which contain a higher proportion of poly-unsaturated FAs (Hilvo M., 2011).

In particular oleic acid (OA) has attracted much attention, especially in the last years, as the "Mediterranean diet", characterized by high olive oil consumption (rich in OA), has been traditionally linked to

a protective effect against cancer (Trichopoulou A., 2000). A wide range of studies have been conducted into breast cancer, where the potential protective effect of olive oil and OA has been clearly demonstrated (Chajes V., 2008). In addition, epidemiological studies suggest that olive oil may have a protective effect on colorectal cancer development (Bautista D., 1997; Stoneham M., 2000). Some animal studies have also shown that dietary olive oil prevented the development of colon carcinomas also in rat models corroborating the idea that olive oil may have chemopreventive properties against colon carcinogenesis (Bartoli R., 2000).

Membrane Lipid Therapy (MLT) is a novel therapeutic approach in which the drugs are designed to target the membrane of tumor cells, modulating its composition and structure and therefore modifying the activity of membrane-interacting protein. (Llado V., 2014).

Minerval (2-hydroxyoleic acid, 2-OHOA) is an orally bioavailable synthetic analog of the oleic acid that selectively modulates sphingomyelin synthase (SMS) activity, thereby increasing the concentration of sphingomyelin (SM), ceramide (Cer) and diacylglycerol (DAG) in the tumor cell membranes and decreasing the levels of phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylserine (PS). This treatment restores the normal, healthy levels and ratios of membrane lipids, inhibiting membrane-protein associated signaling and the aberrant activity of signaling pathways in tumor cells, including the RAS/MAPK and PI3K/AKT pathways, stopping tumor cell proliferation, inducing

tumor cell differentiation and eventually causing selective cancer cell death by autophagy/apoptosis (J Martinez 2005). Minerval exhibits a wide range of action, inhibiting the proliferation rate in various human carcinoma cells, especially lung and glioma (Martinez J., 2005; Llado V., 2010; Barceló-Coblijn G., 2011; Terès S., 2012, Terès S., 2012, Marcilla-Etxenike A., 2012). The European Medicines Agency (EMA) designated 2-OHOA as an orphan medicinal product for the treatment of glioma in October 2011 (http://www.ema.europa.eu).

A novel approach to chemotherapy has the potential to yield novel dietary-drug combinations that can provide additive or even synergistic protection against the progression of cancer. With regard to this novel approach, OA has been reported to act synergistically with cytotoxic drugs, thus enhancing their antitumor effect (Menéndez JA., 2001).

Recently Sujin Jung and colleagues manufactured an alternative complex using liposome as an oleic acid delivery vesicle, called this nano-lipo-complex *LIMLET* (LIposome Made LEthal to Tumor cell). They tested its cytotoxicity against two cancer cell lines, MDA-MB-231 (human breast cancer) and A549 (human lung cancer), demonstrated that *LIMLET* showed distinctive cytotoxicity against A549 and MDA-MB-231 cells. Conversely, empty-liposomes (containing no oleic acid) had no toxicity, even at high concentrations. Also this lipo-complex demonstrated selective, concentration-dependent toxicity against these cancer cells (Jung S.,

2016). Little is known about the molecular mechanisms basing the antitumor effects of oleic acid. Key studies evaluating these are summarized below (Fg. 8).

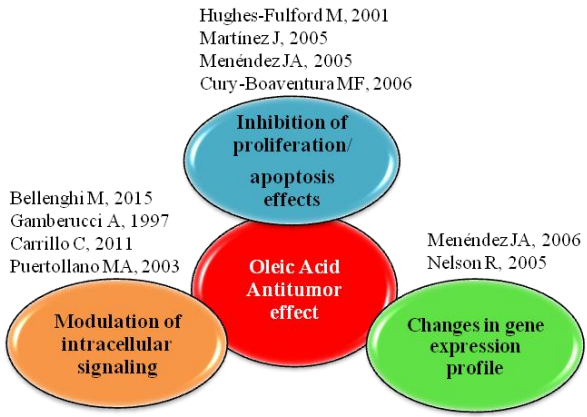


Fig 8: Antitumoral roles of Oleic Acid
(inspired from Carrillo C. Nutr. Hosp., 2012

3. MicroRNAs

Multiple types of small RNAs have evolved in eukaryotes to regulate genetic material and transcripts. These small RNAs are classified in three different family in animal: microRNA (miRNA), siRNA and PIWI-interacting RNA (piRNA).

At present microRNAs represent the dominant class of small RNAs in most somatic tissues, functioning as guide molecules in RNA post-trancriptional regulation. Targeting most protein-coding transcripts these molecules are involved in all developmental an pathological processes. Mature miRNAs produced by two RNaseIII protein, Drosha and Dicer are 21-25 nucleotides in length.

Repression is obtained by miRNA base pairing with mRNA targets in presence of the effector protein AGO. This protein functions by recruiting factors that induce translational repression, mRNA deadenylation and mRNA decay (Huntzinger E., 2011). Binding sites for miRNAs are usually, but not always, located in the 3´ untranslated region (UTR) of mRNAs (Bartel DP., 2009). More than 60% of human protein-coding genes contain at least one conserved miRNA-binding site and, considering that numerous non-conserved sites also exist, most, if not all, protein-coding genes may be under the control of miRNAs (Friedman RC., 2009). Thus, it is not surprising that the biogenesis and function of miRNAs themselves are tightly regulated, and that their dysregulation is often associated with human diseases, including cancer (Lujambio A., 2012) and neurodevelopmental disorders (Im HI., 2012).

3.1. MicroRNAs BIOGENESIS

MiRNA genes are transcribed by RNA polymerase II and their sequences are located within various genomic contexts. In humans, the majority of canonical miRNAs are encoded by introns of non-coding or coding transcripts, and few miRNAs by exonic regions. Often, several miRNA loci are in close proximity to each other, constituting a poly-cistronic transcription unit (Lee Y., 2002). In general, miRNAs belonging to the same genetical cluster are co-transcribed, but each single microRNA can be additionally regulated at post-transcriptional level. Following transcription, the primary miRNAs (pri-miRNA) are submitted to several step of maturation (Lee Y., 2002), typically long over 1kb. The nuclear RNase III Drosha and the cofactor DGCR8 initiate the maturation process by cropping the stem–loop, contained into the pri-miRNAs, to release a small hairpin-shaped RNA of 70-100 nucleotides in length (pre-miRNA) (van Rooij E., 2011). Following Drosha processing, pre-miRNAs ar exported into the cytoplasm, where maturation can be completed. The protein exportin 5 (EXP5), encoded by *XPO5* gene, forms a transport complex with the GTP-binding nuclear protein RAN-GTP and the pre-miRNA (Lund E., 2004). Following translocation through the nuclear pore complex, GTP is hydrolyzed, resulting in the disassembly of the complex and pre-miRNA release into the cytosol, where is cleaved by Dicer near the terminal loop. A small RNA duplex, preferentially loaded into AGO protein, is then delivered to form an effector complex called

RNA-induced silencing complex (RISC) (Bernstein E., 2001; Hammond SM., 2001) which quickly removes the passenger strand to release the mature miRNA (Fig. 9).

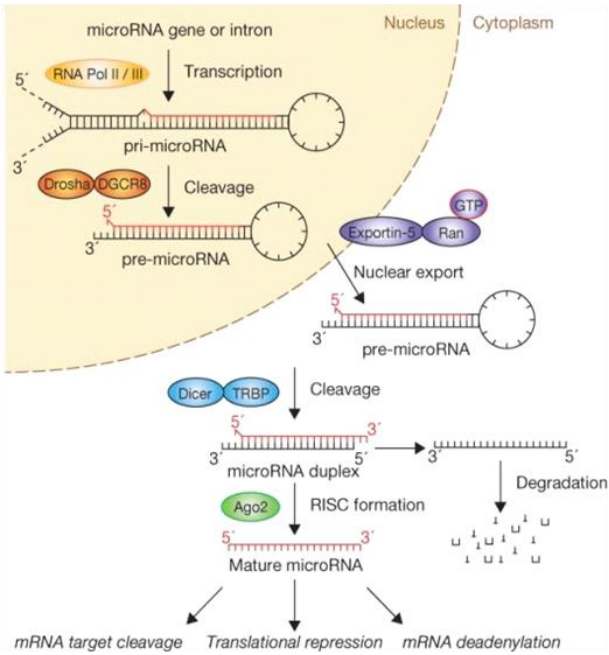


Fig 9: miRNAs biogenesis
(Adapted from Winter J. Nat. Cell. Biol, 2009)

3.2. MicroRNA-221/-222 IN MELANOMA

MiR-221 and -222 are co-transcribed in a common RNA precursor, localized on chromosome X. In addition they share the same seed sequence. Both these characteristics suggest that these two miRs play common functions. Deregulated expression of miR221/-222 has been detected in many different cancers (Ciafrè SA., 2005;

Visone R., 2007; Garofalo M., 2009; Miller Te., 2008; Medina R., 2008; Felicetti F., 2008) where they are mostly upregulated.

Specifically, Felicetti and co-workers reported miR221/-222 increasingly expressed during the multistep process from normal melanocytes to advanced metastatic melanomas (Felicetti F., 2008). To demonstrate their functional role, miR221/-222 have been overexpressed in a moderately aggressive melanoma cell line, selected for its low levels of endogenous miR221/-222 and for its ability to produce melanin. As a direct effect, the enforced miR-221 and miR-222 expression resulted in significant increases of the proliferative growth rate, the invasive and chemotactic capabilities, as well as of the anchorage-independent growth. Their tumorigenic function was also confirmed in an *in vivo* model where miR-221&222 overexpression increased tumor volumes of mice subcutaneously injected with miR-221&222-transduced melanoma cells compared with controls. More important was the opposite effects obtained by silencing of miR221/-222 in metastatic cells, where transfection of chemically modified oligomers (antagomirs) inhibited the main functional properties associated with advanced melanoma.

MiR221/-222 exert their function by repressing a number of antineoplastic genes. In particular in melanoma they inhibit $p27^{Kip1}$ and c-KIT receptor, thus promoting cell proliferation and differentiation blockade (Felicetti F., 2008; Igoucheva O., 2009). $p27^{Kip1}$ is a cell-cycle regulatory protein that interacts with cyclin-

CDK2 and -CDK4, blocking cell cycle progression at the G1/S checkpoint. Regulation of $p27^{Kip1}$ by miR-221 and miR-222 represents an additional oncogenic mechanism underlying the abnormal cell cycle rate of advanced melanoma and of many other tumors (Medina R., 2008; Felicetti F., 2008). MiR221/-222 inhibitory effect on melanogenesis is exerted by repressing the c-KIT tyrosine kinase receptor (Felicetti F., 2008). The stem cell factor (SCF)/c-KIT signaling pathway is essential during melanocytes migration from the neural crest to the skin and it plays an important role in melanogenesis, cell growth, migration, and survival (Alexeev V., 2006). While ubiquitously expressed in mature melanocytes, c-KIT is downregulated in up to 70% of metastases, allowing melanoma cells to escape SCF/c-KIT–triggered apoptosis (Willmore-Payne C., 2005). The transduction signal generated by SCF/c-KIT interaction induces the activation of Microphthalmia-associated transcription factor (MITF) protein, known to regulate a broad repertoire of genes whose functions in melanocytes include development, differentiation, survival, cell-cycle regulation and pigment production. In particular, MITF controls the melanin production directly regulating the main melanogenic enzymes, such as tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1). Reported results showed that, as a consequence of the miR-221&222-dependent suppression of c-KIT, the downstream MAPK signaling cascade is also affected in turn resulting in a moderate downregulation of MITF, TYR and TRP-1

(Fig. 10).

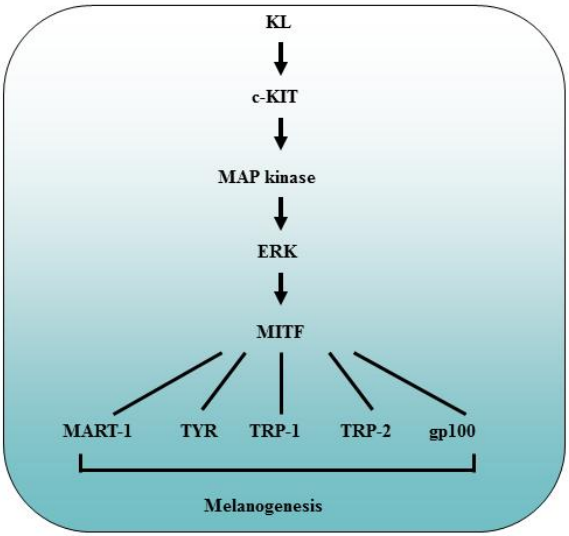


Fig. 10: melanogenesis pathway
In response to the activation of c-KIT signaling, MITF is phosphorylated by MAPK and translocated from the cytoplasm in the nucleus to activate transcription of melanogenic genes.

In accordance, miR221/-222 overexpression in a primary melanoma cell line caused reduction of both differentiation and melanogenesis, as hallmarks of an oncogenic progression (Felicetti F., 2008). The regulation of MITF expression in melanoma cells appears extremely complex as, according to a proposed model, MITF high levels result in cell cycle arrest and differentiation, intermediate levels promote proliferation and tumorigenesis, whereas low amounts lead to cell cycle arrest and apoptosis (Wellbrock C., 2005; Goding C., 2006; Gray-Shopfer V., 2007; Hoek KS., 2010).

3.2.1 REGULATION OF MicroRNA-221/-222 TRANSCRIPTION

Among diverse miRs, particular attention has been paid on the transcriptional regulation of miR221/-222 in different types of cancers, including melanoma, where different groups evidenced both positive and negative regulators of transcription (Garofalo *M., 2012)*

Their regulatory binding sites have been identified in the putative promoter region upstream to pre-miR-222 between -400 and -50 bp (Felicetti F., 2008; Di Leva G., 2010; Mattia G., 2011). Felicetti and colleagues highlighted the first negative regulation system of miR221/-222 transcription played by the Promyelocytic Leukemia Zinc Finger (PLZF) transcription factor in melanoma cell lines. PLZF is a tumor suppressor gene, barely or not detectable in melanomas, but expressed in normal melanocytes. Two putative consensus binding sequences for PLZF are located upstream to premiR-222 and a third site is localized in the intragenic region between the two miR sequences. In advanced melanomas the lack of PLZF allows miR-221/222 up-modulation and, in turn, the activation of two oncogenic pathways involved in melanoma progression, through p27Kip1 and c-KIT deregulation (Felicetti F., 2008) (Fig. 11 A-B).

A more complex function played by ETS-1 transcription factor on miR221/-222 regulation was demonstrated in melanoma (Mattia G., 2011). ETS-1 transcription factor is the founding member of the ETS gene superfamily, encoding a class of phosphoproteins

characterized by a conserved domain that recognizes and binds to a GGAA/T DNA core sequence. The complexity of ETS-1 action has been well demonstrated by the authors that discover its capability of either negative or positive regulation on miR221/-222 transcription, in early and advanced melanoma cells, respectively. ETS-1 post-translational modifications, rather than its total protein content, look functionally relevant to melanoma. In fact, a key role is associated with the status (phosphorylated or not) of Thr38. In low grade malignant cells the presence of significant amounts of barely or not phosphorylated ETS-1 negatively act on miR-221/222 transcription. Conversely, in metastatic melanomas the persistent activation of the MAPK-ERK1/2 cascade increases the fraction of Thr38 phosphorylated ETS-1 inducing miR221/-222 transcription and possibly tumor malignancy (Mattia G., 2011) (Fig. 11 A-B).

Errico and colleagues demonstrated that the HOXB7/PBX2 dimer acts as a positive transcriptional regulator of the oncogenic microRNA221/-222. In addition, demonstrating c-FOS as a direct target of miR-221&222, they identified a HOXB7/PBX2 →miR-221&222 →c-FOS regulatory link, whereby the abrogation of functional HOXB7/PBX2 dimers leads to reduced miR-221&222 transcription and elevated c-FOS expression with consequent cell death. Taking advantage of the treatment with the peptide HXR9, an antagonist of HOX/PBX dimerization, they recognized miR-221&222 as effectors of its action, in turn confirming the HXR9

efficacy in the treatment of human melanoma malignancy, whilst sparing normal human melanocytes (Errico MC., 2013).

More recently Felli and co-workers demonstrate the circuitry connecting the oncomiR221/-222 with the tumor suppressors miR-126/-126* in melanoma development and progression. Accordingly to the inverse correlation between endogenous levels of miR221/-222 and miR-126/-126*, respectively increasing or decreasing with malignancy and looking for a central player in this complex network, they revealed the regulation role of AP2α, which is on one side directly targeted by miR221/-222 and on the other transcriptional activator of miR-126/-126* (Felli N., 2013). These authors showed the chance of restoring miR-126&126* expression in metastatic melanoma to control miR-221&222 levels. Thus, the low-residual quantity of miR-221&222 should assure the release of AP2α expression, which in turn can bind to miR-126&126* inducing their transcription. All together these results point to an unbalanced ratio functional to melanoma malignancy between these two couples of miRs (Felli N., 2015) (Fig. 11 A-B).

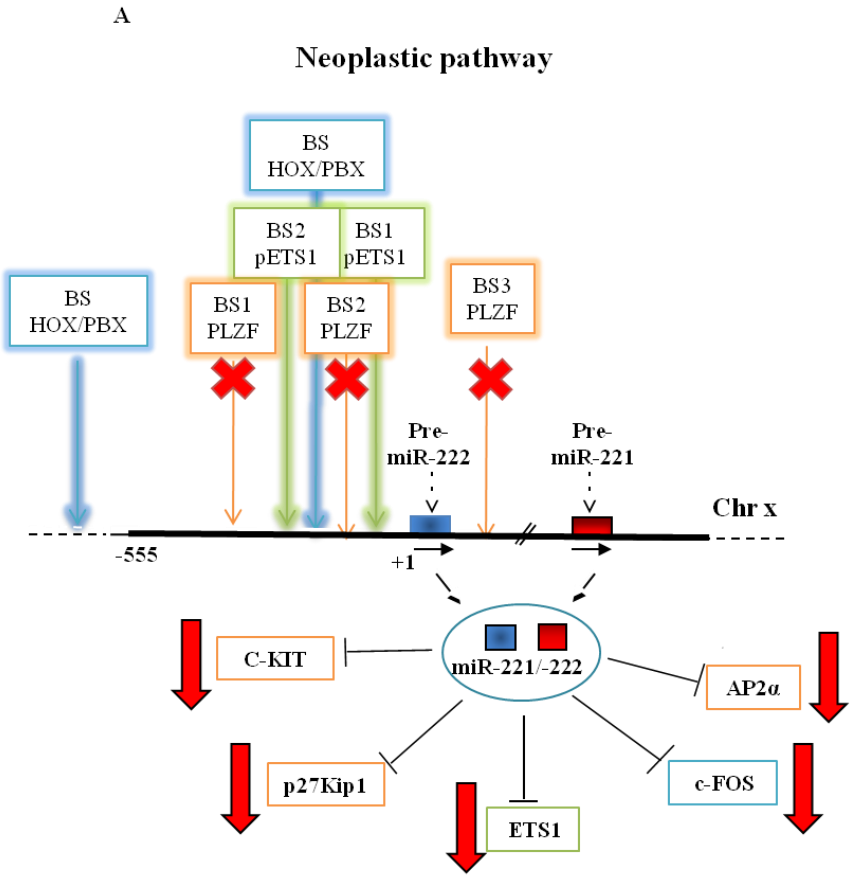
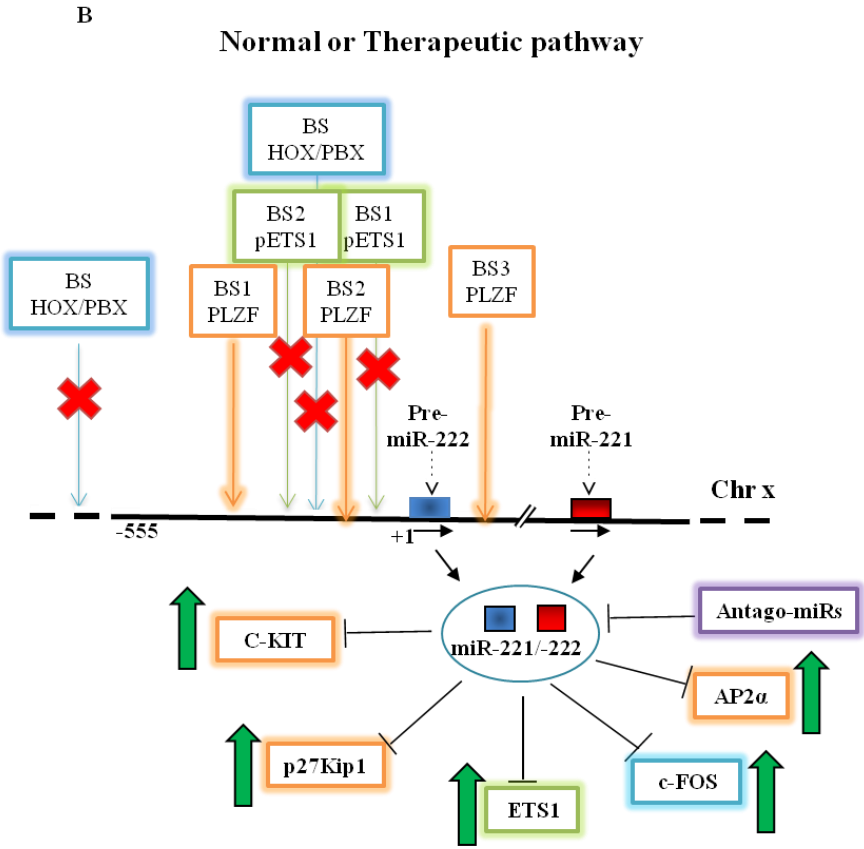


Fig 11: Regulation of miR221/-222 transcription
(A) Neoplastic pathway. (B) Normal or therapeutic pathway

B


Normal or Therapeutic pathway
BS HOX/PBX
BS2 pETS1
BS1 pETS1
BS HOX/PBX
BS1 PLZF
BS2 PLZF
BS3 PLZF
Pre-miR-222
Pre-miR-221
Chr x
-555
+1
C-KIT
miR-221/-222
Antago-miRs
AP2α
p27Kip1
ETS1
c-FOS

AIMS OF THE STUDY

Melanoma is one of the deadliest skin cancers and its incidence is rapidly increasing in the world. Surgical resection is curative only at early stages, but new efforts are needed to contrast the metastatization phase (survival rates 6-8% at 5 years with a median survival of 6-9 months). Thus, the investigation of new therapeutic options must be contextual to the progress of the melanoma biology understandings. Different questions continue to be obscure starting from the high heterogeneity of this tumor and the different metabolic options of melanoma cells that consent them to adapt to and modify the microenvironment, eventually using it for metastatization. In fact, metabolic pathways substantially contribute to human cancer and growing evidences are associating lipids with tumor development and, above all, its progression.

The aim of this study was to reveal the function of the SCD5 Δ9-desaturase enzyme, and eventually of its main biological product oleic acid (OA), in cancer being its pathophysiological role essentially unknown.

We looked at SCD5 role in human melanoma cells, evaluating whether the SCD5-dependent new balance of fatty acids (specifically oleic and stearic acids) could have any effect on tumor progression. In addition, as a second model, we utilized the murine 4T1 mammary carcinoma cell line, as a suitable model for studying spontaneous metastases *in vivo* in syngeneic mouse models.

RESULTS

4. EXPRESSION ANALYSIS OF STEAROYL-COA Δ9 (SCD5) DESATURASE IN MELANOMA.

In view of the increasing role acquired by lipid metabolism in tumor initiation and progression, we focused our study on the role of SCD5 desaturase in melanoma being intrigued by its virtually unknown role in cancer (Igal RA., 2010). We have evaluated by qRT-PCR and/or western blot analyses the expression of SCD5 isoenzyme in normal human epidermal melanocytes (NHEM) from foreskin and in some representative melanoma cell lines derived from tumors at different stages of progression (Fig. 12 A-B).

The expression of SCD5, that was barely detectable in normal melanocytes, resulted significantly higher in primary melanoma cell lines than in metastatic cells, at both mRNA and protein levels.

To confirm the expression pattern of SCD5 observed in melanoma cell lines we decided to run parallel analyses on a series of human bioptic speciments from primary and metastatic melanoma, by using an immunohistochemical approach. Results showed a sharp positivity in primary cutaneous melanomas, whereas SCD5 was virtually absent in the metastatic ones, corroborating the expression data obtained in melanoma cell lines (Fig. 12 C).

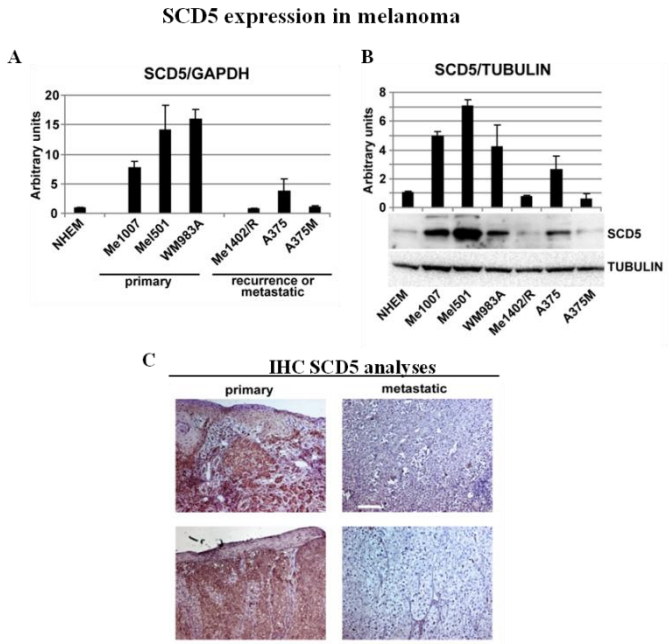


Fig 12: SCD5 endogenous expression levels in differently tumor staged melanoma samples.
Endogenous levels of SCD5 were evaluated in differently staged melanoma cell lines and normal human epidermal melanocytes (NHEM) by (A) qRT-PCR and (B) western blot analyses (mean ± SD of at least three independent analyses). (C) Immunolocalization of SCD5 in primary cutaneous and metastatic human melanoma specimes, representative sections are shown (magnification=10X).

Knowing the biochemical role of SCD enzymes in introducing the first double bond in cis Δ9 position of SFAs (palmitic and stearic acid) to produce MUFAs (mainly palmitoleic and oleic acid, respectively) (Ntambi KM., 2004) (Fig. 13 A), we evaluated by gas chromatography/mass spectrometry (GM/MS) analyses the enzymatic activity of SCD5 on cell lysates from normal human melanocytes as well as from some representative differently staged melanoma cell lines. Interestingly, a close correlation between

SCD5 downregulation and the decreased amount of oleic acid occurring in the more aggressive melanomas was detected, suggesting that SCD5 restored expression or oleic acid supplementation might antagonize the aggressiveness of advanced melanoma (Fig. 13 B).

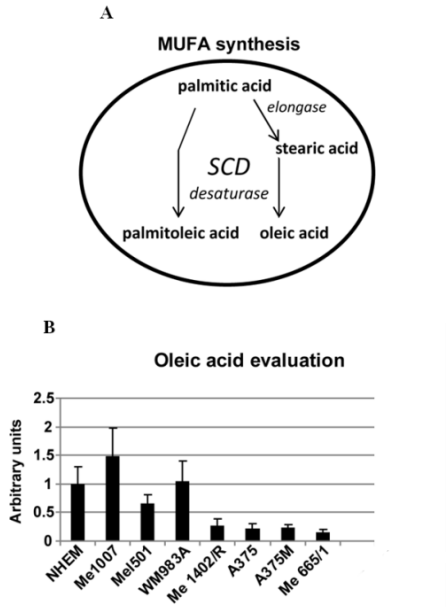


Fig 13: SCD5 biochemical role and oleic acid content in melanoma progression.
(A) Schematic depiction of the SCD5-dependent conversion of saturated fatty acid (FAs) into mono unsaturated fatty acids (MUFAs). (B) GC/MS based quantification of endogenously produced oleic acid at different stages of melanoma progression. Representative experiments are shown and levels of oleic acid are indicated as fold changes compared to normal human epidermal melanocytes (NHEM).

5. REGULATION OF SCD5 EXPRESSION IN MELANOMA.

Our study on Δ9-Stearoyl CoA desaturase 5 showed that its expression was significantly higher in primary than in metastatic

melanoma, being barely or not detectable in normal melanocytes (Fig. 12 A-B) (Bellenghi M., 2015).

Following the idea that SCD5 carries tumor suppressor gene functions, we investigated the possible regulatory mechanisms underlying SCD5 reduction during melanoma progression, looking for i) the option of epigenetic mechanisms; ii) different mRNA/protein stabilities of SCD5, possibly associated with tumor advanced phases iii) possible miR-dependent SCD5 post-transcriptional regulation.

5.1. SCD5 EPIGENETIC REGULATION.

Literature data evidenced lots of tumor suppressor genes epigenetically inactivated by DNA methylation and/or histone modification, as Retinoblastoma 1 (Rb1), tumour protein p53 (TP53), Wilms tumour 1 (WT1), neurofibromin 1/2 (NF1/2), von Hippel-Lindau tumour suppressor (VHL), adenomatous polyposis coli (APC), cyclin-dependent kinase inhibitor 2A (CDKN2A) (Feinberg AP., 2016).

The *UCSC Genome Browser (https://www.genome.ucsc.edu/cgi-bin/hgGateway)* indicates the presence of CpG islands (CpG island n°118) in SCD5 promoter (Chr4: 83719009-83720241), showing the option of promoter methylation.

Epigenetic analyses were conducted by using 5-aza-2'-deoxycytidine (5AzaCdR), a derivative of the most popular inhibitor of DNA (Cytosine-5-)-Methyltransferase 1 (DNMT1),

which is incorporated into DNA during the S-phase of the cell cycle (Egger G., 2004). In parallel experiments we utilized Trichostatin A (TSA), an organic compound that selectively inhibits the class I and II mammalian histone deacetylases (HDACs) families of enzymes.

To assess the possible SCD5 epigenetic regulation we selected Me1007 primary and A375M metastatic melanoma cell lines in view of their different SCD5 expression levels. SCD5 was re-expressed in A375M advanced melanoma by 5AzaCdR treatment, indicating that at least in part SCD5 undergoes promoter methylation regulation. By contrast no striking effects seemed related with deacetylation. As expected no significant effects were produced in Me1007 early primary melanoma cell line neither with 5AzaCdR nor with TSA treatments (Fig. 14 A-B), confirming the suggestion that SCD5 might be inactivated in aggressive melanoma in view of its antimetastatic role.

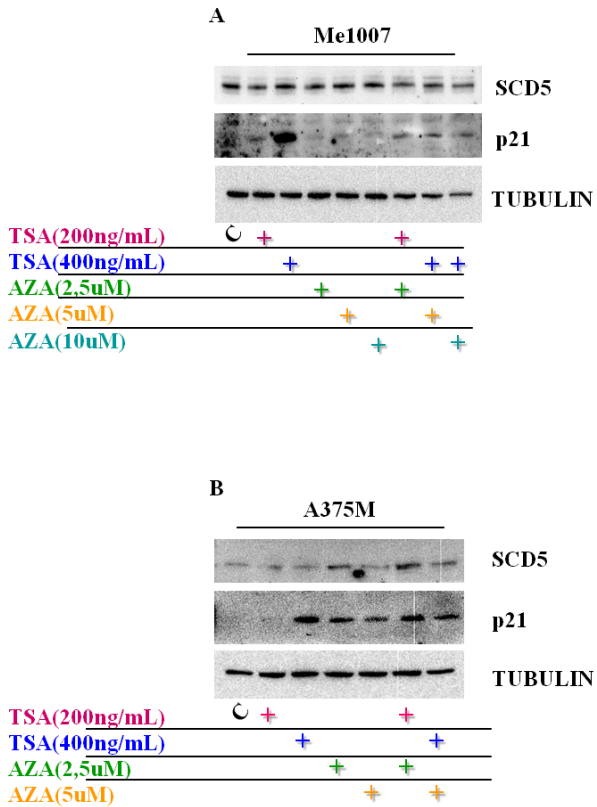


Fig 14: SCD5 epigenetic regulation
Representative western blot analyses of SCD5 epigenetic regulation in (A) Me1007 primary melanoma cell line, which evidenced no significant effects with either 5AzaCdR or TSA treatments and (B) A375M metastatic melanoma cells, which confirmed SCD5 CpG island promoter methylation, as evidenced by SCD5 induction upon 5AzaCdR treatment. P21 induction was used as positive control of treatment effectiveness.

5.2. SCD5 mRNA AND PROTEIN STABILITIES.

In order to check for the possibility of SCD5 different mRNA/protein stabilities along with tumor progression, we selected again Me1007, as a representative of primary melanoma and A375 cells, instead of A375M previously analyzed, as a metastatic cell

line model. This choice because A375M cells, being totally negative for SCD5, are unsuitable for these kind of analyses. Firstly, we evaluated the mRNA stability by treating both melanoma cell lines with Actinomycin D [2µg/mL], which intercalates into DNA thus preventing RNA polymerase I activity. Although basal levels of SCD5 mRNA were obviously different, according to their diverse tumor stages (Fig. 12 A), we did not observe significant modifications in their degradation rates (Fig. 15).

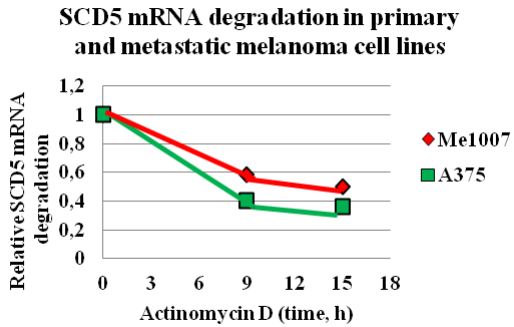


Fig 15: SCD5 mRNA stability
To evaluate SCD5 mRNA stability, Me1007 primary and A375 metastatic melanoma cell lines were treated with Actinomycin D. No significant differences were detected in their mRNA degradation rates.

In the same cell lines SCD5 protein degradation was then evaluated treating them with the protein synthesis inhibitor Cycloheximide (CHX) [50ɣ/mL]. Interestingly, the immunoblot analyses evidenced significantly accelerated protein degradation in the metastatic A375 cell line (half-life ≈ 90 min), compared with the slower rate in Me1007 primary melanoma (half-life ≈ 12-hours) (Fig. 16 A). We

then verified whether the SCD5 different protein half-lives in primary and metastatic melanoma cell lines were depended on a different degradation rate regulated by the proteasome. Notably, treatment with proteasome inhibitor MG132 [10μM] blocked SCD5 degradation in A375 cells, while it did not seem to affect the protein levels in Me1007, at least up to 15 hours (Fig. 16 B). Essentially, we detected a significantly more stable SCD5 protein in primary compared to metastatic melanoma cell lines, according to the expression pattern detected in our panel of cell lines (Fig. 12 A-B).

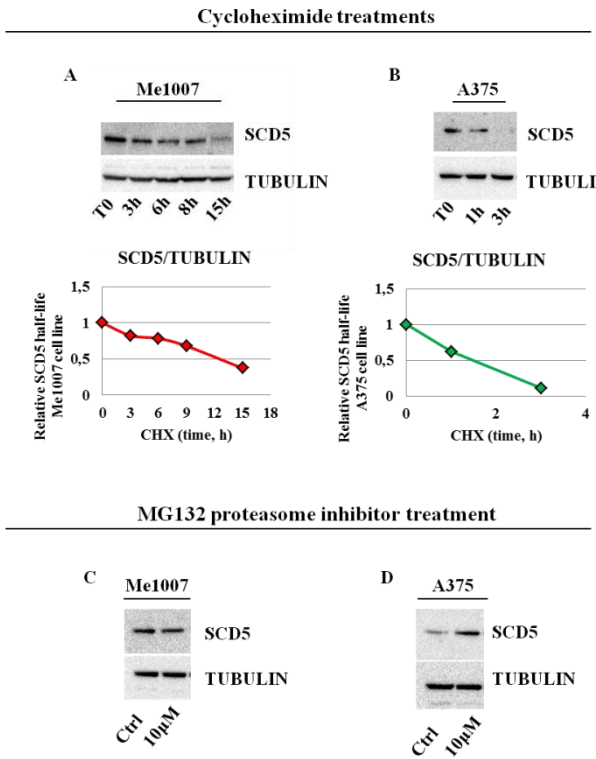


Fig 16: Evaluation of SCD5 protein stability and degradation
Me1007 primary (A) and A375 metastatic (B) cell lines were treated with Cycloheximide (CHX). In all the experiments protein extracts were subjected to western blotting analysis to detect SCD5 at the indicated time points. Results show a significative longer half-life of SCD5 protein in Me1007 compared with A375 cell line. The amounts of SCD5, normalized on Tubulin constant levels, were plotted as a regression curve. (C, D) Western blot analyses show SCD5 accumulation in A375 compared to Me1007 cell lines after MG132 treatment.

5.3. SCD5miRs-REGULATORY SYSTEM.

Considering the inverse relation between SCD5 and miR221/-222 expression patterns, the first decreasing and the latter raising during melanoma progression (Fig. 17 A-B), and the presence of one conserved binding site for miR221/-222 in the 3' Untranslated

Regione (3'UTR) of SCD5 mRNA (http://www.targetscan.com), we hypothesized the involvement of these couple of miRs in SCD5 regulation.

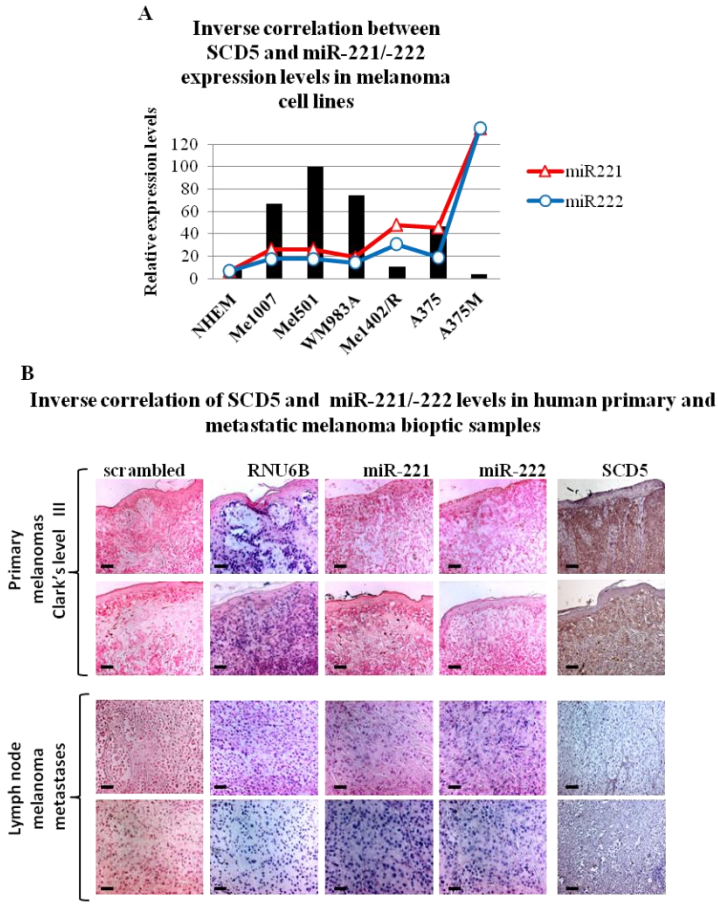


Fig 17: Inverse correlation between SCD5 and miR-221/-222: *in-vitro* and *in-vivo* studies

(A) Densitometric analysis of SCD5 protein and miR-221/-222 levels in normal melanocytes (NHEM) and representative differently staged melanoma cell lines. (B) *In situ* hybridization of miR-221/-222 and immunohistochemistry of SCD5. Bar, 50 µm. Representative sections from two primary and two metastatic human melanoma specimens are shown. Scrambled and RNU6B correspond to negative and positive controls, respectively.

To assess the possible miRs-dependent SCD5 regulation we used two different experimental sets: on the one side we utilized Me1007 primary and A375M metastatic melanoma cell lines for their opposite expression level of SCD5. In fact Me1007 cells, positive for SCD5 and almost negative for miR221/-222, were lentivirally transduced with these two miRs, while A375M, negative for SCD5 but highly expressing miR221-222, were transfected by antagomiRs to block miR221/-222 action. As expected for a miRs-dependent regulatory system, SCD5 protein expression was confirmed to be inversely related to miR221/-222 levels. Western blot analyses showed two opposite expression patterns: a significant miRs-based SCD5 downregulation in Me1007/miR-221 or miR-222 primary melanoma cells and its induction when miR-221 and/or -222 were abrogated after antagomir treatment in A375M metastatic cells (Fig. 18 A-B). To verify the functional binding of miR221/-222 to the putative conserved site present in the 3′UTR of SCD5, we performed a series of luciferase assays. The SCD5 3'UTR, containing either the wild-type or mutated miR221/-222 binding site (Fig 18 C) was cloned downstream to the Renilla open reading frame in a modified psi-Check vector. Constructs were cotransfected into the 293FT cell line with miR221 and/or miR222, or with a non-targeting oligonucleotide as negative control. Results showed a significant miR221/-222 dependent inhibition of the Renilla activity (~50% reduction in 293FT in presence of both miRs). Importantly, mutation of nucleotides in the seed sequence

totally abrogated miR221/-222-dependent repression of Renilla in both cell lines (Fig. 18 D). All together these experiments demonstrate that miR221/-222 directly interact with and repress SCD5, probably suggesting a coordinated function of these couple of miRs in SCD5 post transcriptional regulation.

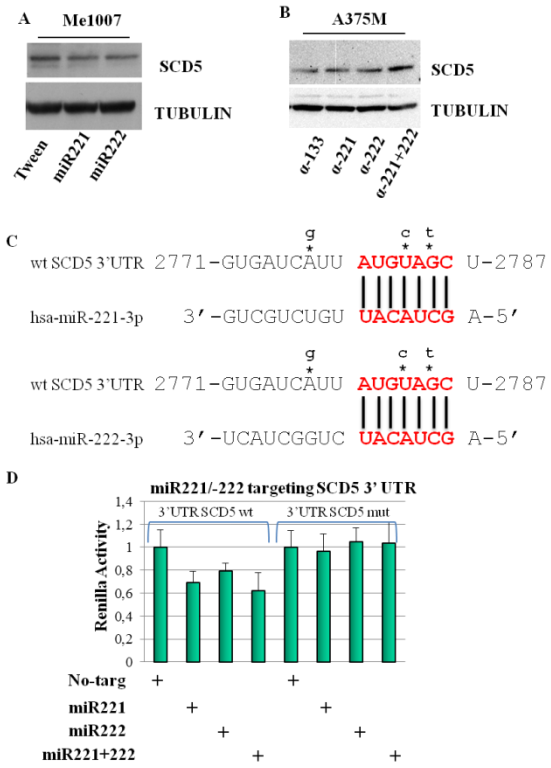


Fig 18: miR-221&222 targeting of SCD5
(A) Representative WB analyses of SCD5 in (A) Me1007 control (Tween empty-vector) and miR221/-222-transduced cells, and (B) in antagomir-221&222-transfected A375M cells. Tubulin was used as loading control. (C) Nucleotide pairing between SCD5 3′UTR and miR-221 and -222 sequences. The seed sequence is indicated in bold (red), while lower case letters with asterisks represent the mutated nucleotides. (D) Luciferase reporter assay performed in 293FT cell line cotransfecting miR-221 or -222 in the presence of the SCD5 3′UTR. As controls, mutated 3′UTR sequences and a non-targeting oligomer were also included.

Literature data established that microphthalmia-associated transcription factor (MITF), in axis with recombination signal binding protein J kappa (RBPJK), binds and represses miR221/-222 promoter thus preventing effectively initiation of melanoma invasion (Golan T., 2015). In fact, MITF high levels are recognized as suppressor of invasion and metastases formation, while low levels correlate with a high metastatic potential. (Cheli Y., 2012; Thurber AE., 2011). MITF is also recognized as a major regulator in "phenotypic switching", thus and explaining at least in part the high plasticity of melanoma cells (Goding CR., 2011; Vandamme N., 2014). A375M metastatic melanoma cell line express barely detectable level of endogenous SCD5 in parallel with low expression levels of MITF and high levels of miR221/-222 (Felicetti F., 2008). This picture correlate with their aggressive phenotype and high metastatic potential. So, we selected this melanoma cell line to enforcedly express SCD5 and evaluate first of all, the effect of reconstituted SCD5 expression on MITF and miR221-222 axis. For this purpose we utilized a lentiviral vector system. SCD5 was correctly transcribed and translated in the transduced cells (Fig. 19 A-B) with approximately 200-fold increase of mRNA paralleled by a 30-fold increment of the SCD5 protein compared with control empty vector-transduced cells. We also evaluated by GM/MS the levels of fatty acids (palmitic C16:0, stearic C18:0, palmitoleic C16:1 and oleic acid C18:1) in SCD5 and control vector-transduced A375M cell line demonstrating that the

stearic to oleic conversion was significantly enhanced by SCD5 overexpression (see the ratio C18:1/C18:0) (Fig. 19 C), reaching a 4-fold increase of oleic acid compared to control cells and comparable to the average level of primary melanomas (Fig. 19 D). Conversely, the ratio between palmitoleic and palmitic acid (C16:1/C16:0) remained almost unchanged (Fig. 19 C). These results demonstrate that the enzymatic action of SCD5 was preferentially addressed in OA synthesis.

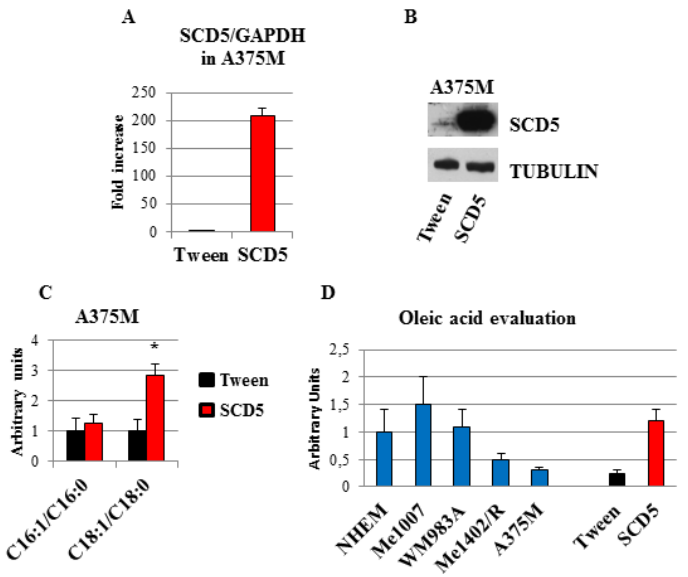


Fig 19: SCD5 enforced expression in A375M metastatic melanoma cells and GM/MS analyses

(A) qRT-PCR and B) Western blot analysis of SCD5- and Tween-transduced controls. (C) SCD5 overexpression significantly increases the desaturation indexes, represented by the palmitoleic:palmitic (C16:1/16:0) and oleic/stearic (C18:1/18:0) acid ratios evaluated by GC/MS in metastatic melanoma cell line ($p<0,01$). (D) GC/MS-based quantification of endogenously produced oleic acid at different stages of melanoma progression; representative experiments are shown; levels of SCD5 and oleic acid are shown as fold changes compared to NHEM.

Western blot and immunofluorescence analyses evidenced that SCD5 transduced A375M cells were able to upmodulate MITF expression (Fig. 20 A-B). Then, we also evaluated whether miR221/-222 expression was also modulated. QRT-PCR confirmed a significant reduction of miR221/-222 transcription compared to control cells (reduction ranging from ≈ 60-40%) (Fig. 20 C) thus demonstrating that SCD5 takes part to MITF/ miR221/-222 axis regulation. We then considered that differentiation, proliferation and survival of melanocytes largely depend on MITF (Park HY., 2009; Sommer L., 2011). According to MITF-dependent re-establishment of differentiation program, we observed the activation of melanogenesis-linked protein tyrosinase (TYR) (Fig. 20 B), eventually underlined by increased melanin content (Fig. 20 D).

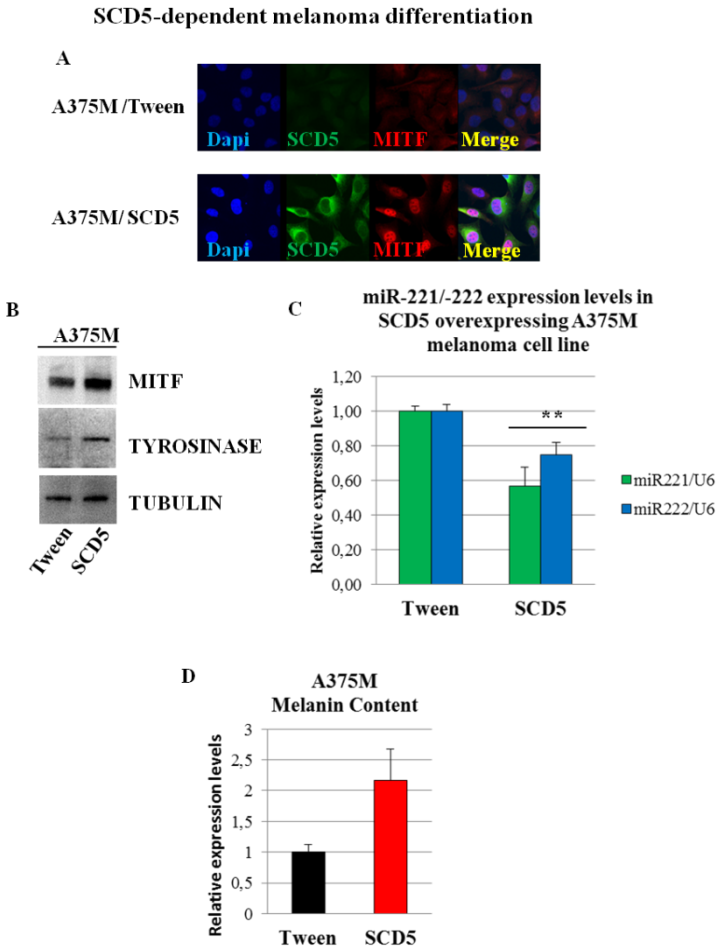


Fig 20: SCD5 favors melanoma differentiation in A375M metastatic melanoma cell line

(A-B) Immunofluorescence and western blot analyses performed on SCD5-overexpressing A375M melanoma cell line evidenced the capability of SCD5 to upregulate MITF expression compared to empty-vector control, eventually associated with increased (B) tyrosinase. (C) QRT-PCR evidenced a significant reduction of miR-221/-222 transcription in SCD5 overexpressing cells compared to control. (D) cellular melanin content.

6. SCD5 ENFORCED EXPRESSION: IN VITRO AND IN VIVO STUDIES

To characterize more in depth the functional role of SCD5 in melanoma, a number of functional analyses were performed either *in-vitro*, as proliferation, invasion, migration and agar forming foci, or *in-vivo* in A375M cells, transduced or not with SCD5.

The proliferative rate of melanoma cells, overexpressing or not SCD5, was performed by using a colorimetric assay XTT-based, demonstrating the lack of significant SCD5-dependent effects on cell growth. To assess whether SCD5 interferes with the dissemination abilities of melanoma cells, we utilized a Boyden chamber based assays, either uncoated or coated with matrigel inserts, to evaluate migration and invasion. We also verify the effects of SCD5 enforced expression on the ability to form foci in semisolid agar. Results showed slightly, but significantly decreased invasion, migration and foci numbers in SCD5-transduced A375M cells in comparison to empty vector controls (Fig. 21 A-B-C).

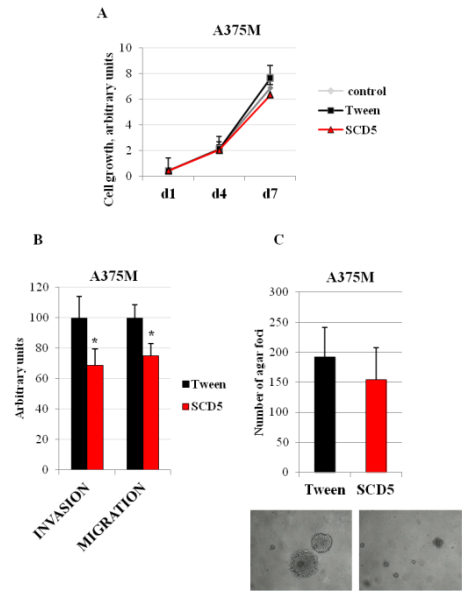


Fig 21: SCD5 overexpression in A375M metastatic melanoma*: in-vitro* studies
Comparison between A375M/Tween and A375M/SCD5 cells for A) cell growth proliferation, B) invasion and migration capabilities and C) foci number obtained in semisolid medium (down, representative images of foci).

The above results support the hypothesis of SCD5 role in preventing melanoma metastatic spread, at least in part via stearic to oleic acid conversion. To confirm such results *in vivo*, SCD5- and empty vector-transduced A375M melanoma cells were injected into immunocompromised mice.

Female of six-to-eight week old athymic Nu/Nu mice were utilized for human melanoma xenograft experiments. Animals were divided into two different groups and injected either subcutaneously (s.c.) (~10^6 cells) in the flanks or intravenously (i.v.) with SCD5- or

empty vector-transduced A375M melanoma cells (see Table 3 for each experimental set up).

Experimental groups	Injection site/n° of mice used	Experimental design
A375M/Tween	s.c./8 mice	Tumor monitoring, excision for WB and IHC analyses
	i.v./8 mice	Metastases formation and count
A375M/SCD5	s.c./8 mice	Tumor monitoring, excision for WB and IHC analyses
	i.v./8 mice	Metastases formation and count

Table 3: *In-vivo* experimental design for A375M metastatic melanoma cell line

Monitoring studies revealed that, despite similar in-tumor take and growth at the primary site, SCD5-transduced A375M, but not Tween control cells, were hampered in metastasis formation at the lung site ($p<0.01$) (Table 4 and Fig. 22 A-B).

day	A375M/Tween Tumor Volume (mm^3)±sd	A375M/SCD5 Tumor Volume (mm^3)±sd
6	30,4	72,2
13	387,8±122	266,8±113
16	684,4±235,6	569,8±130
20	1092,6±308	706,8±294

Table 4: Tumor volume measures of xenografted SCD5- overexpressing tumors compared to Tween-controls

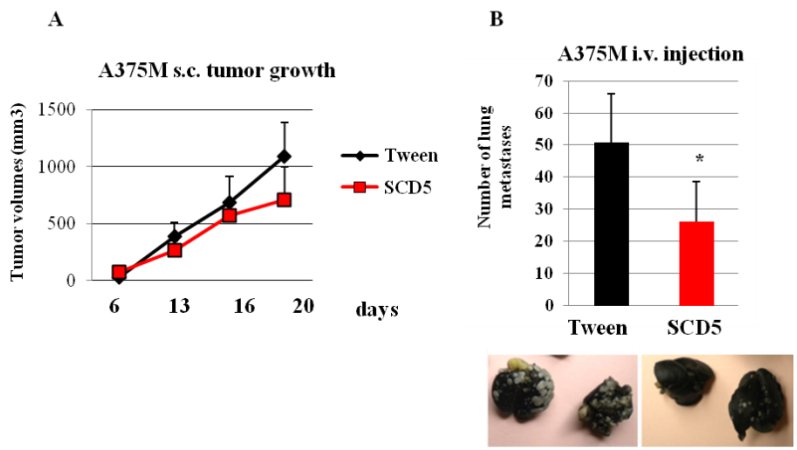


Fig 22: Functional effects of SCD5 enforced expression: *in-vivo* studies
Control Tween- and SCD5- transduced A375M metastatic melanoma cells were evaluated for: (A) tumor growth at the primary site at the indicated time points after subcutaneously (s.c.) injection into athymic nude mice; (B) total number of lung metastases obtained through intravenous (i.v.) injection ($p<0.01$, top) and representative picture of lung metastases (bottom).

Tumor primary nodules were recovered and analyzed by immunohistochemical analyses to assess the number of mitotic cells and Ki67 staining. SCD5- and Tween-transduced cells, showed no

differences in cell proliferation according to *in vitro* results (Fig. 23 A-B-C).

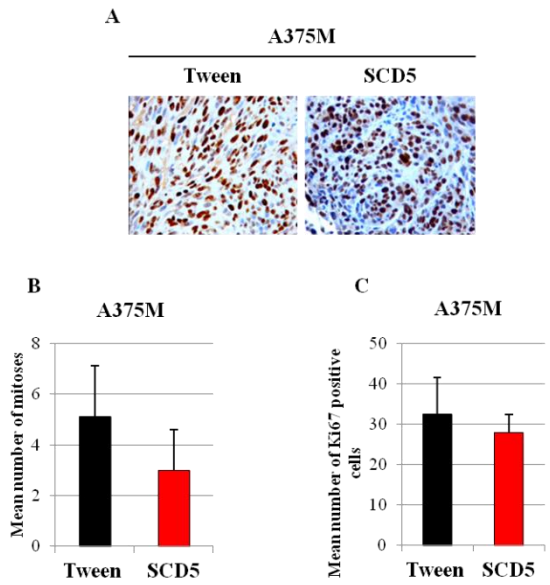


Fig 23: Analyses of tumor nodules excised from Nu/Nu athymic mice subcutaneously injected with A375M/Tween and A375M/SCD5 cell lines.
(A) Ki 67 nuclear staining analysis obtained with the Image J software, (B) mean number±SD of mitosis and (C) Ki67 positive cells. Differences are not statistically significant.

However, the histological analysis of primary tumors indicated the presence of more numerous spindle-like cells in Tween- vs SCD5-transduced tumors, the latter also characterized by a less fibrotic morphology with an intracellular accumulation of collagen, as for Masson's trichromic and Gomori's stainings (Fig. 24), suggesting that a reduced extracellular matrix (ECM) deposition could be associated with less metastases in SCD5-derived tumors.

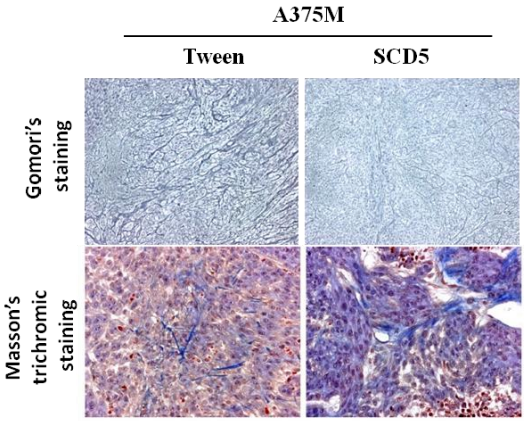


Fig 24: Histological evaluation of primary xenografted tumors derived from A375M/Tween in comparison with A375M/SCD5
Representative images of Gomori's and Masson's trichromic staining showing a less fibrotic morphology associated with intracellular accumulation of collagens in SCD5-transduced tumors.

In melanoma SPARC is required for tumor progression and its expression represents a marker of poor prognosis (Girotti MR., 2011). Considering that the staining of primary tumor nodules evidenced a clear reduction of collagen extracellular deposition, a picture reminiscent of SPARC-deficient mice (Chavey C., 2006), we looked at possible connections between SCD5 and this matricellular protein. To this end, we performed IHC analyses of COLLAGEN IV and of its chaperone protein SPARC (Chiodoni C., 2010). Data clearly showed that SCD5 restored expression was able to favor the intracellular accumulation of both SPARC and collagen IV, associated with their reduced extracellular release (Fig. 25 A).

Western blot analysis performed on proteins extracted from the same tumor nodules confirmed the significant increase of SPARC in SCD5- respect to Tween-transduced A375M tumors (Fig. 25 B).

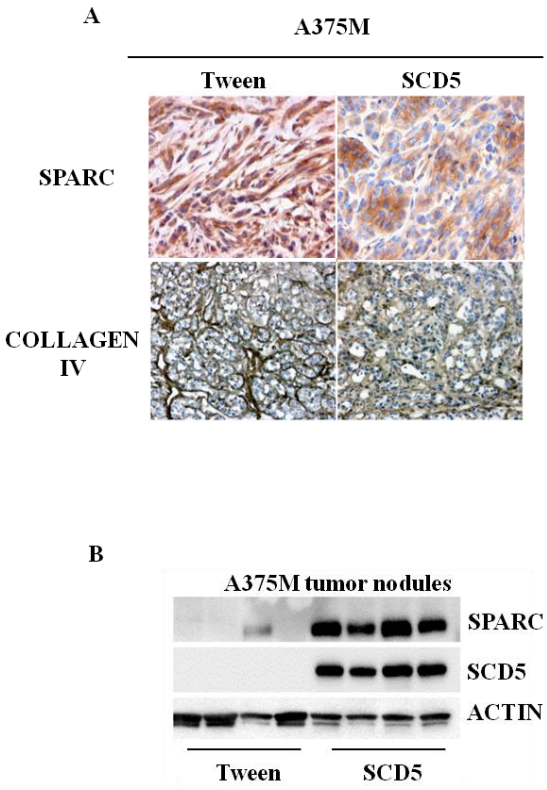


Fig 25: SPARC expression in primary tumor nodules.
(A) Representative IHC shows SPARC expression level and localization in nodules obtained after s.c. injection of Tween- and SCD5-A375M cells; magnification= 40X. (B) Immunoblot of total protein extracted from primary nodules excised from Nu/Nu mice injected s.c. with Tween- or SCD5-transduced A375M cells.

To dissect these data, we reevaluated them *in vitro* by comparing the amounts of secreted SPARC in the conditioned media (CM) of

advanced and primary melanoma cells. We observed that metastatic melanoma cell lines released amounts of SPARC significantly higher than primary ones (Fig. 26 A). We next assessed the effects of SCD5-silencing in the WM983A primary melanoma, which express high endogenous level of SCD5. Notably, the downregulation of SCD5 was significantly associated with increased SPARC release (Fig. 26 B). Conversely, SCD5 enforced expression in A375M metastatic melanoma cells turned out in a considerable intracellular accumulation of SPARC at expenses of its secreted form (Fig. 26 C). Immunofluorescence analysis evidenced that SPARC was mostly retained into the trans-Golgi perinuclear area in parallel with COLLAGEN IV accumulation into the cytoplasm of SCD5-transduced melanoma cells (Fig. 26 D). According to the observed differences in SPARC localization but not in its quantity, qRT-PCR analyses showed no variation in SPARC steady-state mRNA levels in SCD5-transduced compared to control cells (Fig. 26 E).

SPARC is also known to cooperate with CATHEPSIN B protein in favoring invasiveness (Girotti MR., 2011). CATHEPSIN B belongs to the family of lysosomal cysteine proteases and plays an important role in intracellular proteolysis.

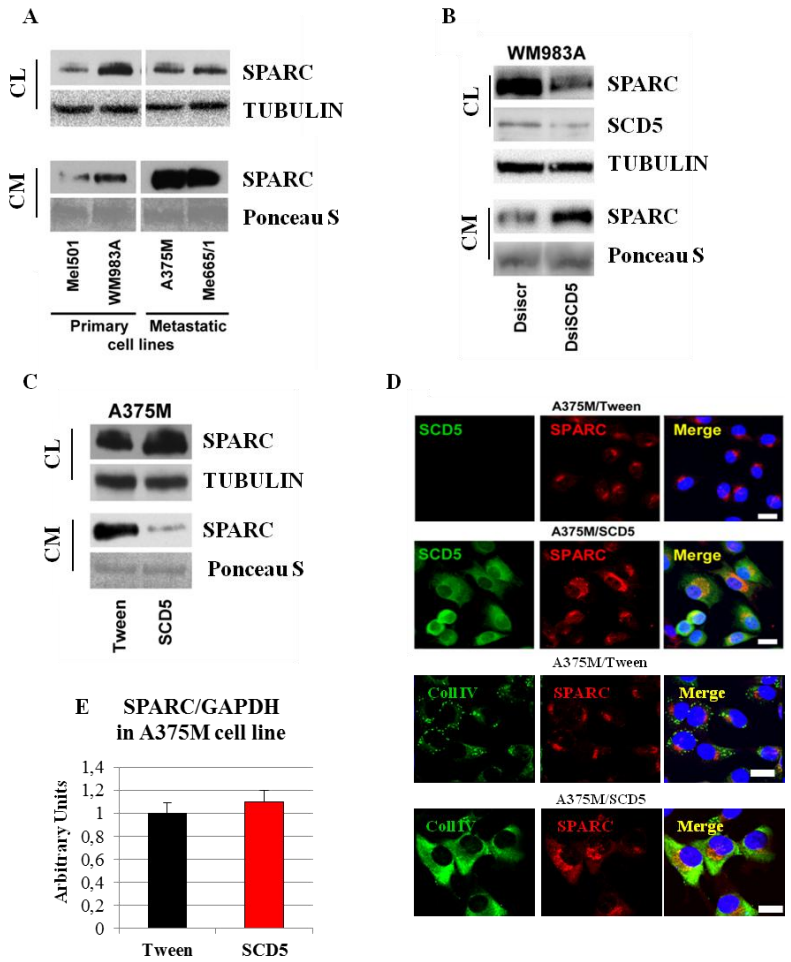


Fig 26: Regulation of SPARC expression

Western blots of endogenous SPARC levels evaluated on: (A) cell lysates (CL) and conditioned media (CM) from representative primary/low-invasive and metastatic melanomas; SPARC immunoblots of (B) SCD5-silenced WM983A primary melanoma and (C) SCD5-over-expressing A375M metastatic cell line. (D) Immunolocalization analysis showing the intracellular accumulation of both SPARC (red) and Collagen IV (green) in A375M/SCD5 compared to A375M/Tween. (E) QRT-PCR of SPARC expression in A375M/SCD5 compared withTween empty vector control. Scale bar=10 µm

Upregulation of this secreted protein can be found in premalignant lesions as well as in other pathological condition, as cancer itself (Tong B., 2014; Lai WF., 2004; Ha SD., 2010; Yang WE. 2016). It is also involved in autophagy and cannibalism, which is advantageous in tumor malignancy and is possibly involved in specific immune resistance (Fais S., 2007). As indicated by SPARC retention, the reduced propensity to secrete proteolytic enzymes and prometastatic molecules by SCD5-overexpressing melanoma cells was also confirmed by the parallel intracellular retention of CATHEPSIN B (Fig. 27 A).

As lysosomes represent the main storage of cathepsins, including CATHEPSIN B, which ~~is~~ are known to localize at the cell periphery in advanced melanoma and at the perinuclear region in less malignant tumors we analyzed the cellular distribution and localization of Lysosome-Associated Membrane Proteins 2 (LAMPs). According to the reduced malignancy of SCD5-transduced cells, confocal analysis evidenced LAMP2 positive vesicles redistributed in the nuclear proximal area, while in control cells these vesicles are scattered into the cytoplasm on the way to the cell membranes. A parallel strong accumulation of CATHEPSIN B, reminiscent of SPARC distribution and mostly colocalizing with LAMP2, was detected in the same area (Fig. 27 B). It is worth noting that most of the accumulated CATHEPSIN B was in its mature form, as demonstrated by the Magic Red assay that specifically detects its activated fraction, indicating that the

reduced secretion of this cysteine protease was not due to a failure in protein maturation (Fig. 27 C).

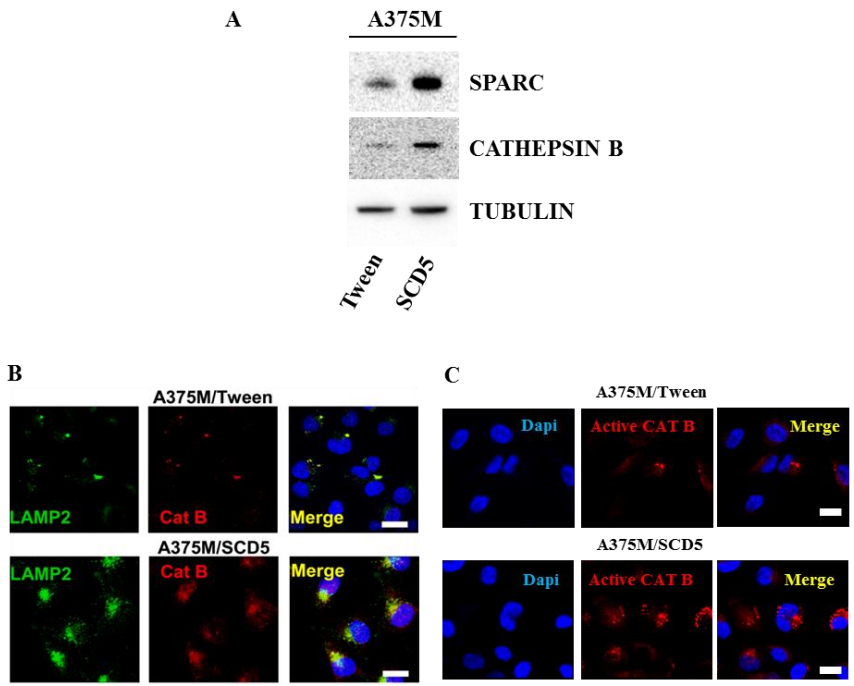


Fig 27: SCD5-dependent regulation of CATHEPSIN B activity
(A) CATHEPSIN B immunoblot in SCD5-overexpressing A375M metastatic cell line. (B) A significant intracellular accumulation of CATHEPSIN B (red) and LAMP2 (green) with colocalization is appreciable. It is notable the change of vesicles distribution in SCD5-compared to empty vector transduced cells, primarily perinuclear in the former and more peripheral in the latter, according to malignant progression. (**C**) Intracellular CATHEPSIN B activity detected by using a Magic Red-(RR)2 CATHEPSIN B fluorogenic substrate demonstrating that a substantial fraction of CATHEPSIN B accumulated in SCD5-transduced A375M is in its active form. Scale bar=10 µm

7. EFFECTS OF OLEIC ACID EXOGENOUS SUPPLEMENTATION

Knowing from literature data the existence of -a direct link between γ-linoleic acid and SPARC secretion in breast and colon cancer cell

lines (Watkins G., 2005) and considering the preferential role of SCD5 in the conversion of stearic to oleic acid (OA), we evaluated whether the exogenous supplementation of OA could reproduce the same effects of SCD5 gene transduction.

The A375M/Tween melanoma cell line cultured in low serum condition (1%) in order to favor the intracellular uptake of exogenous lipids, was subjected to a time course treatment (up to day 6) with different doses of oleic acid (OA) (10 to 100 μM) or left untreated (Fig. 28 A). After 3 days of treatment, with either 50 or 100 μM of OA, the intracellular accumulation, with a parallel reduction of the secreted form of SPARC, which was comparable to that induced by SCD5 gene transduction (Fig. 28 B). This result indicated that most of the SCD5-dependent effects on melanoma cells passes through the production of OA.

The specificity of OA action was confirmed by testing palmitoleic acid (PA) that did not show any effect on SPARC and CATHEPSIN B secretion in A375M cells (Fig. 28 C).

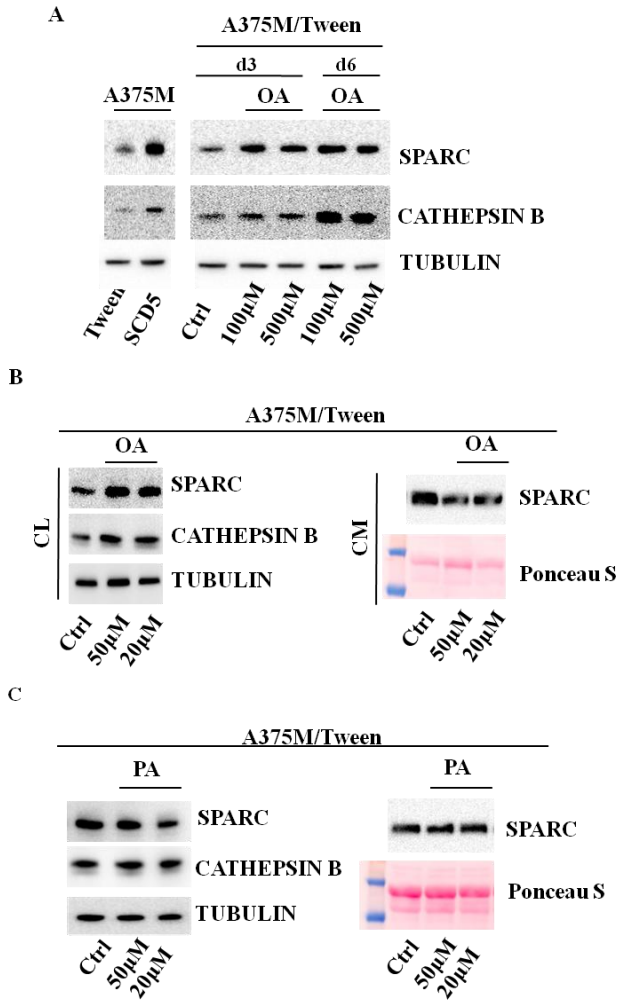


Fig 28: Effects of oleic (OA) and palmitoleic (PA) acids on SPARC and CATHEPSIN B secretion

(A) Dose–response of OA-treated A375M/Tween cells, evaluated by immunoblots at different time points; SPARC and cathepsin B levels were compared with those of SCD5-transduced A375M cells. Immunoblot of cell lysate (CL) and conditioned medium (CM) of A375M/Tween cells treated for 3 days with the indicated concentrations of (B) oleic acid (OA) or (C) palmitoleic acid (PA).

8. SCD5 INDUCES INTRACELLULAR pH ACIDIFICATION

The extracellular pH (pHe) of tumor tissues is often acidic (Warburg O., 1927) and acidic metabolites, e.g. lactic acid, produced by anaerobic glycolysis in hypoxia condition, the so called Warburg effect, seem to be the main underlying cause. Acid pHe not only favors the activation of some lysosomal enzymes, but also induces the expression of some pro-metastatic genes. Lysosomal enzymes have an acidic optimal pH and some tumor cells have the ability to secrete them, such as CATHEPSIN B and cathepsin L (Rofstad EK., 2006). Cathepsin K plays an important role in osteoclast-mediated bone reabsorption (Karsdal MA., 2005; Saftig P., 1998) and its inhibition prevents breast cancer-induced osteolysis and skeletal tumor burden (Le Gall C., 2007). Thus, osteoclast mediated acidic pHe leads to mineral dissolution and activation of cathepsins to digest bone matrix, such as type I collagen. Podgorski and coauthors (Podgorski I., 2009) reported that SPARC, a major non-collagenous protein in bone, is digested by cathepsin K and its fragments are associated with bone-metastasis formation.

As showed by results obtained for SPARC and CATHEPSIN B, which are intracellular accumulated in SCD5-overexpressing cells compared with empty vector controls, we evaluated the competence of SCD5 in modifying the acidification gradient between tumor cells and microenvironment, as reported for some fatty acids which can reduce the intracellular pH, possibly delaying the vesicular

maturation process (Wu ML., 2000). Hence, we evaluated the intracellular acidity of Tween- and SCD5-transduced A375M melanoma cells using a pH-dependent reversible fluorogenic probe and analyzing the emission profiles by a microplate-based fluorimetry. According to our hypothesis, the intracellular pH was lower in A375M/SCD5 cells than in A375M/Tween controls (Fig. 29 A), thus suggesting the existence of a reversed pH gradient associated with reduced malignancy (Whal ML., 2002). The addition of four pH calibration buffers, allowed establishing the intracellular pH, indicated as pH≥7.4 for control cells and ≤7.0 for SCD5-transduced cells. According to the described role of oleic acid in regulating proton extrusion (Grasso EJ., 2011), A375M control cells treated with growing doses of oleic acid displayed a gradual reduction of pH, ranging from 7.4 at 20 µM to 6.5 at 100 µM (Fig. 29 B), confirming the parallel roles of SCD5 and OA in intracellular acidification.

Accumulating evidence shows that an acidic microenvironment is associated with reduced proton extrusion and is regulated by different integrated membrane proteins being. While Na+-HCO3-co-transporter and Cl-/HCO3-exchanger contribute to decrease ~~in~~ the intracellular pH, the Na+/H+ exchanger (NHE) (Chesler M., 1985), the H+-lactate co-transporter, monocarboxylate transporters (MCTs), and the H+-ATPase (H+ pump) are responsible for the secretion of H+ (Nishisho T., 2011).

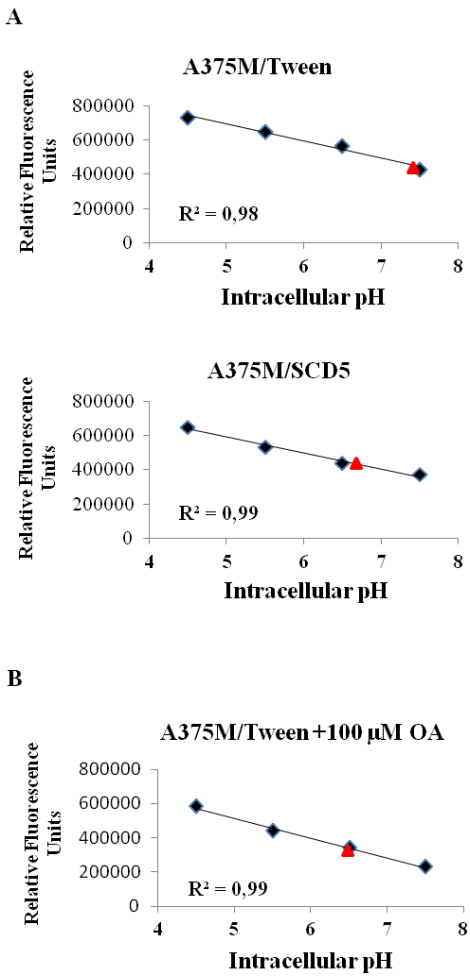


Fig 29: Determination of intracellular pH by using the pHRhodo fluorescent dye

Intracellular pH evaluation in (A) A375M/Tween vs A375M/SCD5 and (B) A375M/Tween cells treated for 48 h with 100 µM oleic acid. The red triangles indicate the intracellular pHs determined according to the reported plots.

As multi-subunit vacuolar ATPases (V-ATPases) are one of the key regulator family of extracellular pH, in that generate the acidic metastatic microenvironment pumping protons outside the cells (Pérez-Sayáns M., 2009), we investigated their possible involvement in the SCD5-dependent intracellular acidification. Our gene profiles studies (Fig. 30 A) indicated the downregulation of five V-ATPase subunits. Among them we focused on the most decreased ones, ATP6V1C2 and ATP6V1G2, reduced 4 and 3.5-fold respectively.

Western blot and IF analyses confirmed ATP6V1C2 downregulation in the A375M/SCD5 cellular model (Fig. 30 B-C). Conversely, the ATP6V1G2 one does not appear significantly modulated.

All together our results indicate a significant role of OA in lowering the intracellular pH at least in part through the reduced activity of the V-ATPase C2 subunit.

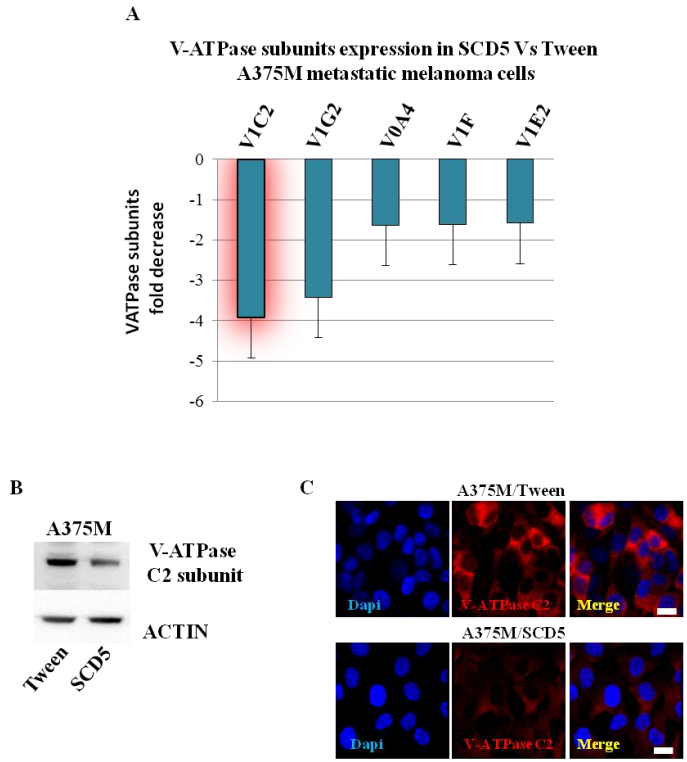


Fig 30: SCD5-dependent regulation of the V-ATPase subunit ATP6V1C2

(A) Gene expression profile indicated the SCD5-dependent decrease of 5 V-ATPase subunits (in red V1C2 we selected for futher analysis). (B) Western blot analysis confirmed the down-regulation of the ATP6V1C2 subunit in A375M cells, without any apparent change in cellular localization, as confirmed by (C) immunolocalization studies.

8.1. ACIDIC CULTURE CONDITIONS RESTORE SPARC AND CATHEPSIN B SECRETION

As a correct acidification appears crucial for proper activation and trafficking of proteins and proteases involved in tumor initiation

and progression, control and SCD5-transduced melanoma cells were cultured at low pH and compared with cells grown at buffered conditions (pH 6.0 vs 7.4). Interestingly, in SCD5 overexpressing cells the extracellular acidic conditions were able to unblock SPARC and CATHEPSIN B intracellular retention, allowing their secretion. A time response analysis, performed by Western blot on proteins, either recovered from cell lysates or from the corresponding conditioned media, showed that after 12h at pH 6.0 the amounts of SPARC released by A375M/SCD5 cells were comparable to those of control cells. An identical expression pattern was observed for the intracellular levels of CATHEPSIN B (Fig. 31 A-B).

Strong evidence of extracellular acidic pH capability to restore the malignant secretory pathway came from confocal microscope analysis of SPARC and CATHEPSIN B in Tween- and SCD5-transduced A375M cells.

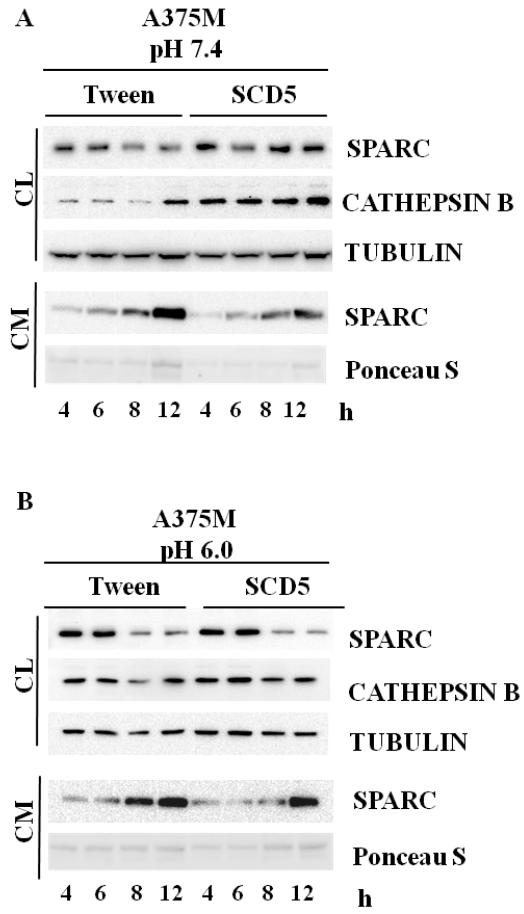


Fig 31: Acidic culture conditions restore SPARC and cathepsin B secretion in A375M/SCD5 cells

Western blots of A375M/Tween and A375M/SCD5 cell lysates (CL) and conditioned media (CM) obtained from cells cultured at (A) pH 7.4 or (B) pH 6.0.

Acidification of the external pH (pHe< pHi) was able to drive both SPARC and CATHEPSIN B toward the cellular periphery (Fig. 32 A-B). It is important to note the different secretory patterns followed by these two proteins, being CATHEPSIN B localized in

discrete punctuate vesicles and SPARC more diffused into the cytoplasm. Unlike SPARC, we were unable to detect the secreted fraction of CATHEPSIN B in the CM, possibly because of its quick degradation (Almeida PC., 2001). Looking for the option of a protein cargo protected from circulating nucleases and proteases, we isolated the microvesicles (MVs) released in the extracellular medium by Tween- and SCD5-transduced A375M, grown at neutral or acidic conditions. Notably Tween-derived microvesicles (MVs) contained comparable amounts of CATHEPSIN B, either at pH 7.4 or pH 6.0, whereas a significant increment was induced by the extracellular acidic culture conditions in A375M/SCD5-derived MVs, once more indicating that the SCD5-dependent impairment could be overcome by low pH. The same expression pattern was observed for SPARC. As representative MV markers, CAVEOLIN-1 and RAB5b protein levels were evaluated too. As expected, their pattern was essentially unchanged (Fig. 32 C).

Finally and according to the functional roles played by SPARC and CATHEPSIN B in the invasion program of melanoma cells, their restored secretion stimulated by the low pH was associated with increased invasion and migration of A375M/SCD5 cells (Fig. 33 A-B).

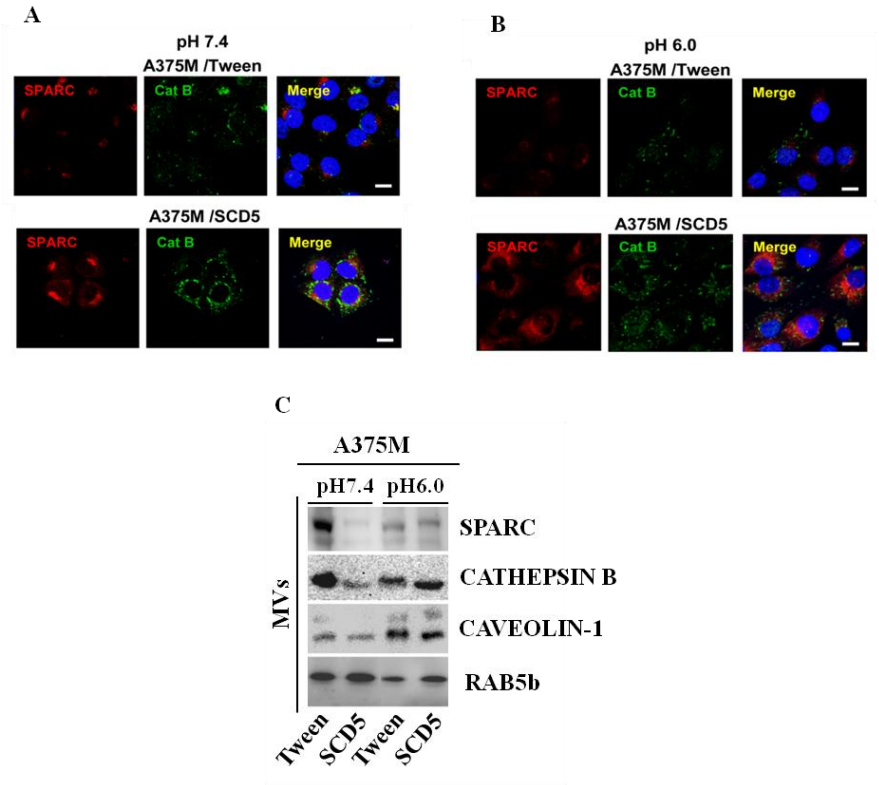


Fig 32: Immunolocalization analyses of SPARC and CATHEPSIN B cellular localization in buffered (pH 7.4) and acidic (pH 6.0) cell cultured condition

(A, B) Confocal microscopy of SPARC and CATHEPSIN B in Tween- and SCD5-transduced A375M cells at the indicated pHs. (C) A375M/Tween- and A375M/SCD5-derived microvesicles from CM of cells cultured at pH 7.4 or 6.0 evidencing a significant increment of secreted CATHEPSIN B when SCD5 overexpressing cells were cultured in acidic conditions.

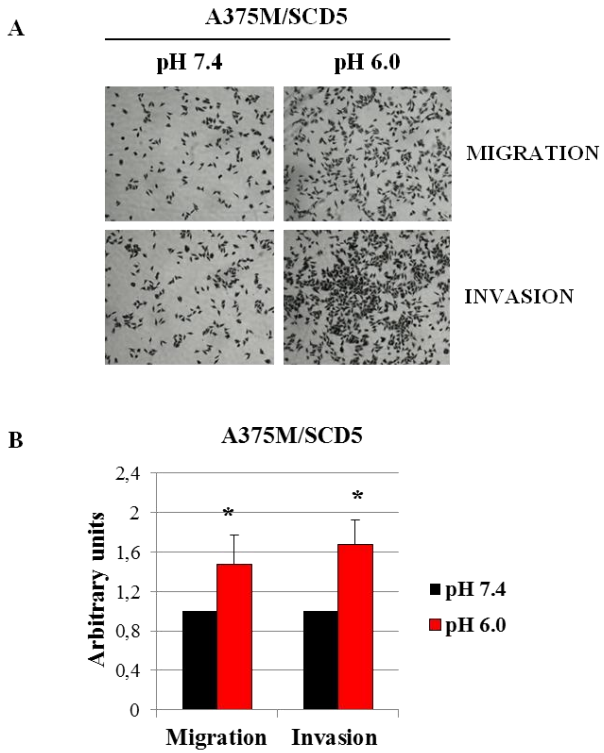


Fig 33: Boyden chamber assay evaluation of migration and invasion
(A) Crystal violet staining of A375M/SCD5 cells able to migrate and invade at neutral (pH 7.4) and acidic (pH 6.0) conditions. (B) Densitometric evaluation shows a significantly increased capability at acidic (pH 6.0) conditions ($p<0.05$).

9. SCD5 FUNCTIONAL ROLE IN THE MURINE MAMMARY CARCINOMA CELL LINE 4T1

In order to further validate SCD5 Δ9 desaturase role, we also utilized the murine mammary carcinoma cell line 4T1, a very useful *in vivo* model of spontaneous metastases. Four desaturases, SCD1 to SCD4, exist in mice (Wang J., 2005), all showing a certain

degree of homology with human SCD5, but no one exactly being its homologous. As for A375M metastatic melanoma model, we overexpressed SCD5 by transducing the 4T1 cells with the lentivirally vector Tween (Fig. 34 A-B).

We then evaluated the fatty acids contents (Palmitic and stearic acids (C16:0/C18:0, palmitoleic and oleic acids (C16:1/C18:1) by using GM/MS analyses evidencing the preferentially induction of oleic acid toward the palmitoleic one also in this cell model (Fig. 34 C).

Accordingly and in agreement with melanomas, SCD5-transduced and oleic acid-treated 4T1 cells displayed SPARC intracellular accumulation (Fig. 35 A). In vitro analysis confirmed the reduced malignancy associated with SCD5 as indicated by reduced cell migration in wound healing experiments (Fig. 35 B).

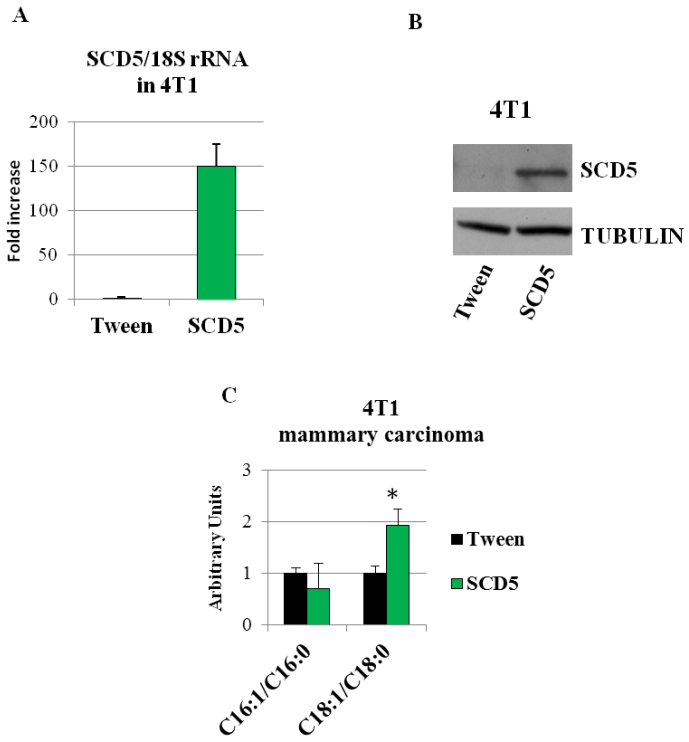


Fig 34: SCD5 enforced expression in 4T1 mammary carcinoma cell line: desaturation index analysis

A) QRT-PCR, B) Western blot comparison between SCD5-transduced and Tween controls 4T1 cells. (C) Increased desaturation indexes, represented by the palmitoleic:palmitic (16:1/16:0) and oleic/stearic (18:1/18:0) acid ratios, evaluated by GC–MS in 4T1 cell lines, transduced with SCD5 ($p<0.01$).

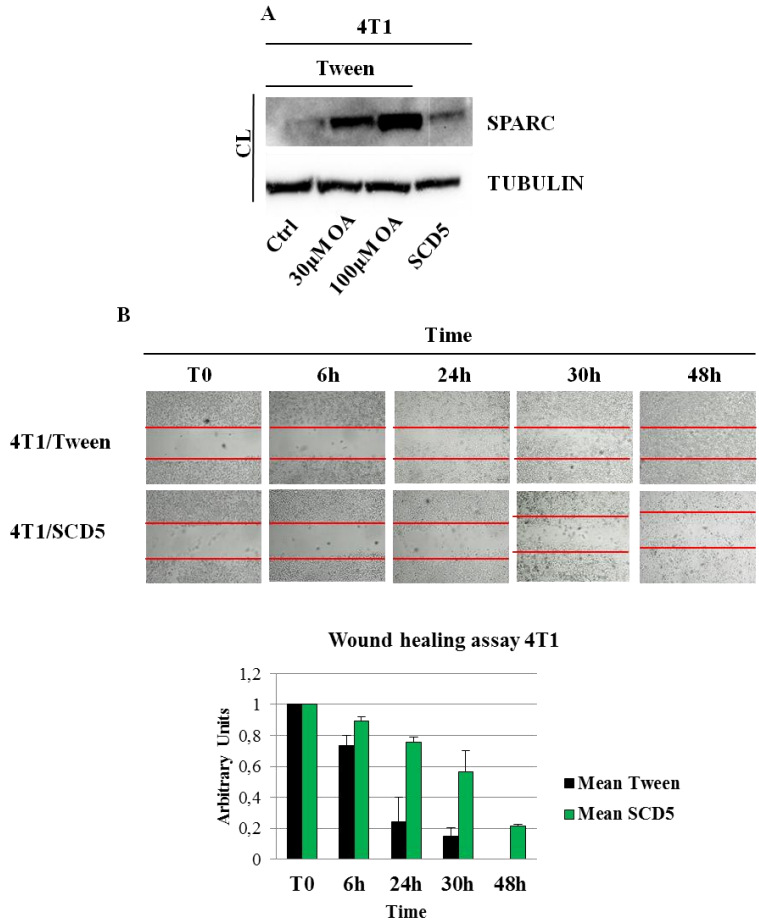


Fig 35: Functional effects of SCD5 enforced expression in murine 4T1 mammary carcinoma cell line

(A) Western Blot analyses of SPARC performed on cell lysates (CL) of SCD5-overexpressing compared withTween control. (B) Wound healing assay to compare migratory capability.

Basing on the capability of 4T1 cells to spontaneously produce distant micrometastases in syngeneic BALB/c mice, we carried out some additional *in vivo* experiments.

The experimental animal groups, subcutaneously injected with SCD5- or Tween-transduced vector control 4T1 cells (~ $7x10^3$) (Table 5) confirmed the SCD5-dependent significant hampering of lung micrometastases formation ($p<0.005$), whereas only a slight reduction of primary tumor volumes was observed (Table 6, Fig. 36 A-B).

Experimental Groups	Injection site/n° of mice used	Experimental design
4T1/Tween	s.c./ 14 mice	Tumor monitoring, excision for IHC analyses, evaluation of spontaneous micrometastases formation
4T1/SCD5	s.c./ 14 mice	Tumor monitoring, excision for IHC analyses, evaluation of spontaneous micrometastases formation

Table 5: In-vivo experimental design for 4T1 mammary carcinoma cell line

day	4T1/Tween Tumor Volume (mm^3)±sd	4T1/SCD5 Tumor Volume (mm^3)±sd
7	0	0
14	180±30	50±10
21	600±190	280±210
27	790±210	410±350

Table 6: Tumor volume measures of syngeneic SCD5-overexpressing tumors compared with Tween- controls

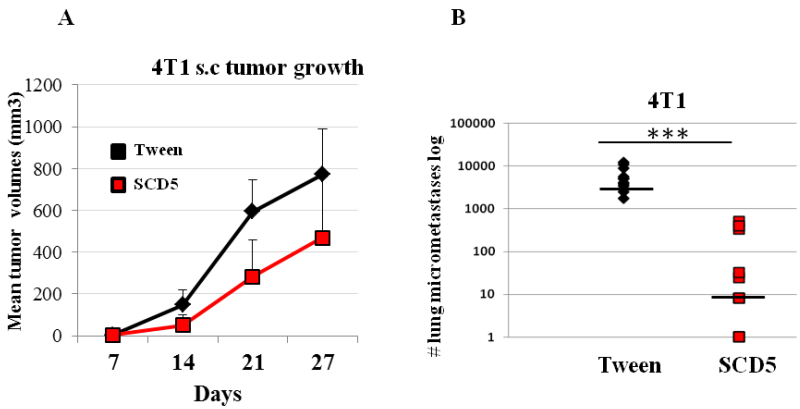


Fig 36 : Functional effects of SCD5 enforced expression in 4T1 mouse mammary carcinoma model: *in-vivo* studies
(A) In-vivo growth of primary tumors after s.c. injection of 4T1 cells in BALB/c syngeneic mice. (B) Lung micrometastases evaluation by clonogenic assay ($p<0,001$).

Masson colorimetric assay and IHC analyses performed on tumor nodules confirmed the capability of SCD5-overexpressing cells in decreasing extracellular collagen deposition, particularly collagen

IV (Fig. 37). It is worth noting that also in 4T1 murine mammary carcinoma model in presence of SCD5 was observed a downmodulation of miR221/-222 (Fig. 38), suggesting the possible regulatory function translational function of SCD5 in different tumor models.

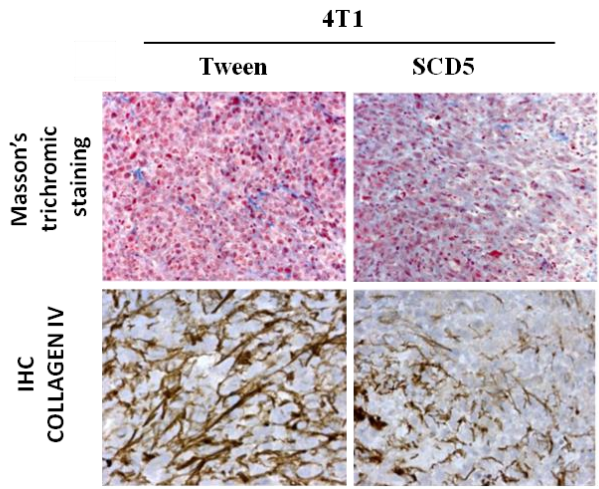


Fig 37: Hystological and Immunohystochemical analyses on primary tumor nodules

Masson's trichrome staining and COLLAGEN IV IHC on primary nodules obtained after s.c. injection of Tween- and SCD5- transduced 4T1 cells; IHC magnification=×20

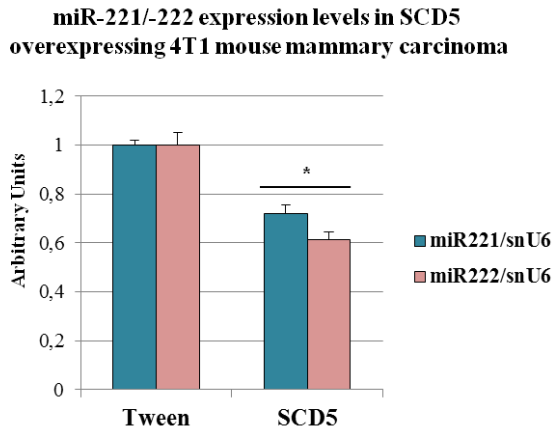


Fig 38: Mir221/-222 expression levels in 4T1 transduced cell lines
QRT-PCR evidenced a significant reduction of miR221/-222 transcription in SCD5 overexpressing cells
compared to control.

DISCUSSION

The deregulated metabolic activities displayed by cancer cells require a constant supply of energy and the availability of appropriate structural membrane components. In fact cellular lipids, including fatty acids, play a fundamental role in sustaining the high rate of proliferation and signal transduction of cancer cells. Also a correct balance between saturated and unsaturated lipids is required for the maintenance of cell homeostasis in normal cells (Ackerman D., 2014). Recent results have clearly recognized the central role played by bioactive lipids and fatty acids as mediators of the crosstalk between cancer cells and stroma (Beloribi-Djefaflia S., 2016).

Mediterranean diet is characterized by high olive oil consumption and a wide range of experimental observation have evidenced its protective role in breast, colorectal, colon and glioma tumor model (Trichopoulou A., 2000Chajes V., 2008; Bautista D., 1997; Stoneham M., 2000; Bartoli R., 2000) possibly through its main component oleic acid (OA). Accordingly, OA induces TNFα production in RAW264.7 macrophages (Liu X., 2010) and impairs tumor neovascularization inhibiting VEGF receptor/MAPK pathway (Lamy S., 2014). On the contrary, palmitoleic acid is synthesized upon stimulation with growth factors, mainly bFGF and PDGF, and is able to amplify mitogenic signals (Koeberle A., 2012).

In humans the main enzymatic activity of SCD5, and of its homolog SCD1, provides the desaturation of two main substrates, palmitic and stearic acid, producing palmitoleic and oleic acid.

Contrary to the virtually unknown role of SCD5, some of the biological effects of SCD1 desaturase activity in cancer are already reported (Nashed M., 2012; Huang J., 2016, Angelucci C., 2015). Over-expression of SCD1 is associated with cell metabolism, proliferation and progression of several solid tumors (Chen L., 2016).

We focused on SCD5 potential role in melanoma, starting from its down-regulated expression in human melanoma cell lines and bioptic specimens during progression (Fig. 12 A-B). Searching for the mechanisms underlying such significant expression decrease, we unveiled the effectiveness of different post-transcriptional regulatory system among primary and metastatic samples. Indeed as confirm of its possible antimetastatic role, we demonstrated the SCD5 epigenetic silencing in advanced melanoma samples compared to primary ones (Fig. 14 A-B). In addition SCD5 protein appears more stable in primary melanomas compared to metastatic ones (Fig. 16 A-B). For long time, both these isoenzymes were considered to play similar desaturation function on saturated fatty acids. It is now important to evidence some specificity discerning SCD1 and SCD5. For example, their distribution appears different in normal tissues, being SCD1expressed in the majority of them, while SCD5 is present only in human pancreatic and brain cells

(Igal RA., 2010). Moreover, at least in melanoma cells, our results evidenced an unexpected specific desaturating function of SCD5 compared to SCD1. In fact, GM/MS analysis on SCD5 overexpressing cells demonstrated an increase of desaturation index compared to Tween control, preferentially evidenced in oleic acid increase (3:1 ratio), indicating a specific action on stearic compared to palmitic acid desaturation (Fig. 19 C). In addition, our experimental evidences show that SCD1 protein was rapidly degraded after cycloheximide treatment both in primary and metastatic melanoma cell lines, according to literature data confirming its proteasome dependent degradation (Kato H., 2006). Despite this high rate of degradation, SCD1 appears highly expressed at all tumor stages indicating that SCD1 levels appear principally determined by its rate of transcription (Igal RA., 2010). Notably, the different protein half-life between SCD5 and SCD1 might be important to increase the oleic acid production at expense of palmitoleic, thus sustaining SCD5 expression in primary melanomas (Fig. 16 A-B and Fig. 19 C) and reducing cell aggressiveness. According to GC/MS in melanoma cells SCD1 does not show the same preferential enzymatic activity toward oleic acid production (our unpublished data), whereas the decline of SCD5 matched with oleic acid reduction during melanoma progression. In general, SCD1 appears to play a pro-tumorigenic role, whereas, our data evidence as SCD5 exert a tumor suppressor function.

In view of our previous studies on microRNA functional roles in melanoma, we made a particular focus on the SCD5/miR regulatory system. We observed an inverse correlation between the expression levels of SCD5 and miR221/-222 a well known couple of tumorigenic microRNAs (Fig. 17 A-B) (Ciafrè SA., 2005; Visone R., 2007; Garofalo M., 2009; Miller Te., 2008; Medina R., 2008; Felicetti F., 2008). Luciferase assays and functional experiments, based on miR221/-222 overexpression or silencing, evidenced the existence of miR-dependent regulatory mechanism on SCD5 (Fig. 18 A-B-C-D), again confirming our hypothesis of the necessity to limit the expression of this desaturase during melanoma progression.

The idea that SCD5 restored expression or oleic acid supplementation might antagonize the aggressiveness of advanced melanoma was sustained by the capability of SCD5 overexpression to diminish *in-vitro* invasiveness and chemotaxis of metastatic melanomas (Fig. 21 B-C). More importantly, this reduction was evident *in-vivo* by intravenous-induced metastasis of human A375M/SCD5 melanoma cell line (Fig. 22-B). The same *in vivo* results were also found in murine mammary carcinoma 4T1 cell line overexpressing the human SCD5 gene (Fig. 36 B-C). This cell line was employed for the capability to spontaneously produce distant metastases in syngeneic BALB/c mice tumor model. The formation of spontaneous metastases was significantly impaired whereas only a slight reduction of primary tumor volumes was observed.

Both the in vivo experiments evidenced the importance of ECM deposition and modification necessary for tumor dissemination and metastatic niche formation. Cancers are not just masses of malignant cells, but complex "organs" where many other cells are recruited to be corrupted by transformed cells (Balkwill FR., 2012). Therefore the complex crosstalk between tumor cells and the microenvironment is one of the factors that limit treatment efficacy (Ruiter D., 2012). We looked for SCD5-dependent regulation of ECM, knowing that the matricellular protein SPARC is necessary for melanoma progression (Ledda MF., 1997) and that its transcript is highly expressed at radial to vertical growth phase transition (Sturm RA., 2002). Either SCD5 transduction or oleic acid supplementation elicited a strong intracellular retention of collagen IV and SPARC (Fig. 26 C-D in advanced melanoma, resembling that observed in *in vivo* studies (Fig. 25 A-B). A similar effect on SPARC was previously described for γ-linoleic acid in breast and colon cancer cell lines (Watkins G., 2005). Contrary to decreased cell survival and proliferation associated with SPARC silencing (FenouilleN., 2011, Fenouille N., 2011) our data, showing reduction of extracellular SPARC in favor of its cytoplasmic form, suggest the possibility of additional unknown roles specifically played by intracellular SPARC. Abnormal accumulation of SPARC was also observed in the endoplasmic reticulum of patients with pseudo-achondroplasia (Hecht JT., 2006), reminiscent of the abnormal retention of collagens observed in other connective diseases (Byers

PH., 2012). Along with SPARC, cathepsin B, another secreted protein involved in migration and invasion of solid tumours (Podgorski I., 2003; Mohanam S., 2001) including melanoma (Szpaderska AM., 2004), was retained intracellularly in the presence of SCD5. The reduced propensity to secrete proteolytic enzymes and prometastatic molecules by SCD5-over-expressing melanomas was supported by LAMP2, a representative lysosomal protein (Glunde K., 2003), which is known to localize at the cell periphery in advanced melanoma and at the perinuclear region in less malignant tumors, including SCD5-transduced cells (Fig. 27 B-C). Insight into the underlying mechanisms came from testing whether SCD5-dependent intracellular oleic acid production might reduce the internal cellular pH, and therefore vesicular trafficking, across the plasma membrane. *In-vitro* growth at acidic extracellular pH increases the rate of experimental pulmonary metastases in athymic nude mice in different melanoma cell lines (Rofstad EK., 2006). This increase likely results from anaerobic utilization of glucose, increased H+ proton efflux and hyperactivation of proton extruder proteins, as V-ATPase and Na+/H+ exchanger (NHE), according to a characteristic ’reverse gradient’ that favours cells able to grow in hypoxic conditions (Estrella V., 2013). Our experimental results indicated that a reduced SCD5- or oleic acid-dependent intracellular pH is associated with a more physiological condition (pHe>pHi) (Fig. 29 A-B). Accordingly, a strong reduction of the extracellular acidity (pH 6.0), besides restoring the

reversed pH gradient (pHe<pHi) characteristic of tumor cells at the microenvironment boundaries, was able to re-establish SPARC and cathepsin B secretion, in turn promoting the tissue remodeling required for tumor cell spreading (Fais S., 2010). As cancer cells depend on the secretion of proteases to invade tissue and form metastases, inhibition of V-ATPase might hamper the secretion profile of cancer cells, therefore interfering with their metastatic properties (Pérez-Sayáns M., 2009**)**. Thereby, SCD5-transduced cells show reduced levels of the V-ATPase subunit C2 (Fig. 30 B-C), possibly explaining the less malignant phenotype associated with a decreased efficiency of this proton pump. Notably, subunit C mediates the binding of V-ATPase to the actin cytoskeleton, linking V-ATPase with actin fibers and in turn with cytoskeleton rearrangement and tumor dissemination (Cai M., 2014).

In conclusion, these data demonstrate the antineoplastic role of SCD5 in melanoma and, more specifically, the increased malignancy associated with its barely detectable levels in melanoma metastatic phase. As oleic acid seems the main product of SCD5 enzymatic activity and one of the main components of the Mediterranean diet, our data can offer an explanation to the beneficial effects associated with the regular consumption of olive oil and recommendation for cancer prevention. Treatment strategies including oleic acid or its derivatives in combination with other drugs and/or immunotherapy should also be considered.

MATERIALS AND METHODS

10 CELLS

10.1. CELL LINES CULTURE

Most of the human melanoma cell lines used in the current study was stabilized from surgical specimens obtained from primary or metastatic tumors at the Istituto Nazionale Tumori in Milan (Italy). The A375 cell line was from the American Type Tissue Collection (Rockville, MD, USA) and its metastatic variant A375M was kindly provided by Dr R Giavazzi (Istituto Mario Negri, Bergamo, Italy). Normal human epidermal melanocytes from foreskin were obtained from Promocell (Heidelberg, Germany). 4T1 is a 6-thioguanine-resistant cell line derived from a spontaneous mammary carcinoma (ATCC-LGC Promochen, Teddington, UK). Table 7 lists the analyzed melanoma cell lines.

Tumor cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (BioWhittaker,Walkersville, MD, USA) and maintained in a humidified incubator (5% CO2, 37°C).

All the cell lines were authenticated according to standard short tandem repeat (STR)-based genotyping (Banca Biologica e Cell Factory, IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy).

Cell line	Mutation	Histological features	References
Me1007	BRAF V600E	Primary tumor VGP*	Colombo MP, 1992; Daniotti M 2004
Mel501	BRAF V600E	Primary tumor VGP*	Supino R, 1992; Benjiam CL, 2007
WM983A	BRAF V600E	Primary tumor VGP*	Schaider H, 2003; Davies MA 2009
Me1402/R	BRAF V600E	Recurrence of primary tumor	Colombo MP, 1992; Daniotti M 2004
A375	BRAF V600G	Metastatic Melanoma	Giard DJ, 1973; Davies MA 2009
A375M	BRAF V600G	Metastatic melanoma	Kozlowski JM, 1984; Davies MA 2009
*VGP vertical growth phase			

Table 7: Melanoma cell lines analyzed in this study

10.2. CLONING AND TRANSDUCTION

Human SCD5 (NCBI Reference Sequence: NM_001037582.2) cDNA, encompassing its complete coding sequence was amplified from normal human genomic DNA using a Taq DNA polymerase high fidelity (AccuTaq) (Sigma-Aldrich, Saint Louis, MO, USA). (Table 8) by Polymerase Chain Reaction (PCR) (GeneAmp PCR System 9700, Applied Biosystem), sequenced (BMR Genomics Padova, Italy) and cloned into the lentiviral Tween-GFP vector .

SCD5 cDNA amplification
forward primer 5' GCC ACG CTA AGA CCC TGG 3'
reverse primer 5' ACA TTG ACT CGT TCC TTC 3'

Table 8: SCD5 cDNA primers sequence (5'-3')

10.3. LENTIVIRUS PRODUCTION AND INFECTION

Lentiviral supernatants were produced by transient cotransfection of (a) DNA (Tween empty-vector or SCD5), (b) pCMV, (c) pMDG, with Lipofectamin 2000 (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions in the packaging human embryonic kidney cell line 293FT. Viral supernatants were collected 48h after transfection, filtered through 0.45μm pore nitrocellulose filters and used freshly on A375M melanoma or 4T1 mammary carcinoma cell lines. 4μg/ml of polybrene was added to the viral supernatants to improve the infection efficiency. After the infection cycles, tumor cell lines were washed twice and fresh medium was replaced. Infection efficiency was evaluated after 48h by flow cytometry FACS Canto (Becton Dickinson, New Jersey, USA)

Overexpression of miR-221 and -222 was obtained in primary melanoma cells by using a lentiviral vector system, as reported by Felicetti and colleagues (Felicetti F., 2008).

10.4. GENE SILENCING AND CELL TREATMENTS

SCD5 was specifically silenced by using a validated small interfering RNA (IDT, Leuven, Belgium). Briefly, 48 h after plating, cells were transfected using Lipofectamine 2000 (Life Technologies) either with DsiSCD5 (HSC.RNAI.N001037582.12.1_1) or with a DsiRNA scrambled control (Dsi-scr #64218602) used both at 200 nM. After 48h of transfection the level of SCD5 protein was analyzed by western blot analyses. Where indicated cells are treated with 5-aza-dC or TSA, actinomycin D, cychloeximide, MG132, palmitoleic or oleic acid, , (Sigma-Aldrich).

10.5. PREPARATION OF CONDITIONED MEDIA IN BUFFERED AND ACIDIC CONDITIONS

Human melanoma cell lines were seeded in 24-well culture dishes (10^5cells/well) in DMEM 10% FCS v/v and maintained in a humidified incubator. After 24 h, the cells were washed once with Phosphate-buffered saline (PBS, Life Technologies, Paisley, UK) and DMEM 0.1% FCS added to each dish. The experiments were performed under either buffered (pH 7.4) or acidic (pH 6.0) conditions. For the latter, the pH of DMEM 0.1% FBS v/v was decreased by adding HCl. Cells and their respective CM were collected at 4–12 h, spun and either used immediately or stored at −80°C.

10.6. MICROVESICLES (MVs) ISOLATION

Melanoma cells were cultured with DMEM medium supplemented with 10% FBS, previously deprived of bovine microvesicles (MVs) by ultracentrifugation (60min at 100 000 × g). MVs were then isolated from supernatants of exponentially growing cells by sequential centrifugations or by utilizing the ExoQuick-TC kit (System Biosciences). Samples were analysed by western blotting for SPARC and cathepsin B expressions, with RAB5b and CAV-1 as positive controls.

11. IN-VITRO ASSAYS

11.1. AGAR FOCI

Base layers of complete DME medium containing 0.5% agar were set in 60 mm plastic dishes. The bottom agar was overlaid with 1.5 ml of 0.33% agar containing suspensions of $5x10^3$cells. Cultures were incubated for 3-4 weeks at 37°C and the colonies counted using an inverted microscope. Two experiments were performed for each cell line and results were calculated as the average ± SE of three dishes for each condition.

11.2. PROLIFERATION

The determination of cellular proliferation and viability was done by using cell proliferation kit II (XTT) (Roche, Mannheim, Germany) according to manufacturing procedures. Briefly the assay is based on the cleavage of the yellow tetrazolium salt XTT to form

an orange formazan dye by metabolic active cell, which is soluble in aqueous solution and is directly quantified using a scanning multiwall spectrophotometer. A proper number of cells were seeded in 96 well culture plate in DMEM 10% FCS v/v in a final volume of 100ul and maintained in a humidified incubator cells. Then cells are incubated with XTT labeling mixture and incubated for 2h at 37°C. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified (Victor X3, Wallac–Perkin-Elmer 2030 software v. 4.00) at 490nm.

11.3. MIGRATION AND INVASION

migration and invasion were assayed, as previously described (Felicetti *et al.,* 2008), using cell culture inserts (Corning Costar Corporation) with 8μm pores uncoated or coated, respectively, with 100g/cm2 of Matrigel growth factor reduced (Becton Dickinson) in order to mimic *in-vivo* barrier. Cells (5 10^5) were placed in the upper compartment in 100 μl of DMEM serum-free, while 600 μl of DMEM supplemented with 10% FBS were placed into the lower compartment of the chamber. Assays were incubated at 37° C in 5% CO2. After 48h cells attached to the upper side of the membrane were removed with a cotton swab; each membrane was fixed (Glutaraldehyde 2.5%) and stained with crystal violet solution 0.1% for 30min at room temperature. After incubation, membranes were washed with distilled water and crystal violet staining was dissolved with acetic acid 10%. Migration and invasiveness were evaluated,

as relative number of cells on the undersurface of the membrane, by a colorimetric assay at 595 nm in a microplate reader (Victor X3, Wallac–Perkin-Elmer 2030 software v. 4.00). The data were expressed as the mean absorbance ± SE for triplicate wells.

12. IN-VITRO ANALYSES

12.1. QUANTITATIVE REAL TIME-PCR (qRT-PCR)

QRT–PCR was performed according to the TaqMan technology (Applied Biosystems, Foster City, CA, USA), using the ΔΔCT method. Assay numbers were: SCD5v1 #Hs01125695_m1, SPARC #Hs00234160_m1, 18SrRNA #4319413E and GAPDH #4326317E.

12.2. QUANTIFICATION OF FATTY ACIDS WITH GC–MS

Melanoma cell lines grown for 72 h in DMEM 2.5% serum were trypsinized, washed three times in phosphate-buffered saline (PBS) and the cell pellets analysed by GC–MS. For either control vector- or SCD5-transduced cells, at least four different samples were evaluated. Quantification of fatty acids was conducted as previously described (Cai Q., 2013). Briefly, lipids were extracted with Folch/BHT reagent and the lower phase was collected and dried under N2. Methyl palmitate, palmitoleate, stearate and oleate (50 µl; 4mg/ml isottane) were added to the extracts as an internal standard. Total tissue lipids were methyl-esterified with BF3/methanol (10%). The fatty acid methyl ester was analyzed by gas chromatography, using a Clarus 500 GC system (Perkin-Elmer) equipped with a

BPX70 capillary column and a flame ionization detector (FID) used to quantify fatty acids.

12.3. WESTERN BLOT AND IMMUNOFLUORESCENCE

Total cell lysates were prepared by using nonidep-p40 (NP-40). In brief cell pellets were resuspended in 30-50ul of NP-40 cell lysate buffer (1% NP40, 200nM NaCl, 50mM Tris pH7.4) plus protease inhibitor (IP) (0.5mM DTT and 0.2mM PMSF), maintained on ice for 30 min, vortexed and then centrifuged at 3000rpm for 10 min at 4 °C. Proteins concentration was measured by Biorad protein-assay (Hercules, CA, USA). Western blot was performed according to standard procedures. Total cell lysates were separated by the precast NuPAGE polyacrylamide gel system (Life Technologies).

The expression levels were quantified using AlphaView software.

Semi-confluent cells, grown in 8-well chamber slides (Nalgene Nunc) were fixed in 4% (w/v) paraformaldehyde (Sigma-Aldrich) for 10 min. After three washes in PBS1X, cells were permeabilized 10 min in 0.1% Triton, and saturated at least for 1 h with 3% BSA (Sigma-Aldrich) at room temperature. Primary antibodies were diluted in PBS1X-0.2% BSA at the desired concentration listed above. Cells were then washed three times in PBS1X and incubated with specific fluorophore conjugated secondary antibodies (Alexa Fluor, Molecular Probes) in PBS1X-0.1% BSA for 45 min. All the incubation were performed in an humidified chamber for 45-60 min

at RT. After last washes in PBS, slides were mounted with SlowFade anti-fade reagent containing DAPI (Molecular Probes).

When indicated Magic Red Cathepsin B activity Kit (ImmunoChemistry Technologies, LLC, Bloomington, MN USA) was used to localize and monitor intracellular Cathepsin B activity over time accordingly to manufacturer's instructions. Briefly Magic Red staining solution was added directly to adherent cells and incubated for 60 min at 37°C. After having removed the medium the cell monolayer surface was rinsed twice with PBS, and eventually stained for nuclei visualization.

Cellular staining was analyzed by Olimpus F1000 laser scanning confocal microscopy.

12.3.1. LIST OF UTILIZED ANTIBODIES

Anti-Microphthalmia (Ab-1) (Calbiochem #OP126L), Osteonectin/SPARC (Takara Clone No.OSN4-2), p21 (Santa Cruz Biotechnology, INC sc-817), Tyrosinase (Santa Cruz Biotechnology, INC sc -20035), human SCD5 synthetic peptide (313-327) (AnaSpec, Eurogentec Group), Rab5B (A-20) (Santa Cruz Biotechnology, INC sc -598), Cathepsin B (S-12) (Santa Cruz Biotechnology, INC sc -6493R), Caveolin-1 (N-20) (Santa Cruz Biotechnology, INC sc -894), CD107b/LAMP2 (BD Pharmigen #555803), Collagen IV (AbCam, ab*6586), V-ATPase C2 (AbCam, ab-176771) were used in accordance to the manufacturer's instructions. β-actin Clone AC-15 (Sigma Aldrich #A5441) and α-

Tubulin (Sigma Aldrich clone B-5-1-2 T5168) were used as western blot loading controls. DAPI (Molecular Probes)

12.4. INTRACELLULAR pH EVALUATION

pHRodo Red (Molecular Probes) is a weakly fluorescent dye at neutral pH that becomes increasingly fluorescent as the pH drops, allowing measurement of intracellular pH in the range 7.5–4.5, and was used as indicated by the manufacturers. Briefly, live cells were washed and incubated with 5 mM pHRodo for 30 min at 37°C. The addition of pH calibrators at 37 ∘ C for 5 min provides a standard curve to assess the intracellular pH of samples. The emission profiles were analysed using microplate-based fluorimetry (Victor X3, Wallac–Perkin-Elmer 2030 software v. 4.00).

12.5. MELANIN CONTENT MISURATION

Melanin Content Misuration was performed in duplicate at least twice for each cell lines. Briefly, after washing the cells with PBS, pellets were lysed with 100uL of 1N NaOH and vortexed repeatedly to homogenize. For analyses, all lysates of each cell extracts were transferred into 96-well plates and the relative melanon content was determined by optical density at 402 nm by using fluorometry (Victor X3, Wallac–Perkin-Elmer 2030 software v. 4.00).

12.6. RENILLA ACTIVITY ASSAYS

For renilla reporter experiments, on the basis of bioinformatic analyses (TargetScanHuman (release 7.1, June 2016)), we identified one conserved putative binding site predicted to interact with miR221/-222 in the SCD5 3'UTR.

The putative SCD5 seed region and its mutated version were respectively amplified (Table 9) by PCR (AccuTaq).

3'UTR SCD5 cDNA amplification
forward primer WT **5' GGT GTA TAA CTC TGA CAT G 3'**
reverse primer WT **5' CAG TTTA CAC ATT ACC AGT G 3'**
forward primer MUT **5' AAG TGA TCg TTA TGc AtC TTC 3'**
reverse primer MUT **5' TCC AGA AGa TgC ATA AcG 3'**
In red are indicated specific point mutations inserted

Table 9: 3'UTR SCD5 cDNA primers sequence (5'-3')

After sequence analysis, these fragments were subcloned immediately downstream Renilla promoter into the psiCHECK 2 modified vector (Promega). The conserved core is indicated in black while red letters represent the mutated nucleotides. 293FT cells (5x104 cells per well) were transfected with: (a) 40 ng of psiCHECK-3' UTRSCD5 plasmid, (b) 50 pmol of either a stability-

enhanced 2'-O-Methyl non targeting RNA control or miR-221 and/or miR-222 oligonucleotides (Dharmacon Inc.), (c) Lipofectamine 2000 (Invitrogen). Renilla activity measured by using the dual luciferase assay (Promega) using Microlite LX1 (dynatech laboratories, Inc). The wt psiCHECK-3'UTRSCD5 cotransfected with the control non targeting oligonucleotide was considered as 100%.

13. IN-VIVO ASSAYS

For the in vivo assays, empty vector- or SCD5-transduced A375M cells in exponential growth phase were injected subcutaneously (s.c.) into the flank and intravenously (i.v.) at the dose of 10^6 cells into adult athymic Nu/Nu mice (Charles River, Calco, Italy), pretreated with rat anti-mouse mAb to IL-2Rb (TMb1, a kind gift from Daniela Mannel, Regensburg, Germany) to deplete NK cells.

Mice were maintained under pathogen-free conditions at the animal facility of Fondazione IRCCS Istituto Nazionale dei Tumori in Milan (Italy).

Tumor growths were monitored twice a week. At different times after the injection, mice were sacrificed for necropsy and macroscopic metastases evaluation. In particular, lung metastases were counted under a stereomicroscope after staining with India ink.

For 4T1 analyses, BALB/c mice were inoculated s.c. into the right flank, equidistant from the inguinal and axillary lymph nodes, with

7×10^3 Tween- or SCD5-transduced cells. Tumor size was monitored twice weekly and the mice were euthanized when the tumors reached ~10 mm in diameter. For surgical excision of primary 4T1 tumors, mice were anaesthetized before surgery and tumors were resected with sterilized instruments. All mice survived surgery. Metastases were evaluated as previously described (Guiducci C., 2004). Briefly, lungs were collected and dissociated in Hank's Balanced Salt Solution (HBSS) containing 1mg/ml collagenase type 4 and 6U/ml elastase for 1 h at 4°C; organs were then plated at various dilutions in a medium supplemented with 6-thioguanine. Individual colonies representing micrometastases were counted after 20–25 days.

All mice were monitored carefully for signs of distress, including behavioural changes and weight loss. Animals were treated and maintained at the Istituto Nazionale Tumouri (Milan, Italy) according to institutional guidelines. Animal experiments were authorized by the Institute Ethical Committee and performed in accordance to institutional guidelines and national law (D.lgs 26/2014).

14. IN-VIVO ANALYSES

Histopathology and Immunohistochemistry

Mice tumor nodules were fixed in 10% neutral buffered formalin, dehydrated and finally embedded in paraffin blocks. Serial sections were cut at 4 µm, dewaxed, rehydrated and stained either with

Hematoxylin and Eosin for morphological analyses or with Masson's Trichrome Staining for collagen fibers detection.

For immunohistochemical staining, heat-mediated antigenic retrieval protocols were used (Tris-EDTA pH 9.0 or Sodium Citrate pH 6.0 buffer, depending on the antibody used) followed by incubation with 3% hydrogen peroxide to quench endogenous peroxidase activity. After saturation for 10 minutes with protein block (Novocastra, Ltd.) the sections were incubated with primary antibodies. For negative controls, mouse or rabbit immune sera were used instead of primary antibodies. Staining was revealed with a polymeric system (Novolink, Max Polymer Detection System; Novocastra Ltd.) and visualized by using AECþ High Sensitivity Substrate Chromogen Ready-to-use (Dako Cytomation Liquid AEC Substrate Chromogen System; Dako).

SCD5 IHC analyses were performed on five cutaneous primary (superficial spreading melanoma, Clark's level III) and five lymph nodal metastatic samples. The bioptic melanoma specimens were obtained with patients' informed consent from the archives of the Human Pathology Section, University of Palermo. Sampling and handling of human tissue material was carried out in accordance with the ethical principles of the Declaration of Helsinki. In particular for SCD5 staining was used a standard ABC technique (Vectastain Rabbit ABC Elite Kit). Slides were counterstained with hematoxylin and evaluated under a Leica DM2000 optical microscope.

14.1. IN-SITU HYBRIDIZATION

Serial sections obtained from the same bioptic human melanoma specimens previously analyzed for SCD5, were subjected to *in-situ* hybridization to evaluate miR221/-222 expression levels by using miRCURY LNA microRNA ISH Optimization kit (FFPE) (Exiqon, Vedbaek, Denmark).

Briefly, sections were (dewaxed) deparaffinized in xylene, rehydrated in ethanol solutions (100% to 70% with water) and then incubated with Proteinase K [5µg/mL] for 10 minutes at 37°C. After two washes in PBS, slides were dehydrated and air-dried. The appropriate double-Digoxigenin (DIG)-labeled LNA probe (specifically designed for miR-221 or miR-222) was prepared at the optimized work concentration of [40 nM] and incubated for 1h at the specific hybridization temperature of 54°C. LNA U6 [5nM] or LNA scramble [40 nM] snRNA probes were used respectively as positive or negative control. Slides were washed in decreasing stringent Saline Sodium Citrare solutions (SSC5X to 0.2X) and finally incubated in a humidifying chamber with blocking solution (PBS 0.1% Tween, 2% Sheep Serum, 1% BSA) for 15 minutes. Then, sections were incubated with the anti-DIG reagent (constituted by the alkaline phosphatase [AP]-conjugated anti-DIG antibody diluted 1:800 in PBS 0.05% Tween-1% Sheep Serum-1% BSA) for 1 hour at RT. After 3 washes in PBS-Tween 0.05%, slides were incubated with the AP substrate NBT-BCIP with Levamisol 0.2mM (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue

tetrazolium) for 2 hours at 30°C. Staining reaction was blocked by washing with KTBT solution (tris-HCl 50mM, NaCl 150mM, KCl 10mM) twice. Nuclear Fast Red was used for nuclear counter staining. Slides were dehydrated and analyzed at the microscopy.

15. STATISTICAL ANALYSIS

Unless indicated otherwise, all data are presented as mean±standard deviation (SD) and results are representative of at least three independent experiments. Statistical analysis was performed using t-test, with $p<0.05$ deemed statistically significant

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PUBLICATIONS Dr. Bellenghi Maria

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