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NEW INSIGHTS ON ESTROGENS AND VITAMIN E-DERIVATIVE TOCOTRIENOLS IN HUMAN MALIGNANT MELANOMA: TOWARDS NOVEL THERAPEUTIC INTERVENTIONS

Tesi di Dottorato di Ricerca:
Dr.ssa MONICA MARZAGALLI
Matricola R10029

Tutor: Prof.ssa Patrizia LIMONTA
Coordinatore: Chiar.mo Prof. Angelo POLETTI

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SUMMARY

Introduction

Cutaneous melanoma

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Epidemiology

Melanoma is among the most aggressive and treatment-resistant human cancers. Although melanoma accounts for less than 5% of all skin cancers, it is responsible for 75% of all skin cancer deaths [1]. Its incidence is still rising worldwide, with 2.6% annual increase over the last decade [2].

The incidence rate of melanoma changes in relation to race, with white populations having an approximately 10-fold greater risk that than black, Asian or Hispanic populations [3], but the 5-year relative survival for black with melanoma is 74.1% compared with 92.9% for whites. The median age at the time of diagnosis is 60 years and although the incidence of melanoma increases significantly with age, it is also one of the most frequent cancers in adolescents and young adults. The incidence of melanoma is higher in men than in women: the risk of a man developing a melanoma during his lifetime is 1.6 times the risk of a woman.

Since melanoma is primarily a disease of Caucasians, phenotype is considered one of the major risk factors: fair skin and associated characteristics, such as blond or red hair, blue eyes, freckles, denote patients with an increased risk of developing the disease [4]. In this context, the inability to tan and sunburns play an important role, an observation traditionally attributed to reduced UV-radiation protection. Epidemiologic studies have implicated intense intermittent UV radiation (UVR) exposure and severe sunburns during childhood in conferring the higher risk of developing melanoma [5]. Natural sunlight exists as a variety of wavelengths filtered by the atmosphere; however, it is largely the UVB radiation that is responsible for the development of melanoma. Longer wavelength, UVA radiation, is the primary light utilized by tanning beds: this type of radiation is known to penetrate more deeply into the dermis then UVB, so that it was felt to be less likely to cause damaging superficial sunburns [6]. Multiple studies now clearly demonstrate a significant correlation between tanning bed use and melanoma [7]. UVR has multiple effects on the skin, including genetic

changes, induction of reactive oxygen species (ROS), alterations in cutaneous immune function, all features that contribute to carcinogenesis.

Testing the hypothesis that sunlight is one of the principal causes of melanoma, studies reported that sites habitually exposed to the sun would have the highest incidence, such as the ears in men and face and neck in both sexes, followed by intermittently exposed sites (back, shoulders, and limbs). Conversely, melanomas were noted to be rare on sites habitually covered by clothing. However, certain types of melanomas were observed to arise in areas that are well protected from UVR, such as the non-hair-bearing skin of the palms and soles and areas under the nails, and still others were recorded at sites that have no UV exposure at all (such as those on mucosal membranes) [8]. To further explore the associations between sunlight exposure and melanoma risk, epidemiologists compared melanoma incidence across occupations (indoor versus outdoor work) as a surrogate measure of historical sun exposure. Unexpectedly, indoor workers experienced higher rates of cutaneous melanoma than outdoor workers [9]. However, more detailed analysis showed that outdoor workers experienced significantly higher rates of melanoma on habitually sun-exposed sites (face, head and neck), whereas indoor workers were found to have a significant excess of melanomas on habitually covered body sites [10, 11]. These findings provided the first epidemiological evidence that melanoma arising on different body sites may be associated with sunlight in different ways.

Among risk factors, family history increases a personal risk of melanoma by 3-8 times, and this risk increases with the number of family members who are affected [12]. Familial syndromes were described, characterized by large numbers of nevi (50-100) and especially dysplastic/atypical nevi [13]. More recently, several genes have been implicated in the development of melanoma: mutations in the cyclin-dependent kinase (CDK) gene *CDKN2A* are thought to account for 20-40% cases of familial melanoma [14]; among the other genes, *CDK4*, and the *BRAF* oncogene [15], but also genes that are associated with nevi and pigmentation traits, such as *MTAP, MC1R* and *TYR* genes are involved in the hereditable contribution to melanoma risk [16]. Moreover, a personal history of melanoma represents an important risk factor: a person who has

already developed one melanoma has a 3-7% chance of developing a second primary melanoma, and this risk is 900 times higher than a person without previous personal history of melanoma [17].

Nevi represent both risk markers and precursors for melanoma: most melanomas arise *de novo*, and therefore are not the result of malignant transformation from existing skin lesions, but they can occasionally develop within certain precursor lesions, such as dysplastic nevi and congenital nevi. In general, a dysplastic nevus is a flat, pigmented skin lesion with indistinct margins and variable color, although the clinical distinction between a nevus with or without dysplasia is often difficult. The risk of a person with congenital nevi to develop melanoma is proportional to the size and number of nevi: small congenital nevi represent a low risk, and giant congenital nevi (defined as greater than 20 cm in diameter) carry a lifetime risk for developing melanoma of up to 10% and therefore should be completely excised [18].

Pathologic description

Melanocytic degeneration to malignant melanoma exists along a spectrum that ranges from mild, moderate, or severe dysplasia to atypical melanocytic proliferation, to melanoma *in situ*, to early invasive melanoma. MIS is considered a premalignant lesion with a significant risk of progression to invasive melanoma, but with a low metastatic potential. Indeed, MIS doesn't develop beyond the basement membrane, so it doesn't have access to blood or lymphatic vessels. Invasive melanoma arises as a proliferation of melanocytes in the basal layer of the skin: they expand radially in the epidermis and superficial dermal layer (Radial Growth Phase, RGP). As the cells proliferate, the growth also begins in the vertical direction, and the lesions become palpable (Vertical Growth Phase, VGP) (Fig. 1).

Histologically, invasive cutaneous melanomas were divided into four major subtypes, based on growth pattern and location, in the first classification system proposed by Clark and colleagues [19].

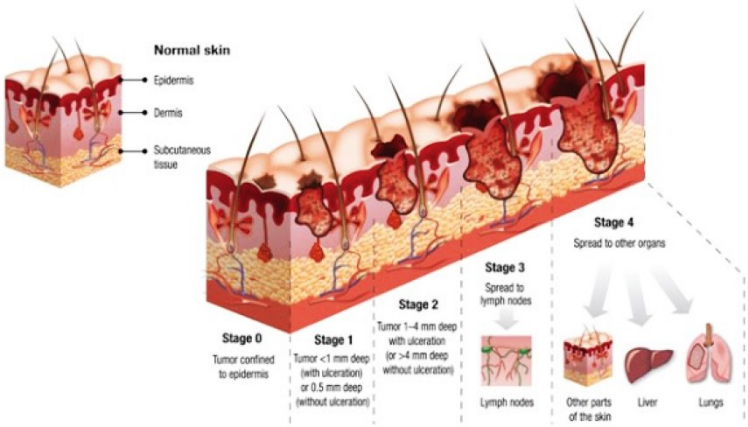

Fig. 1. Schematic description of melanoma progression. Stage 0: melanoma *in situ*. Stages 1-2: invasive melanoma. Stages 3-4: metastatic melanoma.

A prominent feature for classification was the distribution of melanocytes within the early phase of melanoma growth (RGP), identifying superficial spreading melanoma, lentigo maligna melanoma and acral lentiginous melanoma as growing with an intraepidermal pattern. The fourth type, nodular melanoma, was proposed as a separate category because it presented without a significant RGP.

The most common histologic subtype is **superficial spreading melanoma (SSM)** that accounts for 60-70% of all melanomas. It's not specifically associated with sun-exposed skin, but can occur in any dermal tissue. SSM begins as a flat, well-circumscribed polycyclic patch with variegating shades of brown, gray and black growing in the radial dimension, but over time it would develop a vertical growth. Histopathologically, it is characterized by the presence of enlarged melanocytes, often arranged as small aggregates or nests, that display marked upward scatter within the epidermis, in contrast to melanomas that display a lentiginous growth pattern (Fig. 2A).

Nodular melanoma (NM) accounts for 15-30% of all melanomas, and represents an exception to the usual growth pattern: the VGP is present early in tumor development, so the tumor has an elevated metastatic potential. NM are raised, papular lesions that often carry a poor prognosis because of the above-average tumor thickness and frequent ulceration (Fig. 2B).

Lentigo maligna melanoma (LMM) accounts for only 5% of all melanomas. This subtype occurs most commonly on the face of older individuals with sun-damaged skin and presents as a flat, dark, variably pigmented lesion with irregular borders and a

history of sneaky slow development, as the lesion's slow progression may go unnoticed by the patient. Overall, the prognosis of LMM is better than the other melanoma subtypes because the lesion is often superficial, but a delay in diagnosis can thwart this favorable histology (Fig. 2C).

Acral lentiginous melanoma (ALM) is classified as such based on its anatomical site of origin. It develops on the palms of the hands, on the soles of the feet, or in the subungual areas. This is the most uncommon type of melanoma, but it accounts for 45% of melanomas in Asians and 70% in blacks. Histologically, it appears similar to melanomas arising from the mucous membranes. The diagnosis is often made when the tumor is at an advanced stage, because these lesions are often mistaken for subungual hematomas (Fig. 2D).

Both LMM and ALM display a lentiginous growth pattern, in which the melanocytes are arranged as solitary units along the basilar epidermis. This growth pattern typically fades into the adjacent non-lesioned skin, making it difficult to determine the boundary between melanoma and normal skin.

A fifth melanoma subtype has also been identified: **desmoplastic melanoma (DM)** is rare and only accounts for 1.7-4% of all melanomas. Clinically, it presents as an amelanotic lesion, most commonly of the head and neck region. DM is associated with male gender and a history of extensive sun exposure. It exhibits different behavioral patterns than other melanoma subtypes, it often exhibits neurotropism and has a greater propensity for local recurrence with a decreased risk of nodal metastasis [20] (Fig. 2E).

In addition, melanomas can originate from extracutaneous sites such as the mucosal epithelium, or from melanocytes of the uvea.

In general, the histologic subtype of melanoma is not a major factor in determining prognosis; however, some histologic subtypes are more likely to be detected at an advanced stage, thus indirectly affecting prognosis.

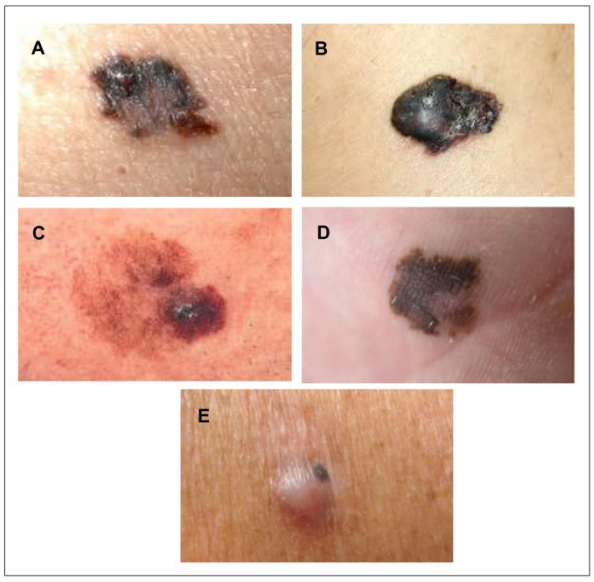

Fig. 2. Clinical aspect of different types of cutaneous melanomas. **(A)** Superficial spreading melanoma. **(B)** Nodular melanoma. **(C)** Lentigo maligna melanoma. **(D)** Acral lentiginous melanoma. **(E)** Desmoplastic melanoma.

Diagnosis

Early detection of melanoma can have a significant effect on patient survival, since the prognosis largely depends on the stage of the tumor at the time of diagnosis: in fact, patients with melanoma *in situ* (MIS) have the same life expectancy as the general population [2].

The majority of melanomas can be diagnosed by history and physical examination. The first includes the assessment of the risk factors previously described, and the history of the lesion itself, that means the evolution or changes in its physical aspect, such as in shape, size and color. Melanoma usually presents as an irregularly pigmented skin lesion with a history of growth and change over time, so a complete head-to-toe skin examination involving all of the body surfaces, together with palpation of lymph node basins, should be the first step for diagnosis. The "ABCDE" clinical warning signs for differentiating melanoma from benign moles were developed since 1980s, and are currently used to guide the decision of which lesions to biopsy. This system identifies five characteristic features that should allow to recognize melanoma onset (Fig. 3):

A: asymmetry

B: irregular borders

C: variable shades of color

D: increase in diameter

E: evolution over time, that might account i.e. also for itching, bleeding or other subjective changes, such as the "ugly duckling" sign, that focuses on identifying individual lesions that do not match a person's typical nevus phenotype [21].

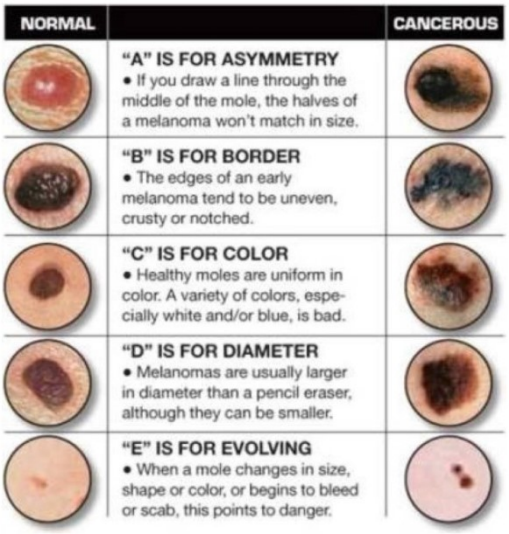

Fig. 3. The "ABCDE" rule for the recognition of cutaneous melanoma.

Although these features are characteristic, not all melanomas follow these rules: for example, primary cutaneous melanomas can also be amelanotic, or non-pigmented. These lesions may manifest as raised pink or flash-colored. A high index of suspicion is needed, because the distinction between a benign nevus or a pigmented lesion and an early melanoma can be quite difficult. Compared to typical nevi, MIS frequently has an atypical pigment network involving more than 50% of the lesion, or the presence of multiple pigment networks. Grey-blue regression is also more commonly observed in MIS lesions. Instead, the comparison between atypical nevi and melanoma *in situ* is more difficult, because both can share similar features [22].

Staging and prognosis

Staging for cutaneous melanoma is systematically defined by the American Joint Committee on Cancer (AJCC) Melanoma Staging Committee. This staging system is continuously evolving and adapting to the new insights about our understanding of the biology of the tumor. In the last edition of this system (7th edition, 2009), important prognostic factors has been redefined: Breslow thickness, ulceration, mitotic rate, nodal status, the presence of other manifestation of lymphatic spread (satellite lesions, in-transit disease) and the presence of distant metastasis [23].

The **status of regional lymph nodes** is the most important prognostic factor for overall survival [24]. Sentinel Lymph Node (SLN) biopsy has become a standard method for stage evaluation, based on the fact that SLN is the first lymph node that receives metastatic tumor cells. The classical technique for pathologic evaluation of the sentinel lymph node is by hematoxylin and eosin staining, with the addition of immunohistochemical staining for melanoma markers S-100 and HMB-45 to increase sensitivity.

Breslow thickness represents a measure of the depth of invasion, in millimeters, as vertical thickness of melanoma, and was described in 1970 by Alexander Breslow [25]. Based on this stratification system, melanoma is considered "thin" (≤1 mm), "intermediate" (1-4 mm) or "thick" (>4 mm), and the prognosis worsen as the thickness increases. A similar depth classification was described previously by Wallace Clark in 1969, based on the level of invasion into the anatomical layers of the skin (**Clark's level**). In this context, level I tumors represent melanoma *in situ* and are limited to the epidermis; level II melanomas extend to the papillary dermis; level III fill the papillary dermis; level IV melanomas extend to the reticular dermis and level V melanomas invade the subcutaneous fat [26]. Over time, Breslow thickness has become the classification system of choice, because of its simpler form and the more accurate prediction of prognosis.

Ulceration has also emerged as a very robust predictor of prognosis. It is defined as the absence of an intact epithelium overlying the lesion. Multiple studies demonstrate

that the presence of ulceration represents a more aggressive tumor phenotype, with a higher likelihood of metastasis and worse prognosis [27].

Mitotic rate is the most recently validated prognostic factor, and it is defined as mitoses/mm^2. A growing body of data shows a significant correlation between increasing mitotic rate and decreased survival [28], and a mitotic rate ≥1 may be the only indication for the evaluation of the regional lymph nodes by SLN biopsy [29].

Other factors affecting prognosis of melanoma are: age (better prognosis for younger patients, despite they are more likely to have nodal metastasis), anatomic location of the lesion (patients with trunk, head and neck melanomas have a worst prognosis compared to extremity tumors), and gender (women have a better prognosis than men) [22].

Staging for cutaneous melanoma, following the Tumor-Node-Metastasis (TNM) system of classification, as defined by AJCC is summarized in Table 1.

Stage I and II refer to localized melanoma. Tumor thickness, mitotic rate, and ulceration are significant independent predictors of survival in this group of patients, and are used in the AJCC melanoma staging system to define T categories.

Stage III comprises patients with regional metastasis (i.e., regional lymph node, satellite and/or in-transit metastasis). In these patients, the number of nodes harboring metastatic disease is the most important predictor of survival, and this parameter is used to define N categories, together with the distinction between macro- and micrometastasis in regional node. Microscopic disease refers to metastasis detected with histologic analysis following lymph node dissection/biopsy, while macrometastasis are clinically or radiographically detected.

Stage IV melanoma refers to disease with distant metastasis. The prognosis for these patients is generally poor, with 5-years survival rates lesser than 10%. Patients are categorized with respect to the site of metastasis, because the anatomic location of distant disease influences the survival rate. Patients with metastasis to distant skin, subcutaneous tissues, and/or lymph node basins have the highest one-year survival rate (62%), patients with pulmonary metastasis have an intermediate prognosis (53% one-year survival rate), and those with non-pulmonary visceral metastasis and/or

elevated serum LDH have the worst one-year survival (33%). Although the exact pattern of elevated LDH isoforms is nonspecific in melanoma patients, and the mechanism of LDH elevation is not fully understood, a growing number of clinical data supports its use as a prognostic factor in patients with stage IV disease [23].

T classification	Thickness	Ulceration Status
Tis	NA	NA
T1	≥1.00 mm	a: w/o ulceration and mitosis ≥1/mm2
		b: with ulceration or mitoses ≥1/mm2 n/mm2
T2	1.01 – 2.0 mm	a: w/o ulceration
		b: with ulceration
T3	2.01 – 4.0 mm	a: w/o ulceration
		b: with ulceration
T4	>4.0 mm	a: w/o ulceration
		b: with ulceration

N classification	# of Metastatic Nodes	Nodal Metastatic Burden
N0	0	NA
N1	1	a: micrometastasis *
		b: macrometastasis **
N2	2–3	a: micrometastasis *
		b: macrometastasis **
		c: in transit met(s)/satellite(s) without metastatic nodes
N3	4+ metastatic nodes, or matted nodes, or in transit metastases/ satellites with metastatic nodes	

M classification	Site	Serum LDH
M0	No distant metastases	NA
M1a	Distant skin, subcutaneous, or nodal metastases	normal
M1b	Lung metastases	normal
M1c	All other visceral metastases	normal
	Any distant metastasis	elevated

Abbreviations: NA, not applicable; LDH, lactate dehydrogenase

From Balch CM, Gershenwald JE, Soong S, et al, J Clin Oncol 27(36): 6199-206, 2009; with permission.

* Micrometastases are diagnosed after sentinel lymph node biopsy.

** Macrometastases are defined as clinically detectable nodal metastases confirmed pathologically.

Table 1. AJCC TNM classification of cutaneous melanomas (7th edition) [23].

Molecular aspects of melanoma development and progression

Melanoma arises from malignant transformation of melanocytes, the melanin-producing cells of the skin, eye, mucosal epithelia, and meninges, that are responsible for pigmentation and photoprotection. Melanocytes produce two main types of pigment: brown/black eumelanin and red pheomelanin. The first is the photoprotective pigment that provides UV radiation attenuation. In response to UV radiation, factors regulating survival, differentiation, proliferation and motility of melanocytes are secreted from keratinocytes, that in turn stimulate melanocytes in melanin production. The first step in melanin synthesis is catalyzed by the enzyme tyrosinase, which converts tyrosine to dihydroxyphenylalanine (DOPA). The biosynthetic pathways for the two pigments diverge downstream DOPA, and the choice of pathway is determined by the signaling activity of the melanocortin receptor MC1R. The biosynthetic intermediates of melanogenesis are toxic, and that's the reason why the synthesis occurs in a specialized organelle, the melanosome. Most melanocytes do not retain melanin, but the melanin granules are transported along microtubules to the tips of dendrites, from where they are transferred to keratinocytes [30], so that melanin can protect cells form the damaging action of UVR, thus preventing skin cancers [31].

Pigment synthesis is stimulated by binding of α-melanocyte-stimulating hormone (α-MSH) to MC1R on melanocytes; this process activates intracellular cAMP-signaling pathways leading to the activation of microphtalmia-associated transcription factor (MITF), a key transcription factor responsible for melanin production. MITF is also crucial for melanocytes differentiation during development, together with other transcription factors such as c-KIT [2]. The melanocyte lineage is derived from the neural crest, and has the same embryonic origin of neurons and glial cells. The triggers of the melanocyte lineage are PAX3 and SOX10, acting synergistically to activate MITF transcription, and the downregulation of FOXD3 and SOX2 [30].

Mutations in genes involved in melanocytes development are found in melanomas, thus leading to the possibility that differences in the susceptibility of melanocytes to

became cancerous might be related to differences in developmental origin of melanocytes, different states of embryonic development or differences in environment through which melanocytes migrate and differentiate.

Over the last decade, significant progresses has been made in uncovering critical somatic genetic alterations, and it has become clear that specific genetic mutations are associated with particular clinical and histopathological features of melanoma.

The MAPK pathway has been implicated as a central player in the pathogenesis of melanoma, because mutations in NRAS and BRAF cause constitutive activation of the pathway leading to increased cell proliferation and survival, and have been found in more than 90% of clinical cases of melanoma [32].

RAS mutations

The first melanoma oncogene to be identified was *RAS* [33], that encodes for a small GTPase located at the plasma membrane. Mutations that cause the constitutive activation of the protein lead to the hyperactivation of downstream effector pathways, including the MAPK and the PI3K pathways, inducing cell cycle progression, cellular differentiation and cell survival. NRAS is the most frequently mutated protein of the RAS family members in melanoma (15-30% of melanomas), and particularly frequent are mutations in the codon 61 (glutamine/arginine Q61R or glutamine/lysine Q61K). Some studies reported an association between NRAS mutations and melanomas on sun-exposed sites, while other studies did not confirm this association; similarly, some studies reported an association with certain histopathological subtypes, such as nodular melanoma and lentigo maligna melanoma, compared with superficial spreading melanoma [5].

BRAF mutations

Approximately 60-70% of primary and 40-70% of metastatic melanomas have an activating mutation in BRAF [34], and 80% of these mutations involve a single substitution of glutamate for valine (V600E): this modification mimics phosphorylation on the regulatory domain of the protein, thus leading to enhanced kinase activity and sequential phosphorylation and activation of MEK and ERK, even in the absence of RAS

activation [35]. The activation of the MAPK pathway triggers the stimulation of G1/S transition of the cell cycle, because ERK1/2 activates c-Myc, cyclin D1 and negatively regulates proliferation inhibitors such as p27 [36]. Multiple studies have shown that patient with BRAF-mutated melanomas are younger than patients with melanoma without BRAF mutation, and that BRAF-mutant melanomas are more commonly superficial spreading melanomas and arise on intermittently sun-exposed areas of the body. BRAF mutations are also commonly found in acquired melanocytic nevi [37]: this suggests that the mutation itself is insufficient to cause melanoma, and further genetic alterations are required within a nevus to result in tumor formation. In general, some phenotypic features are characteristic of BRAF-mutant melanomas, such as the presence of upward scatter of intraepidermal melanocytes, the predominance of melanocytes arranged in nests rather than single cells, and the degree of melanization, since BRAF-mutant melanomas are more pigmented than non-BRAF mutant [8].

KIT mutations

KIT is a transmembrane receptor tyrosine kinase that is crucial in melanocytes development, influencing their proliferation, migration, differentiation and survival, by acting through downstream signaling pathways such as the MAPK, PI3K/Akt and JAK/STAT pathways. KIT is activated by point mutations in 5-25% of mucosal and acral melanomas [34], and in skin with cumulative sun-induced damage (CSD-melanomas) [8]. In the melanoma types in which KIT mutations are found, BRAF mutations are relatively uncommon, and therefore the two mutations represent a mirror image of each other. Acral and mucosal melanomas and CSD-melanomas share several features, that are opposite to BRAF-mutant melanomas: for example, their incidence rises with age and they frequently show a lentiginous growth pattern, and tend to be poorly circumscribed. This characteristic is likely to be due to an increased lateral mobility of the neoplastic melanocytes, which can be functionally linked to KIT pathway activation [38]. Indeed, this pathway is essential during melanocytes migration from the neural crest to the skin via the dorsolateral pathway, and its reactivation may induce a histopathological growth pattern that reflects increased cell motility.

Akt3/PTEN mutations

As the MAPK pathway, the PI3K pathway is crucial for cell growth and survival. Although PI3k itself is not mutated in melanomas, selective activation of downstream factor Akt3 occurs in 53% of primary and 67% of metastatic melanomas [39]. Activated Akt stimulates proliferation and tumorigenesis through upregulation of cyclin D3, which controls G1/S cell cycle progression, and through downregulation of the cell cycle inhibitors p21 and p27 [36]. The increasing expression of this protein during melanoma progression is due to several mechanisms, including the amplification of the gene itself and various alterations, that decrease the expression of PTEN, a tumor suppressor protein that normally negatively regulates the PI3K pathway. Deletions and mutations of PTEN occur in approximately 30% of melanoma cell lines that frequently contain concurrent mutations in BRAF, but not in NRAS. Indeed, activating mutations in NRAS can lead to the parallel activation of both the PI3K and MAPK pathways, abrogating the need for additional mutations in PTEN or BRAF.

CDKN2A mutations

CDKN2A is the primary familial high-risk melanoma susceptibility locus identified in families with multiple cases of melanoma. This gene encodes two different suppressor proteins, $p16^{INK4A}$ and $p14^{ARF,}$ which are both involved in cell cycle regulation. $p16^{INK4A}$ normally functions by inhibiting cyclin-dependent kinase 4 and 6 (CDK4 and CDK6), thus preventing phosphorylation of the retinoblastoma protein (pRb) and leading to G1/S cell cycle arrest. $p14^{ARF}$ acts by blocking the degradation of p53, enhancing apoptosis. Genetic or epigenetic inactivation of this tumor suppressors leads to uncontrolled cell growth. Even though the prevalence of *CDKN2A* mutations in primary melanomas is only 1.2% in the general population, germ-line mutations in this locus have been observed in approximately 20-57% of families with at least three cases of melanoma [40]. In addition to its role as a high-risk allele, *CDKN2A* also displays frequent deletion in the progression from dysplastic nevus to melanoma [41].

MITF mutations

As previously described, microphtalmia-associated factor (MITF) is centrally involved in melanocyte differentiation, cell growth and survival and is known as the master regulator of melanocyte development. The expression of MITF is regulated by multiple signaling pathways, such as α-MSH/MC1R/CREB, Wnt/Frizzled/β-catenin, and PAX3 and SOX10 during melanocyte development. After translation, MITF may be activated by c-KIT and the MAPK pathway. MITF simultaneously stimulates the expression of proliferation-promoting genes, as well as tumor suppressor genes, and its potential dual effects in melanoma cells depends on the balance between cancer-promoting and cancer-suppressing activity [36]. Amplification of *MITF* copy number occurs in 10% of primary and 20-40% of metastatic melanomas, but is not observed in benign melanocytic nevi [42]. Somatic mutations in *MITF* have also been identified in 8% of melanoma samples that do not express its concomitant amplification, demonstrating that these genetic alterations are mutually exclusive.

G-protein mutations

Recently, mutations in G-proteins of the $G\alpha_q$ family have been described in certain subsets of melanomas, such as uveal melanomas. In this type of melanocytic neoplasia, mutations in the MAPK pathway are extremely rare, although the upregulation of this pathway is frequently seen [43]. Two G-proteins have been identified as early oncogenes in uveal melanoma: G_q and G11, encoded by the genes *GNAQ* and *GNA11*, respectively. Mutations in the first protein lead to its constitutive activation and enhanced expression of downstream effectors such as ERK [44]. While *GNAQ* mutations are common in blue nevi and less common in malignant tumors, *GNA11* mutations are most common in uveal melanoma metastasis, with a lower mutation frequency in blue nevi, suggesting that it may have more powerful oncogenic effects than *GNAQ* [8].

Aberrant DNA methylation

The DNA methylation status of cutaneous melanoma has been extensively studied and has been demonstrated to have prognostic and therapeutic significance. Changes in

DNA methylation patterns are important characteristics of most human cancers, and act as an alternative or complementary mechanism to gene mutations in the alteration of gene expression and function. Hypermethylation of tumor suppressor genes, as well as those involved in cell cycle regulation, DNA repair, cell signaling, transcription and apoptosis have been well described in cutaneous melanoma, while specifically hypomethylated genes have been less documented.

CDKN2A and *PTEN* are tumor suppressor genes, previously described because found to be mutated in melanomas, that are frequently silenced by DNA hypermethylation. Other genes hypermethylated in melanoma cells are *RASSF1A*, encoding for a Ras effector promoting various cellular events including apoptosis, migration and mitosis, and *RAR-beta2,* encoding for the Retinoic Acid Receptor β2. There is convincing evidences that RARβ2 acts as a putative tumor suppressor, despite its exact mechanism in tumor suppression still needs to be defined. However, the frequency of aberrant methylation of this gene is high as 70% in melanomas, and in particular it seems that it might play a role in initiation and progression of primary lesion rather than in metastasis formation [45].

Despite the increased aberrant promoter hypermethylation, it has been shown that tumor cells are globally and paradoxically genome-wide hypomethylated when compared to normal cells [46]. There are suggestions that hypomethylation can contribute to carcinogenesis through two distinct mechanisms, that means the re-expression of oncogenes and triggering of chromosome instability. In malignant melanoma, hypomethylation has been shown to lead to activation of a number of Cancer Testis (CT) genes, such as MAGE (Melanoma Antigen Genes) or BAGE (B melanoma Antigen), normally expressed in adult testicular germ cells, that can also be aberrantly activated and expressed in a range of human cancers. Though the biological role of CT genes in cancer remains poorly understood, it has been demonstrated a correlation between a high expression of these genes and worse outcome [45]. Among the hypomethylation of specific promoters, a global DNA hypomethylation is observed in cancers, and also in melanoma, that has been shown to contribute to chromosomal instability through the demethylation of transposons and pericentromeric regions.

Indeed, DNA hypomethylation in tumors occurs prevalently at repetitive sequences, including short and long interspersed nuclear elements and LTR elements, segmental duplications and subtelomeric regions [47]. Moreover, some studies demonstrated that, in particular, the loss of 5-hmC is correlated with the parameters of poor prognosis in melanoma, including Breslow thickness, mitotic rate, ulceration as well as with lower overall survival [48].

Therapeutic strategies

The current management for melanoma *in situ* is represented by surgical removal of the clinically apparent tumor. As melanoma forms discontinuous nests of tumor cells in the dermal lymphatics adjacent to the primary tumor [49], the surgical therapy might include the excision of surrounding unaffected tissue to prevent local recurrence. Based on the observation that, initially, melanoma spreads predominantly to regional lymph nodes, it was proposed that lymphadenectomy should be considered to obtain cure, and preventing metastasis dissemination, since metastatic melanoma is mostly an incurable disease. Metastasectomy continues to be the standard of care for patients that present with a solitary metastasis, such as for lung, gastrointestinal, pancreatic or brain metastatic disease. While there's clearly a benefit for resection of solitary lesions, the ability to completely resect all known sites of disease is a critical determinant of long-term survival, and the potential for combining surgical resection with other adjuvant therapies, that means that treatments given in addition to the primary or main treatment, remain a topic of interest. Currently, the only adjuvant therapy approved by the US Food and Drug Administration (FDA) is high-dose interferon α-2b (IFNα-2b), but unfortunately the dosing regimen is near to what is maximally tolerated. Side effects include severe flu-like symptoms, fatigue, malaise, anorexia, neuropsychiatric side effects, and potential hepatic toxicity. Patients that may benefit from this regimen have node-negative disease, micrometastatic nodal disease or palpable nodal disease, and clinical trials suggested that with adjuvant IFNα-2b treatment there is a modest 5-10% improvement in disease free-survival [22].

Adjuvant radiation therapy for melanoma is not routinely used, but it might be useful in case of CNS disease.

Patients with unresectable metastatic disease should be considered for systemic therapy.

Until 2010, all major chemotherapeutic and immunological approaches as well as radiotherapeutic interventions have failed to increase survival rates of stage IV patients in randomized clinical trials [50], and prior to 2011 the only agents approved by the US FDA for the treatment of metastatic melanoma were dacarbazine and interleukin-2 (IL-2).

Dacarbazine and its prodrug, temozolomide (TMZ), are alkylating agents that were approved by the US FDA for the treatment of metastatic melanoma since 1974. However, less than 5% of patients achieve a complete response with dacarbazine and there is only a 2-6% survival reported at 5 years [51]. TMZ has an advantage over dacarbazine in being able to cross the blood-brain barrier, so that it's used for the treatment of CNS disease, but no differences in overall survival has been observed [52]. Dacarbazine has remained the standard chemotherapy of choice for metastatic melanoma, because other single and combination chemotherapy regimens have failed to demonstrate any overall survival benefit. A common chemotherapy regimen is composed by cisplatin, vinblastine and dacarbazine or TMZ, while another combination strategy is adding carboplatin to paclitaxel, a regimen used in the treatment of non-small-cell lung cancer and advanced ovarian cancer.

IL-2 is a cytokine that mediates the homeostatic function of T and natural killer (NK) cells, and emerging data have supported its role in expanding regulatory T cells (Tregs), which have innate suppressive functions. It was approved by the US FDA for metastatic melanoma in 1998. However, high-dose IL-2 therapy has shown very marginal response rates, at approximately 6%, and no benefit in overall survival. Moreover, IL-2 is associated with substantial toxicity, requiring administration in an intensive care unit setting [22]. Adverse effects include capillary leak syndrome, characterized by hypotension, tachycardia, peripheral edema, reversible multi-system organ failure, and

cardiac arrhythmias. Additional toxicity consists of fever, pruritus, electrolyte abnormalities, cytopenias, and coagulopathy.

Another therapeutic approach has been to combine chemotherapy with immunotherapy in a regimen generally referred to as "biochemotherapy", such as the combination of dacarbazine with IL-2 or IFNα-2b: an improvement of progression-free survival was demonstrated, but not an advantage in overall survival. Additionally, this regimen is associated with severe toxicity [22].

IL-2 belongs to the tumor immunotherapies, which are the modalities that utilize the immune system to recognize and eradicate cancer. Among the others, adoptive cell transfer (ACT) is a form of passive immunotherapy in which patients are infused with a large number of melanoma-specific T cells, with objective response rates as high as 70%. T cells are selected for their ability to produce IFNγ in the presence of tumor or specific antigens and subsequently expanded in culture using IL-2 stimulation, to produce a large number of cells to be reinfused into the patient [53]. The source of tumor-specific T cells might be the tumor-infiltrating lymphocytes or the transgenically modified peripheral blood T cells that express a melanoma-specific T cell receptor. Adverse effects are related to autoimmune events such as the destruction of normal melanocytes, or to the lymphodepleting preconditioning regimen of patients.

Recent advances is our understanding of the genetic profile of melanoma cells and the molecular factors that drive malignant transformation have resulted in the identification of new therapeutic targets and the development of new targeted therapies.

Targeting immunity pathways

Regulatory pathways that limit the host's antitumor immune response have become particularly interesting molecular targets for the treatment of metastatic melanoma.

To this purpose, regulatory T cells (Tregs) inhibitors and Toll-Like receptor (TLR) agonists have been developed. Tregs suppress the activated effector T cells, so they can inhibit antitumor immunity in animal models. An increase of Tregs number is observed in both the peripheral circulation and tumor microenvironment of melanoma patients. These cells are characterized by high expression of the IL-2 receptor CD25, so

this protein might be exploited as a molecular target. Ontak, a fusion of the IL-2 protein and diphteria toxin, selectively eliminates CD25-expressing Tregs, but the unwanted depletion of antitumor effector cells, such as CD4+ helper and CD8+ cytotoxic T cells that also express CD25, might occurs.

The activation of the innate immune system (macrophages and dendritic cells) through TLRs promotes the activation of naive B and T cells, and may mediate the antitumor immunity. Activation of these receptors by TLRs agonists, such as imiquimod, induces tumor regression, and the tumors that regressed following TLRs agonist treatment have been associated with a significant immune response, including an increase in CD8+ T cell infiltration in the site of application [54].

Several targeted therapies implicate the development of recombinant, humanized monoclonal antibodies against specific proteins of the immune cells, such as CTLA-4 and PD-1.

The cytotoxic T-lymphocyte antigen 4 (CTLA-4) is an inhibitory checkpoint receptor that blocks T cell activation and helps regulate the balance between immune activation and tolerance. T cell activation is regulated by a series of signals from antigen presenting cells (APCs): the first signal is delivered through antigens, that are recognized by the T cell receptors (TCRs); the second is a costimulatory signal, provided by CD80 and CD86 located on the surface of APCs, that bind to CD28 on the surface of T cells. In this setting, T cells are stimulated to enter the cell cycle, differentiate and produce cytokines. Under normal conditions, CTLA-4 is then mobilized to the surface of the activated T cells, replacing CD28 in the binding to CD80 and CD86: this interaction reverses T cell activation and provides an autocrine regulatory mechanism for preventing uncontrolled T cell activation and autoimmunity. An anti-CTLA-4 monoclonal antibody (ipilimumab) has been developed to avoid this negative feedback, and to prolong the antitumor activation of T cells. This molecule was approved by the FDA for the treatment of advanced melanoma in 2011, despite it's associated with side effects related to autoimmune events, such as dermatitis, colitis, drug-related hepatitis, endocrinopathies and neuritis [54].

Similar to CTLA-4, the programmed death 1 (PD1) receptor is a T cell coinhibitory protein that binds to the PD-1 and PD-2 ligands (PD-L1 and PD-L2) on APCs and suppresses T cell activation. Nivolumab, a specific anti-PD1 monoclonal antibody has been developed, and appears to be well tolerated without significant toxicity. Combination strategies with ipilimumab and nivolumab antibody might be evaluated, and are supported by preclinical studies that demonstrated a synergistic effect on melanoma [55].

Targeting proliferative pathways

Melanoma is a heterogeneous disease that presents different genetic alterations: the identification of the key signaling molecules that are implied in melanoma development and progression is crucial for the development of new targeted therapeutic strategies.

The most common mutation found in melanomas is in the *BRAF* gene: about 50% of melanomas harbor this mutation, of which 90% carrying the V600E point mutation. Vemurafenib, the first targeted drug for melanoma, is a selective $BRAF^{V600E}$ inhibitor that was approved by the FDA in August 2011. This antibody inhibits the kinase domain of the mutated protein, decreasing cell proliferation through reduced activation of MEK and ERK, but it doesn't have antitumor effects against cells with $BRAF^{wt}$. Compared with dacarbazine, vemurafenib induces a significant improvement in overall survival and progression-free survival: six-month survival is as higher as 20% for vemurafenib-treated patients [56]. The toxicity profile includes skin reactions, such as photosensitivity, rash, pruritus, hyperkeratosis, keratocanthomas, and squamous cell carcinomas. Additional adverse effects are fatigue, arthralgias, alopecia, headache, nausea, vomiting and diarrhea. Despite the encouraging results, the duration of response is limited because tumor quickly develops resistance through molecular alterations in the same or other signaling pathways [57]. Dabrafenib is another monoclonal antibody developed to target mutated BRAF. It's an ATP-competitive inhibitor efficient against both $BRAF^{V600E}$and $BRAF^{V600K}$, with response as similar as vemurafenib in terms of mechanism of action, timing of responses, side effects and development of resistance [57].

Other inhibitors of the MAPK pathway are directed against MEK. Selumetinib is a selective MEK inhibitor that is efficient in $BRAF^{V600E}$ melanoma cell lines and animal models; at the same extent, trametinib inhibits cell growth by the inhibition of pERK1/2, inducing cell cycle arrest in cell lines with mutant BRAF and RAS. For these reasons, MEK inhibitors might be used for the treatment of melanomas that become resistant to BRAF inhibitors. However, in some cases, administration of trametinib resulted in low activity in patients previously treated with vemurafenib or dabrafenib, so resistance to BRAF inhibitors could be associated with resistance to MEK inhibitors [57]. Sorafenib is a potent multi-kinase inhibitor that targets also RTKs (receptor tyrosine-kinases), such as c-KIT. It is effective against melanomas that carry rare mutations of BRAF, but it doesn't show a significant benefit in melanoma patient harboring the $BRAF^{V600E}$ mutation, so that this drug might be administered together with MEK inhibitors in the treatment of the more aggressive forms of melanoma [57].

Activating mutations in c-KIT are frequent in melanomas. In contrast to BRAF mutations, which appear to be highly restricted to a single locus, c-KIT mutations have been reported across several exons with some more likely to be associated with the emergence of drug resistance, so that mutation analysis is recommended for enrollment of patients for clinical trials of c-KIT inhibitors. This receptor is emerging as an important target, since many melanomas harboring c-KIT mutations are often resistant to other therapeutic strategies. Actually, specific c-KIT inhibitors such as imatinib demonstrate antimelanoma activity [36, 54].

The PI3K/Akt pathway has also been shown to harbor activating mutations that cooperate with BRAF mutations to promote tumor pathogenesis. Moreover, the tumor suppressor PTEN is frequently lost in melanomas. The PI3K inhibitors GSK2126458 and BEZ235 are under evaluation, but monotherapy doesn't show advantage in clinical response, suggesting their use in combination with other drugs [57]. mTOR is a master regulator of protein synthesis, proliferation, autophagy and apoptosis, and it serves as a bioenergetic sensor of abundance of metabolites and energy depletion. It is located downstream of PI3K and Akt, so that inhibitors of mTOR might be used to inhibit this proliferative pathway. Monotherapy with rapamycin and its analog temsirolimus has

limited efficacy, but rapamycin synergistically increased sensitivity of melanoma cells to both chemotherapics and BRAF inhibitors [36].

Finally, bevacizumab, a VEGF inhibitor currently used for different types of cancer, has been tested in patients with metastatic melanoma. Melanoma, as many other tumors, produces high levels of VEGF that have been correlated with poor prognosis, immune suppression, neovascularization and tumor progression. Clinical trials suggest the potential of VEGF as a target but fail to confirm a significant improvement in the clinical management of melanoma [54].

Overcoming resistance

Despite the very encouraging results obtained with targeted therapies, most patients experience relapse. For example, in most patients treated with MAPK pathway inhibitors, metastases recur after 5-7 months after initial complete or partial tumor shrinkage. Mechanisms of resistance might be due to: 1- an intrinsic, genetic resistance that drives clonal evolution; 2- an acquired resistance that determines the reprogramming/reactivation of signaling networks.

Resistance to vemurafenib might occur because of mutations in the MAPK or in other proliferative pathways. Intrinsic or acquired genomic alterations of the MAPK pathway occur in up to 70% of all patients with BRAF inhibitor resistance. Melanoma cells can maintain high level of ERK phosphorylation despite the presence of the BRAF inhibitor, as a consequence of the expression of the constitutive active $NRAS^{Q61K}$ mutant, even if BRAF and RAS mutations are normally mutually exclusive in melanomas. Mutated NRAS promotes dimerization of $BRAF^{V600E}$ with other wild-type RAF isoforms such as ARAF or CRAF, which restores strong activation of the MAPK pathway. Somatic mutations in NRAS have been detected in 8-18% of BRAF inhibitor-resistant patients. Resistant cells either amplify the number of $BRAF^{V600E}$ copies, or express $BRAF^{V600E}$ splice variants, and promote dimerization irrespective of the RAS status. An additional way to escape from vemurafenib is located downstream BRAF: MEK mutations can be detected in 3-16% of BRAF inhibitor-resistant patients [50]. Such ERK activating resistance mechanisms seem to be deleterious to resistant tumor cells in the absence of BRAF inhibitor treatment, in which the excess of activation of the pathway induces a

paradoxically reduction in cell proliferation. This observation led to the design of an intermittent dosing schedule that was found to delay or prevent the onset of drug-resistant disease in patient-derived melanoma xenograft [58]. However, combination therapy represents a more traditional approach to prevent disease. In general, response to BRAF inhibitor treatment is highly variable: some patients derive no benefits, others have a complete response, and in this case the duration of response varies greatly. BRAF mutation genotype seems to be important, since patients with $BRAF^{V600E}$ mutation have superior response rates and longer progression-free survival than do those with $BRAF^{V600K}$ mutation [59]. The combination therapy with BRAF inhibitors and MEK inhibitors represents a strategy to double-target the same signaling pathway to overcome single-drug resistance, thus giving a superior efficacy in both V600E and V600K mutations: clinical studies evidence that the combination regimen of dabrafenib plus trametinib results in a response rate of 76%, compared with a 54% response rate of dabrafenib only; moreover, combination therapies seem to reduce toxicities associated with the paradoxical activation of the MAPK pathway due to BRAF inhibitor monotherapy [58]. Unfortunately, similar to that reported with BRAF inhibitor monotherapy, a high heterogeneity exists in response and progression of melanoma with combination of BRAF and MEK inhibitors, and tumor recurrence may occur. A possible approach is then to target other signaling pathways together with the MAPK pathway, to overcome drug resistance. The PI3K/Akt pathway plays an important role, since loss of functional PTEN, alterations in Akt3, mutations in PI3K, hyperactivation of mTOR are observed in melanomas, and might be implicated in the resistance to vemurafenib and dabrafenib [60]. Non-ERK dependent acquired resistance can also arise through upregulation of growth factor receptors such as the PDGFR-β or IGF1R, upstream to the PI3K pathway. Given the evidence indicating that the MAPK and PI3K pathways cooperate in melanomagenesis, the extensive crosstalk that exists between the pathways and the role of each pathway in resistance to inhibition of the other, a strong rationale exists for combined pathway inhibition in melanoma, and hope is raised that future triple targeted therapies could overcome double resistance.

Concurrent with the development of combination of MAPK inhibitors, advanced have been made in immunotherapy, because only 10% of patients treated with the CTLA-4 antibody ipilimumab have long-term improvement in overall survival compared with dacarbazine chemotherapy [59]. Additionally, ipilimumab seems to have little efficacy when used after unsuccessful treatment with a BRAF inhibitor. These findings make the choice for initial treatment difficult, because on the one hand BRAF inhibitors determine a high likehood of response, but variable durability with early recurrence, and on the other hand ipilimumab induces a small chance of response, but a high possibility of long term survival. Because of the ipilimumab's slow onset of action, some researchers suggested a first-line therapy with this drug, and patients with aggressive disease would be best treated with BRAF inhibitors [59]. Antibodies that block the PD1/PD-L1 axis belong to the second-generation checkpoint inhibitors and, unlike ipilimumab which has no real predictive prognostic biomarkers, tumor expression of PD-L1 could be a potential biomarker for response. The use of BRAF inhibitors with immunotherapy has been proposed, because clinical evidence suggests that longer survival with BRAF inhibitors might be associated with immune activity: BRAF inhibition leads to a rapid increase in melanoma antigen expression, and to an invasion of tumor-infiltrating lymphocytes soon after the initiation of treatment. In this case, immune escape might be prevented by blockage of the compensatory PD1/PD-L1 tumor defense [59]. The trial assessing the combination of vemurafenib and ipilimumab was stopped because of the overlapping toxicities; so another approach is sequential therapy. Biased retrospective data suggest that ipilimumab is of little benefit after BRAF inhibitor failure, but patients whose disease progresses on ipilimumab do benefit from BRAF inhibitors [59].

"Phenotypic drug resistance" is a definition of an adaptive program of the cells that is related to energy metabolism and cell cycle. Cancer cells rely on an increase in glucose uptake, the enhancement of glycolysis and lactate production along with the absence of oxidative respiration despite sufficient oxygen concentrations, a process that is called "Warburg effect". The higher induction of anaerobic metabolism is due to the requirement of rapidly proliferating tissue for production of bioenergy and generation

of biomass for cell duplication, so that this process is characteristic of proliferating cells. On the other hand, differentiated cells do not require a rapid metabolism, so that they exploit OXPHOS (oxidative phosphorylation) to survive. The slow-cycling, differentiated subpopulation was found in melanoma irrespective of the cells' genetic origin, and this phenotype could also be acquired by rapidly proliferating cells. This kind of "phenotype switching" might be a determinant of drug resistance, irrespective of the agent used: it has been recently demonstrated that vemurafenib induces features of stress-induced senescence in addiction to apoptosis, that is characteristic of quiescent cells [50].

Cancer Stem Cells (CSCs)

The "cancer stem cell concept" is an attractive model for explaining a number of clinical observations, including tumor dormancy and treatment resistance. An early definition implies that carcinomas are a mixture of malignant stem cells and their differentiated daughter cells: in fact, the classical, idealized notion holds that cancer stem cells (CSCs) are characterized by their limited number and their ability for self-renewal through asymmetric cell division. The tumor mass would thus be consecutively formed and continuously repopulated by the progeny of rapidly proliferating cells and post mitotically differentiated daughter cells. In 2009, a consensus conference of the American Association for Cancer Research (AACR) postulated that a CSC is a tumor cell that have the capacity for self-renewal, the ability to generate all heterogeneous tumor cell lineages and to recapitulate continuous tumor growth [61].

Two models have been used to explain how tumors grow and progress. In the stochastic model, all tumor cells are equivalent and have the potential to behave like a CSC, and retain plasticity to go from a non-stem cell to a stem cell-like precursor: in this case, intratumor heterogeneity appears to depend on subclonal differences that result from differential genetic and epigenetic alterations during development of cancer. In the hierarchical model, the cancer stem cells represent a rare, slow cycling population of cells that are biologically distinct, can renew themselves, and give rise to

various progeny cells that lack the ability to self-renew: CSCs reside at the top hierarchy, while the other cell types comprise the bulk of the tumor mass.

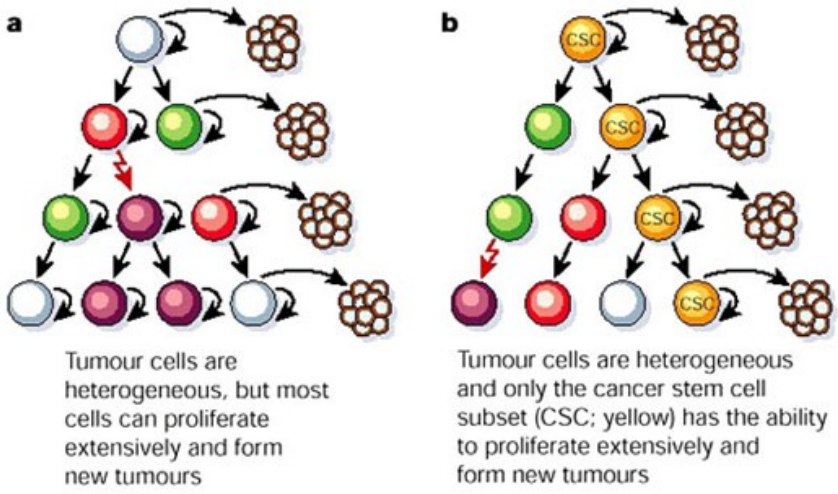

Fig. 4. Symmetrical (stochastic, **a**) or asymmetrical (hierarchical, **b**) models for cancer stem cells growth and progression.

CSCs are more resistant to standard cancer therapies (both chemo- and radiotherapies) than non-CSC populations, because of their quiescence, or dormancy, and their ability to metastasize. Identifying CSCs and characterizing their cellular origin, phenotype and the molecular mechanisms that confer their tumor-initiating capacity is crucial to develop most effective cancer therapies, aimed to target the cancer cell subpopulation with the ability for self-renewal, metastasis and drug and radiation resistance.

CSCs are commonly defined based on the following distinct properties *in vitro*:

- CSC-enriched subpopulations can be isolated primarily based on differential expression pattern of surface markers, compared to the non-stem counterpart [62];
- They have the ability to generate spherical colonies in suspension cultures [63];
- They exhibit increased survival upon treatment with conventional chemotherapies or radiations [64].

Furthermore, CSC-enriched subpopulations have greater tumorigenicity, that means a greater potential for tumor initiation/formation when xenotransplanted in immunodeficient mice.

The first evidence that the spheroid cell culture could enrich for CSCs in melanoma came in 2005, when cells propagating as melanospheres exhibited the capacity to undergo multi-lineage differentiation and they exhibited a ten-fold increase in tumorigenicity compared to adherent cells [65].

Different surface markers have been identified to characterize melanoma cancer stem cells [66].

CD271, also known as low-affinity nerve growth factor receptor (NGFR) or p75NTR, has been identified as a marker of neural crest cells, so it represents a de-differentiation marker for the melanocyte lineage and melanoma cells.

CD133, also known as Prominin-1, is a cell surface protein of unknown function first found on immature hematopoietic and neural stem cells, and has been linked to cancer stem cells in different tumor types.

ABCB5 is a member of the ATP-binding cassette (ABC) transporter family that has known roles in multidrug resistance to cancer therapeutics, so it may confers to melanoma stem cells drug resistance and enhanced survival, compared to the non-CSCs population.

CD20 is a cell surface marker normally found on B cells, and has been associated with melanoma cells having stem cell characteristic.

Other CSCs markers are linked to totipotent or pluripotent cell lineages, and this reflects the similar features of CSCs with embryonic stem cells, in term of self-renewal and plasticity: these markers comprise Oct4, Nanog and Sox2 [67].

Given their ability to initiate tumor, CSCs have been hypothesized to be responsible for metastasis formation: it is estimated that approximately one million of cells are shed per gram of a tumor every day; however, only a few of these cells have the ability to metastasize [68]. Circulating tumor cells (CTCs) bear the ability to initiate primary tumor and metastases formation, and are enriched of CSCs markers [69]. Moreover, they also possess the capacity to interact with and condition the local

microenvironment, to promote neovascularization or to evade the host antitumor immune response, and have plasticity in cell motility.

One major factor that drives metastases is epithelial-to-mesenchymal transition (EMT), a process seen in normal embryogenesis and adopted by epithelial cancers to acquire a more aggressive phenotype. In this process, polarized epithelial cells lose their associations to the surrounding environment and attain migratory properties of mesenchymal cells. The reversal of EMT, mesenchymal-to-epithelial transition (MET), is the acquisition of an epithelial-like phenotype, a mechanism by which neoplastic cells colonize target tissues. There is evidence that EMT may also be an important process underlying melanoma progression and metastases [70]. Another phenotype transition involved in metastasis is the mesenchymal-to-amoeboid transition: amoeboid motility is characterized by squeezing movements that allow cancer cells to glide through matrix barriers, without the use of matrix metalloproteinases (MMPs) or integrin engagement [71].

Because of the therapeutic and prognostic implications of detecting early metastases, characterization of the subset of cells with metastatic capacity has become a focus of active research. Along with eliminating the tumor mass through conventional or targeted methods, new combination therapies should also address cancer stem cell populations that may show various mechanisms of resistance. Irrespective of which pathway is exploited to eliminate CSCs, the objective should be to eliminate the entire tumor mass, to prevent drug resistance and then tumor recurrence.

Estrogen receptor β

Structural and functional features of estrogen receptors

Estrogens are steroid hormones that are principally produced by gonads (ovaries and testes), but also by extra gonadal tissues (adipose tissue, brain, vascular endothelial cells, bone cells, breast tissue and fibroblasts). Estrone (E_1), estradiol (E_2) and estriol (E_3) are the three main endogenous estrogens, with E_2 the predominant and most biologically active form and they are synthesized from testosterone and androstenedione through the enzyme aromatase. The physiological role of these hormones includes sexual maturation and fertility, regulation of lipid and carbohydrate metabolism, skeletal development and integrity, and homeostasis of the cardiovascular and central nervous system [72].

Estrogenic effects are mediated through two estrogen receptor (ER) subtypes, ERα and ERβ. The first estrogen receptor (ERα) was cloned in 1986, and until 1995 it was assumed that it was the only receptor responsible for estrogen's action. However, in 1995, ERβ was cloned from a rat prostate cDNA library, and then the human counterpart was discovered [73, 74].

ERα and ERβ are encoded by two different genes, *ESR1* and *ESR2*, respectively, that are located on different chromosomes. They belong to the steroid/thyroid hormone superfamily of nuclear receptors, members of which share the same architecture, characterized by three independent but interacting functional domains:

- The N-terminal (NTD) or A/B domain. This region contains a transactivation domain with ligand-independent function (AF1) and a co-regulatory domain that is responsible for the recruitment of co-activators and co-repressors.
- The DNA-binding (DBD) or C domain. This region contains zinc fingers required for the binding to specific estrogen response elements (EREs) in the proximal promoter region or at a distal regulatory elements of estrogen-responsive genes.
- The ligand-binding (LBD) or D/E/F domain. This is the carboxy-terminal region, that has a ligand-dependent transactivation function, and is also responsible for

the binding to co-regulatory and chaperone proteins, as well as for receptor dimerization and nuclear translocation (AF2 domain).

The two ERs share about 97% similarity in their DBD, 59% in LBD and only 16% in their NTD (Fig. 5).

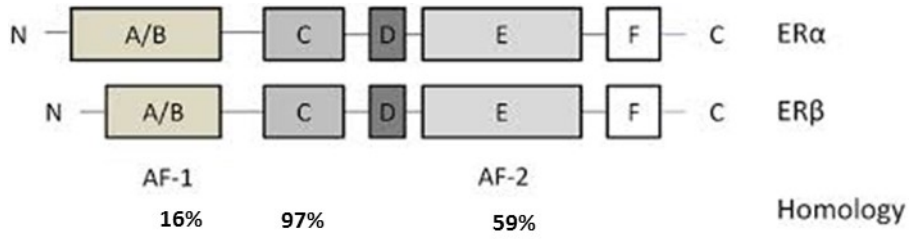

Fig. 5. Schematic representation of the estrogen receptors structure. The three main domain (N-terminal, DNA-binding and ligand-binding), are shown. The DBDs of ERα and ERβ are highly conserved, and the NTDs are the least conserved.

Differences in the LBD influence the shape of the ligand-binding pocket, so that specific ligands for each receptor have been synthesized. Furthermore, the same ligand may have different effects on ERα or ERβ. For example, tamoxifen, raloxifen and ICI-164,384 are synthetic antiestrogens that are partial E_2 agonists/antagonists with ERα, but are pure antagonists with ERβ. Positioning of helix 12 is the key event that permits discrimination between agonists and antagonists: when the ERα LBD is complexed with pure agonists (E_2 and diethylstilbestrol), helix 12 is positioned over the ligand-binding pocket and forms the surface for the recruitment and interaction of coactivators. In contrast, in the ERα- and ERβ LBD complexes with raloxifen or tamoxifen, helix 12 is displaced from its agonist position and occupies the hydrophobic groove formed by helix 3, 4, 5: in this position, helix 12 foils the coactivator interaction surface [75]. Dissimilarity in the AF1 N-terminal domain between ERα and ERβ may also account for the different responses to various ligands, indeed, this domain is very active on ERα on a variety of estrogen receptor promoters, but under identical conditions, the activity of AF1 in ERβ is minimal [76].

ERs primarily reside in an inactive state in the cytoplasm in complex with heat-shock protein (Hsp) 50, 70 and 90, which stabilize the receptor. The so called “classical” mechanism of ERs action implicates that, after ligand binding, the receptors go through Hsp dissociation, conformational changes, dimerization and binding to DNA at a

sequence-specific response elements known as estrogen response elements (EREs), located in the regulatory region of estrogen responsive genes. Once bound to EREs, the receptors interact with coregulatory proteins (chromatin modulators, coactivators, corepressors or basal transcription factors), forming multiprotein complexes, on the basis of the shape of the ligand-receptor complex. This machinery influences the activity of the receptors, which activate or repress gene transcription. The nature and concentration of ligand determines whether homodimers (ERα-ERα or ERβ-ERβ) or heterodimers (ERα-ERβ) are formed, or a combination of the two. Ligands that selectively bind ERβ promote the formation of ERβ homodimers only; by contrast, selective ERα ligands stimulate the formation of both ERα homodimers and ERα-ERβ heterodimers, leading to distinct patterns of gene regulation. In particular, most studies support that ERβ acts as a dominant negative regulator of estrogen signaling and demonstrate repressive effects on ERα-mediated transcriptional activity. With regard to chromatin binding sites, if ERα or ERβ are present alone, there is a substantial overlap in their chromatin binding sites, but when they are present together, ERα becomes dominant in competing for them. Activating ERα with selective agonists, such as PPT, the pattern of binding sites is similar to that of E_2. By contrast, in the presence of ERβ selective agonists, such as ERB-041, the pattern of binding sites is different compared to E_2: this suggests that different ligands induce a differential conformation of this receptor, that influences ERβ binding site occupancy [72].

Beside dimerization, the conformational changes induced by agonists allows the AF2 domain to form a stable interaction with coactivator proteins (such as SRCs) through the nuclear receptor (NR)-box leucine-rich motif (LxxLL, in which “x” represents any aminoacid). It has been demonstrated that there is both an ER subtype-selective and a ligand-specific recruitment of NR-boxes to the receptor, confirming that different agonists induce a distinct receptor conformation when bound to ERα or ERβ. Instead, estrogen antagonists induce a receptor conformation that leads to association with corepressors (i.e., NCOR1), shutting off target gene transcription [77]. Additional cofactors with histone modifying and chromatin remodeling activities can bind ERs. Histone acetyltransferase (HAT), CREB-binding protein (CBP), histone

methyltransferases CARM1 and PRMT1 are tethered to ERs through interactions with the SRC family of coactivators; while corepressors can recruit SIN3 and histone deacetylases (HDACs) to form a large corepressor complex that contains histone deacetylase activity, implicating histone deacetylation in transcriptional repression [76]. Importantly, selective cofactor recruitment by ERα and ERβ may serve as a mechanism for differential transcriptional activities between the two receptors.

On ligand binding, ERs can also regulate gene transcription by interacting with and activating other direct DNA-binding transcription factors, so they can be tethered for example with AP1, Sp1 or NFκB, and influence the transcription of genes normally regulated by these factors. During this interaction, ERs can bind to full or imperfect ERE motifs, such as ERE half-sites, or can influence gene expression without binding themselves to DNA [78]. ERα and ERβ can have opposite effects at these sites in activating or repressing gene transcription; moreover, while ERα is mostly found at EREs, ERβ is mostly found at AP1 sites. The action of ERβ at these sites is dependent on the presence of coactivators, such as Tip60 [79].

Taken together, these structural and functional features might explain the different biological effects of the two ERs.

ERs can also be activated by a ligand independent way via crosstalk with other signaling pathways. Both receptors can be activated via phosphorylation by MAPK and PI3K; furthermore, hyperactive growth factor receptors, such as EGFR or IGF1R can stimulate protein kinase cascade that phosphorylates and activates ERs in their genomic action. In addition, membrane and cytoplasmic ERs can mediate the rapid, “non-genomic” effects of estrogens. MAPK, PI3K, HER2, EGFR, IGF1R, SRC and G proteins are among the components that are activated by ERs within 3-15 minutes after estrogen treatment and that can signal to regulate gene expression through the activation of other transcription factors [78]. The mechanism by which the ERs become associated with the plasma membrane is not fully understood, but palmitoylation of the receptors is one mechanism that has been shown to allow their association with caveolae rafts [72]. Recently, an orphan G protein-coupled receptor (GPR30), located

at the plasma membrane, has been reported to mediate non-genomic estrogen signaling [77].

Each ER subtype exists as several isoforms that are derived from alternative splicing and differential promoter usage. ERα and ERβ splice variants differ from wild-type receptors in structure, so they might have different functions or lack in functionality. Many of these isoforms have been detected by PCR, and the lack of specific antibodies makes the corresponding protein detection difficult.

ERα is produced from 8 exons and has a molecular weight of 66.6 KDa. Splice variants of ERα include deletions at different exons. The ERαΔ3 variant is a receptor lacking exon 3, which encodes the second zinc finger on DBD, preventing the protein from binding to ERE: as a result, the protein has a dominant negative activity, suppressing estrogen-induced transcriptional activity. Another example is an exon 5-deleted variant (ERαΔ5), that lacks the hormone-binding domain, but possesses the AF1 and DBD functions, leading to a constitutively active receptor [80]. The truncated ERα isoform ERα-36 is localized in the plasma membrane and cytoplasm and mediates the rapid, membranous effects of estrogens. Its expression in the presence of the wild-type isoform has been reported to confer endocrine resistance in breast cancer, and to promote tamoxifen agonist action in endometrial cancer. Furthermore, following expression in cancer cell lines, ERα isoforms modified the transcriptional activity of the wild-type receptor and influenced cell growth through both genomic and non-genomic signaling [78].

Full-length human ERβ protein, also named “ERβ1”, is produced from 8 exons and includes 530 aminoacids with an estimated molecular weight of 59.2 KDa. Four alternative splice variants of ERβ (ERβ2, ERβ3, ERβ4 and ERβ5) exist as a result of alternative splicing of the last exon (Fig. 6): these variants are expressed in a cell-type and tissue-specific manner, with the ERβ3 expressed only in the testis, so that ERβ2, 4 and 5 are the well-studied isoforms. An *in silico* model by Leung et al shows that, in comparison with ERβ1, each of the other isoforms has a shortened helix 11 with variable lengths, and may not have a complete helix 12. Only ERβ2, also called “ERβcx”, is the result of an alternative splicing where exon 8 is exchanged for an alternative

exon. Compared with ERβ1, ERβ2 has a complete helix 12, but it assumes a totally different orientation, likely due to the restriction imposed by a shorter loop between helices 11 and 12. This unusual orientation may hinder ligand access to the binding pocket; moreover, a reduction in the coregulator binding surface is observed. ERβ4 and ERβ5 transcripts are generated with an early stop codon, so they contain only part of exon 7 and different exon 8 sequences, that leads to a significantly truncated helix 11 and the complete absence of helix 12, so that they cannot bind to ligands or recruiting coregulators. As a consequence of truncations:

- The molecular weight of ERβ2, 4 and 5 are 56, 54 and 53 KDa, respectively.
- ERβ splice variants have lesser affinity for E_2, compared to ERβ1, except for ERβ2 that is not able to bind ligands.
- None of the alternative isoforms have intrinsic transactivation activity.

Despite the fact that ERβ2, 4 and 5 don't form homodimers, they are able to interact with ERβ1 and ERα in the presence of E_2: given their lack of functional helix 12, this implies that E_2 binding to ERβ1 or ERα is sufficient to induce heterodimer formation [81]. Such interactions contribute in modulating ERβ1/ERα activity, in a specific cell context.

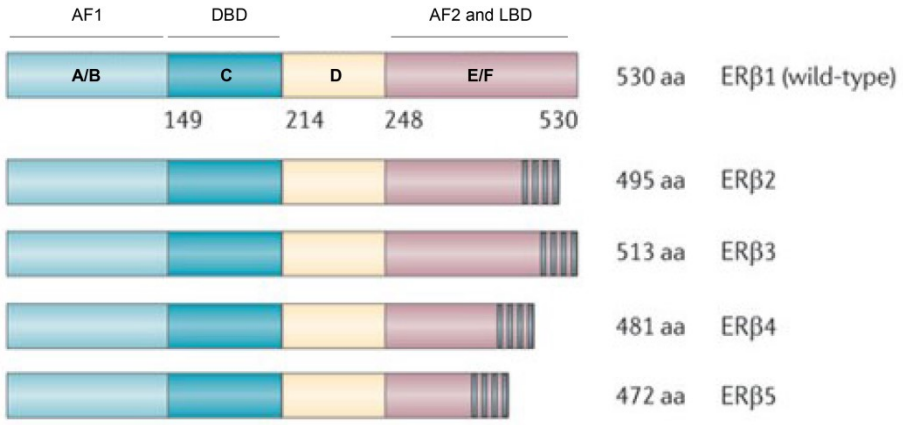

Fig. 6. Structure of the main ERβ protein (ERβ1), in relation with its four isoforms. Dotted bars represent the divergent C-terminal regions among the isoforms.

Estrogen receptor β and cancer biology

Many lines of evidence suggest a relationship between the perturbation of estrogen signaling and cancer initiation, progression and response to treatment, and especially the variation of ERα/ERβ ratio in tumor tissues indicate that the ER subtypes have different functions in cancer biology and therapy. This notion has been further supported by *in vitro* and *in vivo* studies in which the use of specific ligands and methods to disrupt ER subtype-specific expression has shown that ERα and ERβ have opposite effects on cell proliferation and apoptosis.

The role of ERα in cancer is well documented and this receptor has been associated with the development of different, primarily hormone-related, types of tumor such as breast, prostate, ovarian and endometrial cancer. Overall, ERα seems to contribute to tumorigenesis stimulating cell proliferation [78]. On the other hand, ERβ seems to exert antiproliferative and apoptotic actions in tumors both related and unrelated to hormones.

Breast cancer

In the normal adult mammary gland, ERα is expressed in <10% of epithelial cells; instead, ERβ is expressed in 70-80% of them and in the stroma and immune cells resident in the breast. ERα$^{-/-}$ mice revealed that the α receptor subtype is essential for branching morphogenesis, while ERβ$^{-/-}$ mice indicated that this receptor is not required for ductal growth of the breast, but for terminal differentiation and for maintaining the organization and differentiation of the epithelial tissue [82]. Prolonged exposure to estrogens is a well-established risk factor for breast cancer in postmenopausal women: the combination of gene mutations (*BRCA1, TP53, ERBB2*), together with the expression of ERα determines the final course and progression of the tumor.

The involvement of ERβ and its isoforms in breast cancer is still being investigated, and controversial issues have been addressed. Published data clearly demonstrate that ERβ1 has an antiproliferative function when introduced into ERα-positive breast cancer cells [83]. Interestingly, while E_2 has no effect on cell proliferation, the ERα-selective agonist PPT stimulates proliferation, while the ERβ-selective agonist diarylpropionitrile (DPN) inhibits cell growth and induces apoptosis [84]. Furthermore, it has recently

been shown that ERβ1 inhibits epithelial-to-mesenchymal transition (EMT) and invasion in triple-negative breast cancer cells [85]. Clinically, a general finding is that total ERβ levels declines during breast tumorigenesis, and higher levels of ERβ expression have found to be associated with the expression of good prognostic markers or better clinical outcome: it is inversely correlated to HER2 expression and associated with increased disease-free survival and overall survival, even if it seems to depend on the molecular characteristics of the tumor.

Less clear is the role of ERβ2 in breast cancer: some *in vitro* studies show that it exerts an antiproliferative effect in ERα-expressing MCF7 cells, probably inducing ERα degradation, but some other studies show no effects on cell proliferation. Moreover, the relation between ERβ2 expression and clinicopathological parameters is confused, because in some studies its expression is correlated with the ERα expression and low histological grade; in other studies it was associated with lymphovascular invasion and in some other studies ERβ2 isn't associated with any clinicopathological parameter [86]. Among other isoforms, ERβ5 expression has been positively correlated with longer relapse-free survival and its nuclear staining with overall survival, suggesting that ERβ5 expression may be a prognostic marker for breast cancer. In addition, recent studies show that it sensitizes breast cancer cells to the induction of the apoptosis process [87].

Prostate cancer

Both androgens and estrogens play an important role in normal prostate growth and development and for the maintenance of adult prostate homeostasis. ERα is required for the development and branching morphogenesis of the prostate, and in the adult mouse prostate there is very little ERα expression and most is in the stromal compartment. On the other hand, ERβ is abundantly expressed in the epithelium of the adult mouse and human prostate, and it is also expressed in the stroma and the infiltrating immune cells. The antiproliferative action of ERβ in the prostate epithelium has been demonstrated generating male βERKO (ERβ knock-out) mice, that show increased epithelial proliferation, decreased apoptosis and accumulation of incompletely differentiated cells that lead to prostatic hyperplasia. Declining levels of

this receptor subtype have been observed with progression from benign prostatic hyperplasia to malignant disease, with a further decrease associated with increasing Gleason grade of prostate cancer. *In vitro* studies show that the activation or overexpression of this receptor can inhibit proliferation of both androgen-dependent (LNCaP and 22RV1) and androgen-independent (PC3) prostate cancer cell lines, because of the decrease in the expression of oncogenic factors, such as cyclin E, PI3K and c-Myc) and the increased expression of tumor suppressor genes, such as $p21^{WAF1}$, $p27^{Kip1}$ and PTEN. Moreover, it has been demonstrated that ERβ promotes apoptosis through the upregulation of PUMA (p53-upregulated modulator of apoptosis), via transcriptional activation of FOXO3a [88]. In addition to the antiproliferative actions, ERβ also inhibits migration and EMT, as it does in breast cancer.

A contradicting result obtained in clinical studies is that ERβ expression is then high in lymph node or bone metastases [89]. This fact may depend on the isoform expressed: there's increasing evidence that expression of ERβ2 is increased in high grade and metastatic prostate cancer [90]. With ERβ2, ERβ5 has been shown to correlate with poor prognosis in prostate cancer: specifically, coexpression of nuclear ERβ2 and cytoplasmic ERβ5 are independent prognostic marker for biochemical relapse, postoperative metastasis and time to metastasis following radical prostatectomy for localized prostate cancer [91].

Gynecological cancers

Gynecological cancers comprise ovarian and endometrial cancer, both related to the action of female sex hormones.

The two estrogen receptors are differentially expressed in ovaries, also depending on the reproductive age. In premenopausal women, ERα is present in the ovarian stroma and thecal cells, ovarian surface epithelium and corpus luteum; in postmenopausal women this receptor subtype is found in the ovarian surface epithelium, in epithelial inclusion cysts and stroma, but its expression decrease with ageing. In contrast, ERβ is localized predominantly in the granulosa cells [92]. In the majority of reports, ERβ expression declines in ovarian epithelial tumors compared to normal ovarian tissue, and the expression of this receptor is inversely correlated with stage of disease and

positively correlated with disease-free and overall survival. Moreover, during the transition from normal epithelium to ovarian malignancy, a shift from nuclear-to-cytoplasmic localization of ERβ is observed, so it has been hypothesized its loss in the ability to antagonize the proliferative activity of ERα. The antiproliferative role of the β receptor subtype in ovarian cancer is also confirmed by *in vitro* studies: its expression slows the growth and motility of SK-OV-3 ovarian cancer cells, through downregulation of cyclin A2 and cyclin D expression and upregulation of $p21^{WAF1}$, and activates apoptosis. This effect is seen in both ERα-positive and negative cells. ERβ2 and 4 expression is decreased, and, on the other hand, ERβ5 expression is significantly elevated in ovarian cancers compared to normal ovary, suggesting a possible distinct function of the splice variants in ovarian carcinogenesis [93].

Both ERs are expressed in normal endometrium, but levels of ERβ are lower than those of ERα. There are only few studies that support the tumor-suppressor function of ERβ in the endometrium; in contrast, the majority of reports suggest that this receptor has a tumor-promoting role in endometrial cancer [93]. In many cases, during malignant transformation of the endometrium, a decrease of the ERα/ERβ ratio has been observed, and patients that exhibit this low ratio have a shorter disease-free survival. In uterine carcinosarcoma, a rare and highly aggressive form of endometrial cancer, ERβ expression is elevated and continues to increase with tumor progression. With regard to ERβ isoforms, ERβ2 is unchanged, while ERβ5 is significantly overexpressed in endometrial cancer, and its expression correlates with tumor stage [92].

In summary, ERβ seems to play different roles in gynecological cancers, with a tumor-promoting action in endometrial cancer and a tumor-suppressive function in ovarian cancer.

Hormone-independent tumors

Colon cancer

Clinical and animal studies show that hormone replacement therapy reduces the risk of colon cancer; furthermore, men are more likely to develop this disease compared to women. These findings suggest that estrogens may lower the risk for colorectal cancer

development [94]. ERβ is the predominant ER in the human colonic epithelium, with the higher expression level in the ascending colon. The expression of this receptor is reduced in colorectal cancer compared with normal tissue, and this seems to be related to loss of differentiation and advanced stage of disease. Effects of estrogens on colonocytes may be related to the direct effects of the estrogenic ligand and its interaction with the receptors, or may be related to changes in ERs ratio and function independent of the ligand. For example, the rise in incidence of colon cancer in postmenopausal women, when circulating amount of estrogens is decreased, could be related to the loss of cell cycle regulatory effects of ERβ; alternatively, the decrease in estrogen levels results in a change in the ratio ERα/ERβ. Supporting this notion, timing to start of hormone replacement therapy may play a crucial role: early start prevents changes in the ERs ratio, and results in lower rate of colon cancer through maintaining ERβ receptors [95]. However, the protective role of ERβ in colon cancer progression has further been confirmed in *in vivo* studies with mice spontaneously developing intestinal adenomas ($Apc^{Min/+}$): in this mouse model, deletion of the receptor leads to an increase in size and number of adenomas, while the treatment with the ERβ-selective agonist DPN results in a significant reduction in polyp multiplicity in both male and female experimental groups [96]. In particular, ERβ regulates the expression of proteins related to apoptosis, cell cycle control, cell differentiation and proliferation, and stimulates antiinflammatory networks in colon cancer cell lines [97]. In contrast, disruption of ERα signaling has no effects, and as the cancer progresses, the expression of ERα increases, suggesting its possible role in colorectal cancer progression.

The expression of the ERβ isoforms 2 and 5 follows the trend of ERβ1: they are highly expressed in normal colon tissue with less expression in early disease stage and the lowest expression in colon adenocarcinomas [98].

Lung cancer

Estrogens are thought to play a role in lung carcinogenesis. However, the exact role of ERβ is still controversial in some cases.

ERβ is the major mediator of estrogen in the lung, indeed ERβ knockout mice display lung abnormalities such as a decreased number of alveoli and reduction in the

expression of key regulators of surfactant homeostasis. Its expression is found both in normal lung tissue and in cancer tissue. Non-small cell lung cancer (NSCLC) is one of the most aggressive tumors, and has been principally related to tobacco smoking, despite there is a significant proportion of never smokers that develop lung cancer. The nonsmoking related lung cancer is more common in women, and premenopausal women develop less differentiated lung cancers compared with postmenopausal women. Independently from gender, local production of estrogens has been observed, and both ERs have been detected in NSCLC cells, but their role remains poorly understood because of contradictory data. Some clinical studies demonstrate a positive correlation between ERβ positivity and better prognosis and reduced mortality in patients, and an inverse correlation with lymph node metastasis and tumor size [99]. Moreover, *in vitro* studies show that ERβ overexpression or specific activation inhibit growth and induce apoptosis in NSCLC cells, by significant induction of the cell cycle inhibitors p21 and p27, and through the reduction in the activity of ERK by inactivating mutant Ras [100]. On the other hand, some other reports indicate a negative effect of ERβ on patient survival, independently from gender. In particular, in these studies, cytoplasmic ERβ1 staining has been evaluated as a negative prognostic factor, while nuclear positivity has been found to be a favorable prognostic indicator in early stage, but not in metastatic lung cancers. In contrast, ERβ2 and 5 isoforms have been linked to a better outcome [101].

Malignant pleural mesothelioma is an asbestos-related malignant tumor, for which female gender has been identified as a positive prognostic factor. ERβ staining has been detected in normal pleural specimens and in tumor samples, while, differently from other lung cancers, none of these tissues stain positive for ERα. Clinical reports are supported by *in vitro* studies, where ERβ activation lead to the inhibition of malignant mesothelioma cell proliferation through a G_2-M cell cycle arrest [102]. Furthermore, ERβ activation sensitizes cancer cells to gefitinib, an EGFR inhibitor for the treatment of malignant mesothelioma, via EGFR inactivation and internalization [103].

Lymphoid malignancies

Estrogens significantly affect both the innate and the adaptive immune system, exerting a suppressive function on both B and T lymphopoiesis and on T cell-dependent immune reactions, while enhancing antibody production from B cells. Furthermore, it has been shown that estrogens inhibit the production of proinflammatory cytokines, such as IL-2, TNFα and IFNγ, but stimulate the production of antiinflammatory cytokines, such as IL-10, IL-4 and TGFβ. Because of the role played by estrogens in the immune system, ERs are expressed in these cells, with ERβ appearing to be the dominant subtype in mature leukocytes from peripheral blood, tonsils or spleen in healthy individuals. In addition to the cells of lymphoid origin, ERs are expressed in myeloid cells such as monocytes, macrophages and dendritic cells, and modulate estrogen's action on their maturation, differentiation and migration [104]. ERs have been found to be expressed also in several human leukemia and lymphoma cell lines (chronic lymphocytic leukemia, Hodgkin lymphoma, Burkitt lymphoma, multiple myeloma), that abundantly express ERβ while ERα expression is very low or non-detectable [105].

Epidemiological data show an association between reproductive hormonal factors with a reduced risk in developing lymphoid malignancies, with lower incidence in females compared to males. Supporting this notion, when grafting mice with murine T cell lymphoma cells, male mice develop larger tumors compared to female mice, a difference that is abolished following ovariectomy [106]. Since ERβ is the predominant ER subtype to be expressed, the protective role of estrogens might be related to this receptor. In-fact, ERβ-knockout mice has been shown to develop myeloproliferative disease with lymphoid proliferation, and prostatic hyperplasia. Furthermore, ERβ selective agonists such as DPN or KB9520 exert antiproliferative and proapoptotic effect on murine and human lymphoma both *in vitro* and *in vivo*, while the selective ERα agonist PPT has no effect [106]. Given the additional antiinflammatory activity of ERβ, selective agonists might be useful not only for the direct antiproliferative and proapoptotic effect on tumor cells, but also for tumor surveillance, as lymphocyte infiltration occurs frequently in cancers.

The only ERβ isoform that has been found in cells from CLL patients is the ERβ2, and patients with cytoplasmic only or nuclear and cytoplasmic ERβ2 have worse overall survival than those with ERβ2 solely nuclear [107].

Brain neoplasms

Several lines of evidences suggest that the incidence of brain tumors is significantly higher in males than in reproductive-aged females, thus leading the hypothesis of a protective role of estrogens in the development of brain neoplasms.

Gliomas originate from glial cells, that means astrocytes or oligodendrocytes. Several studies have documented ER expression in gliomas, glioblastomas and astrocytomas and, in contrast to ERα, ERβ is expressed in glial neoplasms as well as in non-neoplastic astrocytes. Moreover, recent studies report that this receptor subtype is highly expressed in non-neoplastic astrocytes and low grade neoplasms, where its localization is preferentially confined to the nucleus. In contrast, most of the high-grade tumors show a reduced and mostly cytoplasmic ERβ expression, and this downregulation correlated with the histological malignancy, so that the expression decreases with tumor progression [108]. These observations are supported by *in vitro* and *in vivo* studies. When human glioma cell lines are transplanted into nude mice, tumor growth is markedly greater in males compared with females, females survive longer than males, and estrogen treated animals (both males and females) survive longer than ovariectomized mice [109]. Furthermore, ERβ selective agonists have been reported to reduce proliferation of glioma cells, by cell cycle blockade in the G2/M phase [110].

Medulloblastoma (MB) is a highly malignant primitive neuroectodermal tumor of the cerebellum. As for other CNS neoplasms, male gender is a risk factor for this type of tumor; conversely, female gender is a favorable prognostic factor, with girls having a better outcome. Both ERs are expressed in the cerebellum and their expression varies with age, during cerebella development. With regard to medulloblastoma, studies on MB cell lines show that, while ERα is undetectable, ERβ is expressed in each cell line, and translating these *in vitro* studies in animal models, the involvement of ERβ, rather than ERα, in modulation of MB development is observed. Xenotransplantation of MB

cell lines in female or male mice show that tumors in females have a lower proliferative potential than males, the expression of ERβ is higher in female than male tumors and it has been shown a significant negative correlation between ERβ1 expression and the levels of the proliferation marker Ki67. Moreover, female tumors have a more differentiated phenotype, as shown by a decrease in the stem cell population [111]. This finding is in line with the fact that well differentiated human medulloblastomas tend to have higher levels of nuclear ERβ compared to poorly differentiated medulloblastomas [112].

All these observations suggest ERβ has a tumor suppressor role also in brain tumors.

Non-melanoma skin cancers

Non-melanoma skin cancers include basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The principal etiologic factors are UV and ionizing radiations, together with chemical carcinogens and human papilloma virus infections. The skin is an estrogen-responsive tissue, and ERβ is the predominant estrogen receptor in the adult human scalp skin, where it is strongly expressed in the stratum basale and stratum spinosum of the epidermis [113]. Several studies have clearly indicated a link between estrogens and skin cancer, and particularly the protective role of these hormones in developing BCC and SCC has been reported. In mouse models that are susceptible to develop skin cancer under carcinogenic agents (such as radiations or chemical agents), ovariectomy determines a dramatic increase in tumor induction compared to controls and male mice. Moreover, malignant progression of benign papillomas to SCC occurs almost exclusively in ovariectomized mice, and in this experimental group a change in the ERα/ERβ ratio is observed [114]. *In vitro* studies have evidenced that the expression of ERα is significantly increasing through transition from the initiation stage (immortalized non-cancerous cells), to promotion stage (papillomas), to aggressive stages (squamous and spindle cells). On the other hand, the protein level of ERβ only slightly changes, with the lowest levels in the advanced stages of progression [115]. The ERs ratio seems to be determinant for the expression of genes involved in cell cycle regulation, such as cyclin D: a ratio>1 determines the

induction of cyclin D, with cell proliferation, while a ratio<1 determines the repression of the expression of cyclin D, leading to cell cycle blockade.

Other studies report the role of ERβ in protecting against UV radiation-induced skin photocarcinogenesis, probably via immunological pathways, and specifically by inhibiting photoimmune suppression, a known risk factor for skin cancer development [116].

Estrogens and melanoma

The role played by estrogens in melanoma susceptibility and malignancy has remained controversial due to contradictory findings of laboratory and clinical studies.

Epidemiological data show a significant divergence of melanoma incidence between sexes during the past three decades: the Surveillance Epidemiology and End Result (SEER) data indicate that the annual rate of increase in cutaneous malignant melanoma is 0.8 in males and 0.46 in females per 100000 individuals, that means a nearly twofold greater rise in male compared with female incidence over this time period. Gender differences are also observed regarding the age-dependence of melanoma onset, with slightly higher rates in women aged 20-45 and progressively higher rates in males after 45-50 years of age, and dramatically higher rates in males aged 50-85. On the contrary, after the age of 45 years, the incidence rates levelled off in women [117]. However, women have a significant survival advantage over men [118].

A number of studies have focused on possible relationships between the characteristics of female endocrine status, as well as assumption of exogenous estrogens, and the risk of cutaneous melanoma, but these links remain controversial. In some cases, estrogen use with oral contraceptive or hormone replacement therapy is associated with an increased incidence of cutaneous melanoma; however, some other studies are not in accordance with this finding. A meta-analysis by Gandini *et al* summarizes the role played by endogenous *versus* exogenous estrogens, and takes into account reproductive factors, such as age at menarche, fertility, parity, menopausal status or pregnancy as well as use of oral contraceptive or hormonal replacement therapy. Some clinical studies documented that pigmentary and nevi

change during pregnancy, and that melanoma patients during pregnancy present worsened outcomes, but these findings seem to depend from age at first birth and parity, with a reduced risk of melanoma among women with both earlier age at first birth and higher parity. On the other hand, an association between use of oral contraceptive or hormonal replacement therapy has not been found, so that exogenous female hormones do not seem to contribute significantly to increased risk of cutaneous melanoma [119]. A recent study supports this finding, with no association between developing melanoma and exogenous hormones administration for ovarian stimulation for assisted reproduction technologies [120]. Another population-based study suggests that antiestrogen therapy for breast cancer can modify the risk of subsequent melanoma, with a 60% increased risk of melanoma among breast cancer patients who did not receive antiestrogens, whereas no excess of risk among women treated with these drugs [121]. When interpreting all these observations, one must consider the limitations of the studies. Information on oral contraceptives, hormonal replacement therapy and adherence to therapy, as well as phototype, nevi number, history of sun exposure and genetic melanoma susceptibility are often not available, so that could be one of the reasons for data contradiction.

Studies conducted in a melanoma fish model (*X. couchianus*) show a two-fold higher incidence of melanoma in males compared with females in response to UVB irradiation, but no significant difference between male and females in spontaneous melanoma frequencies (without UVB irradiation) [122].

ERα is the principal estrogen receptor in human skin, but studies based on immunoreactivity failed to demonstrate a role of this receptor in the pathophysiology of either melanoma precursor lesions or melanomas. On the other hand, ERβ is the predominant ER subtype in melanocytic lesions, and in particular the distribution and degree of ERβ immunoreactivity is markedly different among various classifications of the lesions. ERβ expression in human samples decreases during transformation of melanocytes and progression of malignant melanoma. Benign melanocytic lesions and dysplastic nevi with low-to-mild severe atypia show the higher expression of the receptor, that is predominantly localized in the nucleus of the cells; dysplastic nevi

with several atypia and melanomas *in situ* show immunopositivity in nucleus and cytoplasm, while in malignant melanomas ERβ expression decreases with increasing Breslow depth [123]. Moreover, ERβ immunoreactivity varies in melanoma cells depending on their local microenvironment, that means keratinocyte-associated melanoma cells, stroma-associated melanoma cells or melanoma-associated melanoma cells. In particular, melanoma cells in the epidermis, the papillary dermis and at the periphery of the dermal aggregates show increased ERβ expression compared to melanoma cells in large dermal nodules deeper in the dermis. In the same study no gender differences are observed in the expression of ERs, although some other studies report different results: in a study by Zhou JH et al, ERβ has been found to be more frequently expressed in melanomas of pregnant women than in men, and a trend to a higher expression in women than in men has been observed. This fact leads to speculate that ERβ might explain the generally favorable prognosis of melanoma in women [124].

Nutraceuticals and cancer

Phytochemicals

Natural products provide a useful resource for drug development. About 36% of the small molecule compounds approved by FDA between 1999 and 2008 are natural products or their derivatives [125]. Moreover, the role of dietary factors in preventing cancers has been investigated in a large body of epidemiological studies. "Nutraceuticals" is the term used to identify such classes of natural molecules or dietary elements that might be exploited for the treatment or prevention of different diseases, especially cancer, enforced by lesser toxicity, high availability and reduced costs compared with standard therapies. A number of phytochemicals have been shown to decrease cell proliferation, induce apoptosis, retard metastases and inhibit angiogenesis, and some of these plant-derived compounds, such as taxanes, vinca alkaloids and podophyllotoxin analogues, are currently used for chemotherapy of cancerous patients.

Several dietary factors have been found to possess chemopreventive activity, and most of them exert antiproliferative, proapoptotic, antioxidant and antiinflammatory effects on cancer cell lines.

Epigallocatechin-3-gallate (EGCG) is the most abundant polyphenol in green tea. It is able to induce apoptotic cell death in cell cultures and animal models, and several ongoing clinical trials show its ability to synergistically increase the efficacy of conventional chemotherapy against prostate carcinoma and colorectal cancer [126].

Curcumin is a yellow polyphenol derived from the rhizome of turmeric (*C. longa*). Its beneficial effects are related to its antiinflammatory, antioxidant and cytoprotective properties, and it possesses anticancer effects on mutagenesis, cell cycle regulation, apoptosis, oncogene expression and metastases. It has also been reported to exhibit synergistic chemopreventive effects with other diet-derived polyphenols and to increase the efficacy of many cancer drugs [126].

Resveratrol is a phytoalexin found in the skin of red grape and, therefore, it is an important constituent of red wine. It is known to induce potent antioxidant and

antiinflammatory effects and inhibit the proliferation and induce apoptosis in a variety of cancer cells [127].

Lycopene is a carotenoid, a natural antioxidant found in red fruits and vegetables, such as tomatoes, watermelon, and pink grapefruit. Epidemiological studies have shown that high intake of this dietary factor is inversely associated with the incidence of some cancers. A combination of vitamin E, selenium and lycopene has been shown to inhibit prostate cancer development and to increase disease-free survival [126].

Polyunsaturated omega-3 fatty acids (PUFAs) are antiinflammatory molecules that have been shown to increase apoptosis, promote cell cycle arrest and decrease tumor growth and metastatic disease, and to promote recurrence-free survival [128].

Numerous cell signaling pathways are activated by dietary phytochemicals, and the same compound may activate different pathways, depending on the cell type. Some compounds can interact with specific proteins/receptors, due to their structural similarity with endogenous ligands. "Phytoestrogens" are a large family of plant-derived estrogenic analogues possessing significant estrogen agonist or antagonist activity. The main classes of phytoestrogens are the isoflavones (genistein, daidzein, glycitein, equol), the lignans (enterolactone, enterodiol), the coumestanes (coumestrol), the flavonoids (quercetin, kaempferol), and the stilbenes (resveratrol). The most studied phytoestrogens are the isoflavones genistein and daidzein from soy food and lignans from foods such as cereal brans, legumes and vegetables, and the first attention was put on their utility as an alternative treatment for relief of menopausal symptoms [129].

As described above, the two estrogen receptors are expressed in different types of tumor, irrespective of their hormone-dependence. Since ERα and ERβ mediate opposite effects on cancer cell proliferation, and they can be activated by different ligands, several efforts have been made to investigate the binding and activation of the receptors and the expression of ERE-regulated genes induced by the more common phytoestrogens such as genistein, daidzein, equol and liquiritigenin. These studies show that botanical estrogens are preferential ligands of ERβ, since their binding to this receptor is 20- to 300-fold higher compared to the binding to ERα. Moreover, their

ERβ preferential potency becomes progressively reinforced at downstream levels: the chromatin binding, the recruitment of coactivators and the expression of ERβ *vs* ERα-regulated genes [129]. However, this preferential ERβ activation is lost when cells are treated with higher concentration of these compounds: in this case ERα and ERβ are equally well recruited to gene regulatory sites on chromatin. This fact implies that, while at lower concentrations only the ERβ-induced effects on cell proliferation are stimulated, at higher concentration the resulting effects depend on the relative ERα/ERβ expression ratio. Furthermore, as for other phytochemicals, the cytotoxic effect of phytoestrogens follows a hormetic mechanism, in which a specific chemical compound induces opposite effects at different doses: most commonly, there is a beneficial effect at low doses and a toxic effect at high doses, depending on the cellular context [126].

Triggering cell death with natural compounds: ER-stress related apoptosis

Many natural products are known to cause apoptosis in cancer cells by triggering endoplasmic reticulum stress.

The endoplasmic reticulum (ER) is a membranous system composed by a network of branching tubules and sacs that is present in all eukaryotic cells. It is mainly responsible for the synthesis, folding, post-translational modifications and delivery of proteins, and it also plays a pivotal role in Ca^{2+} homeostasis, being one of the most important Ca^{2+} stores. The mantainence of Ca^{2+} gradients is warranted by the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA): the Ca^{2+}-rich environment is fundamental because many ER chaperon proteins, crucial for correction of protein folding, such as BiP, calnexin, calreticulin and PDI, are Ca^{2+}-dependent.

Several signals can alter ER homeostasis: physiologic or pathologic stimuli might increase the demand of protein folding, while stressful stimuli might disrupt protein folding. An increment in protein synthesis due, for example, to proliferative signaling or overexpression of oncogenes, must be coupled with an efficient protein folding: if not, an excess of unfolded protein accumulates in the ER. On the other hand, stressful conditions due to hypoxia, glucose deprivation and oxidative stress may cause an

unfavorable environment for a correct protein folding and maturation. In both cases a protein overload of the ER might occurs, so that the accumulation of unfolded or misfolded proteins in the ER lumen causes ER stress and ER stress responses, a condition referred to as "unfolded protein response" (UPR). The UPR comprises mechanisms through which cells can keep the balance between protein synthesis and protein folding, to overcome ER protein overload and to restore normal conditions (Fig. 7). If endogenous or exogenous stimuli are too hard, or if the UPR is not sufficient, cells undergo apoptosis.

Three major proteins are known to act as stress sensors in the ER: double-stranded RNA-dependent protein kinase PKR-like ER kinase (**PERK**), inositol-requiring 1α (**IRE1α**) and activating transcription factor 6 (**ATF6**). Each of these proteins displays an ER-luminal domain that senses unfolded proteins, an ER-transmembrane domain and a cytosolic domain that transduces the signals to the cytoplasm. In normal physiological conditions, these sensors are inactivated by the association with the chaperon protein immunoglobulin-heavy-chain-binding protein (BiP, also known as GRP78). On the other hand, when BiP is dissociated to aid in the folding of nascent polypeptides, it triggers the activation of the three sensors and the induction of the UPR: several emergency systems are activated in order to avoid stressful and potentially harmful consequences of protein folding impairment, and to re-equilibrate protein homeostasis. Each sensor is coupled with a cytosolic pathway, and each pathway converges to this goal [130].

Dissociation of BiP from PERK triggers the homodimerization and subsequent activation through autophosphorylation of the kinase, that in turn activates, via phosphorylation, eIF2α (eukaryotic translation-initiation factor 2α), which inhibits protein synthesis.

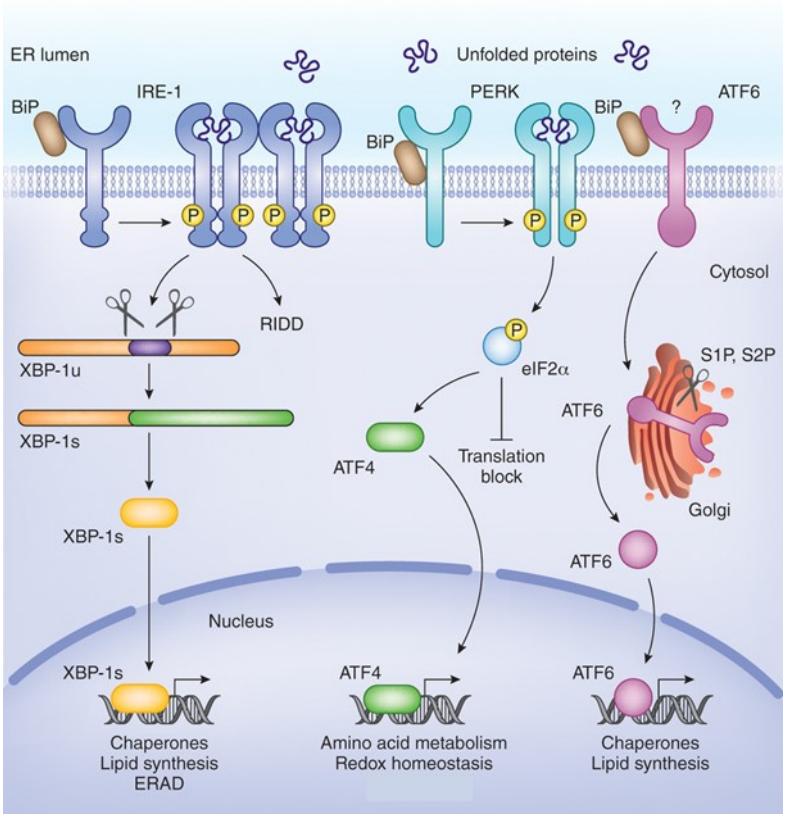

Fig. 7. UPR regulatory mechanisms of adaptive responses to ER stress.

In a similar way, the dissociation of BiP from IRE1α leads to its activation through dimerization and autophosphorylation. IRE1α possesses also an endoribonuclease (RNase) domain, and once activated it removes a 26-nucleotide intron from the mRNA encoding the X box binding protein 1 (XBP1). The spliced XBP1 (sXBP1) encodes a transcription factor that activates the transcription of UPR target genes. The RNase domain also leads to decay of other mRNAs, which further contributes to decreased protein translation during ER stress [131].

After BiP release, ATF6 is translocated to the Golgi complex and cleaved by the proteases S1P and S2P, giving rise to a functional fragment of ATF6 that translocates to the nucleus where it activates the transcription of UPR genes.

Furthermore, activation of the UPR promotes ER-associated degradation (ERAD) of misfolded proteins: this mechanism involves the retrograde translocation of proteins out of the ER, and subsequent degradation by cytosolic 26s proteasomes.

All these pathways go towards the attenuation of protein synthesis, the induction of ER chaperones to fold newly synthesized or misfolded proteins, the degradation of

misfolded proteins, just to improve cell survival. However, when these mechanisms are not sufficient to suppress the induced ER stress, cell death is triggered by apoptosis (Fig. 8).

The principal pathway involved in the activation of the apoptosis process during ER stress is the upregulation of the DNA-damage-inducible-gene 153 (GADD153), also known as C/EBP homologous protein (**CHOP**), a key transcription factor that determines the switch from prosurvival-to-death significance of the UPR. Upregulation of CHOP is achieved downstream of the PERK pathway: despite the eIF2α-mediated translational attenuation, some genes are upregulated, rather than downregulated; among them, ATF4 is involved in proapoptotic pathways via upregulation of CHOP. Among the targets of CHOP, GADD34 can initiate apoptosis due to the restoration of protein synthesis and overloads of the ER; ERO1α contributes to the hyperoxidation of the ER for proper disulfide bond formation, and is involved in apoptotic cell death through the activation of IP3R1 (inositol 1,4,5 triphosphate receptor 1)/calcium/CaMKII (calmodulin-dependent protein kinase II) pathway, with generation of ROS and then cell death [132]. Bcl2 expression is suppressed by CHOP, and this disturbs the equilibrium between pro- and anti-apoptotic proteins. Then, Bax and Bak are released from the ER and translocate to the mitochondrial membrane, where they induce the formation of pores that lead to an alteration of the mitochondrial outer membrane potential (MOMP) and, subsequently, trigger the classical intrinsic apoptosis.

Although IRE1 arm has been characterized as a primarily prosurvival pathway, it has also been implicated in apoptosis following prolonged ER stress. This is mainly achieved through interaction of IRE1 with the tumor necrosis factor receptor associated factor 2 (TRAF2), the complex can then stimulate JNK-mediated suppression of the antiapoptotic activity of Bcl2 and activation of BH3-only proteins [132].

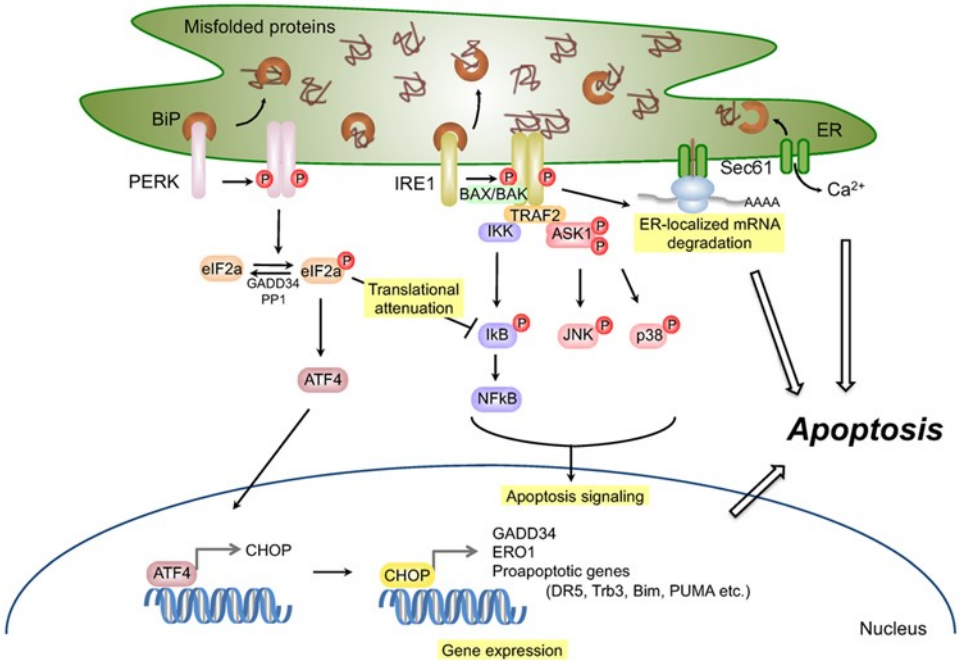

Fig. 8. UPR regulatory mechanisms of proapoptotic responses to ER stress.

Changing in the balance between proapoptotic and antiapoptotic factors, and Ca^{2+} homeostasis perturbation, cause the mitochondria outer membrane permeabilization (MOMP). This process leads to the cytoplasmic release of intermembrane space proteins that drive caspase activity. Cytochrome *c* is the most important protein to be released after MOMP: once in the cytoplasm, it transiently binds the key caspase adaptor molecule Apaf-1. This interaction triggers conformational changes in Apaf-1, with the exposure of caspase activation and recruitment domain (CARD) that in turn binds to CARD domains of the initiator caspase procaspase-9, forming the apoptosome. Dimerization of procaspase-9 leads to its activation and consequently to the activation of the caspase cascade by caspase-3 and -7 cleavage, and then to a rapid cell death [133].

Caspase-4 is also a major player in ER stress. This caspase is bound to the ER membrane where it is kept in an inactive state, and is cleaved, that means activated, following ER stress: its activation seems to be independent of the assembly of the apoptosome [130]. The activation of caspase-4 has been shown to be regulated by either ER-localized Bax and Bak proteins or by calpains, after intracellular calcium storage perturbation.

A possible anticancer strategy might take advantage from the ER stress-induced apoptosis, so new anticancer agents should be developed to change the balance between prosurvival and proapoptotic signals in cancer cells, in favor of the induction of cell death.

Several natural products might be exploited for cancer treatment, due to their activation of the ER-stress related apoptosis. For example, bortezomib is a boronate peptide, a synthetic molecule that is structurally related to naturally-occurring peptide aldehydes. It is a proteasome inhibitor that is used for the treatment of multiple myeloma and mantle cell lymphoma, while some others related molecules, such as delanzomib and carfilzomib, are actually in clinical trials [130].

Other natural compounds have been investigated in preclinical settings.

Thapsigargin is derived from the Mediterranean umbrelliferous plant *T. garganica.* It is an irreversible SERCA inhibitor, thus disrupting Ca^{2+} homeostasis. Following thapsigargin incubation, Ca^{2+} is released from the ER, and part of it is taken by mitochondria, leading to the dissipation of MOMP, with the release of cytochrome *c* and activation of the intrinsic apoptotic pathway [134].

Resveratrol has been shown to cause accumulation of misfolded proteins in cancer cells, triggering ER stress and ER stress-related apoptosis [135].

Fisetin is a flavonol that can be found in strawberries and mangoes; in the human A375 melanoma cell line it triggers ER stress with the activation of both the intrinsic and extrinsic apoptosis [130].

Several flavonoids such as apigenin, quercetin, myricetin and kaempferol, have been described as proteasome inhibitors [136].

Finally, tunicamycin is a mixture of nucleoside homologues originally isolated from *S. lysosuperificus*. It has the ability to inhibit N-linked protein glycosylation, because it is an inhibitor of N-acetylglucosamine transferase, contributing to the endoplasmic reticulum protein overload [137].

Tocotrienols

Tocotrienols (TT) are natural compounds that belong to the vitamin E family, together with tocopherols. The main sources of vitamin E are lipid-rich plant products and vegetable oils; tocotrienols are found in palm oil and rice bran oil, coconut oil, cocoa butter, soybeans, barley, wheat germ and annatto seeds. The most abundant source of tocotrienols is represented by palm oil, that is made of 30% of tocopherols and 70% of tocotrienols.

Four different tocopherols and tocotrienols are known, consisting of α, β, γ and δ isomers. Their chemical structure is composed by a chromanol ring and a side chain that is different between tocopherols and tocotrienols: tocopherols are saturated form of vitamin E, whereas tocotrienols are unsaturated and possess an isoprenoid side chain. The four different isomers differ each other depending upon the number and position of methyl groups. The α and β isomers are trimethylated on the chromanol ring; the γ isomers are dimethylated and the δ are monomethylated (Fig. 9). The unsaturated side chain of tocotrienols allows a more efficient penetration into tissues that have saturated fatty layers such as brain and liver [138].

Tocotrienols are absorbed, as other member of vitamin E, along with fat, in the small intestine, bile salts are necessary for their absorption. They are packaged into chylomicrons and transported in the lymphatic system to the liver. The hepatic α-tocopherol transfer protein (α-TTP), together with the tocopherol-associated proteins (TAP) is responsible for the endogenous accumulation of natural α-tocopherol. Tissue uptake for both classes of compounds occurs with the help of lipoprotein lipases, or by receptor-mediated endocytosis of lipoproteins [138]. The bioavailability of tocotrienols is lesser than α-tocopherol, and this might be due either to a better cell uptake of tocotrienols, given their unsaturated fatty chain, or to their better antioxidant activity in the bloodstream.

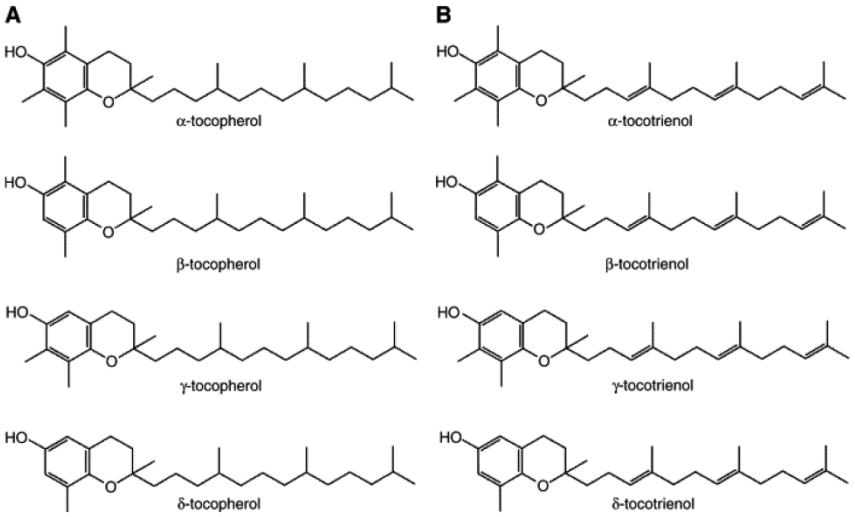

Fig. 9. Chemical structure of vitamin E derivatives. **(A)** Tocopherols. **(B)** Tocotrienols.

The biological activity of vitamin E is associated with its antioxidant properties, because the phenolic group in the chromanol ring quenches and scavenges free radicals, specifically against lipid peroxidation in biological membranes. Tocotrienols are thought to have more potent antioxidant activity than tocopherols, due to the unsaturated side chain that allows their better distribution in the lipid layer of the cell membrane [139]. Furthermore, the antioxidant activity of tocotrienols is also mediated through the induction of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase [138].

Most of tocotrienols' health benefits come from their antioxidant properties; however, some other tocotrienols effects are independent from this activity. Tocotrienols protect against cardiovascular diseases, and this effect is mediated through their antioxidant activity, their ability to suppress inflammation, to reduce the expression of adhesion molecules and consequently to reduce monocyte-endothelial cell adhesion. Furthermore, it has been shown that tocotrienols reduce serum levels of LDL and triglycerides, together with the oxidative modification of LDL; they also suppress HMG-CoA reductase activity through post-translational mechanisms and then reduce cholesterol levels. All these features contribute to the prevention of atherosclerosis, and α- and γ-tocotrienols are the isoforms that possess higher cardioprotective abilities [138].

Another risk factor for the development of cardiovascular diseases is diabetes mellitus. Especially α-tocotrienol significantly prevents oxidative damage in diabetic rats and causes a decrease in serum advanced glycosylation end-products, blood glucose and glycated hemoglobin. Moreover, it improves dyslipidemia, prevents diabetic neuropathy and has an insulin-sensitizing effect [140].

Various reports suggest that tocotrienols are neuroprotective. It has been demonstrated they have activity against Parkinson disease [132] and to protect against glutamate-induced cell death by an antioxidant-independent mechanism. In particular, α-tocotrienol blocks glutamate-induced death by suppressing glutamate-induced early activation of pp60 (c-Src) kinase [141]. The antioxidant properties of tocotrienols prevent polyunsaturated fatty acids (PUFAs) oxidation. A number of neurodegenerative conditions are associated with disturbed PUFA metabolism of arachidonic acid (AA), including acute ischemic stroke: α-tocotrienol has been shown to attenuate both enzymatic and non-enzymatic mediators of AA metabolism and neurodegeneration [142]. All these observations on the central nervous system suggest that α-tocotrienol represent the most potent neuroprotective form of vitamin E.

Anticancer activity

During the past decade much evidence has accumulated demonstrating the anticancer effects of specific forms of vitamin E, despite the antitumor activity of the whole vitamin E is still controversial. Individual tocotrienols and tocopherols display differential potencies in suppressing tumor cell growth and inducing apoptosis, that are generally indicated as to:

δ-tocotrienol = γ-tocotrienol > α-tocotrienol > δ-tocopherol > γ-tocopherol > α-tocopherol

with tocotrienols having more antitumor activity than tocopherols [143].

This anticancer effect may be unrelated to their antioxidant properties, and can be addressed through at least three mechanisms: 1- antiproliferative; 2- proapoptotic; 3- antiangiogenic.

The precise mechanisms for the **antiproliferative** properties of tocotrienols are still under investigation, and seem to involve different pathways. Most of the studies have been conducted on breast cancer. Inhibitory effects have been observed on PI3K/Akt and NFκB signaling; γ-tocotrienol inhibits EGF-dependent activation of mitogenic pathways via reduction of the ErbB/HER receptors autophosphorylation [144, 145] and reducing cyclin D1, CDK4, CDK2 and CDK6 expression and upregulating the cell cycle inhibitor p27 [146]. These findings demonstrate that the antiproliferative effects of γ-tocotrienol result in part from cell cycle blockade at G1. Based on these observations, combined treatment with conventional antiproliferative chemotherapic drugs should be beneficial. Early studies have shown that combined treatment with the ER antagonist tamoxifen and the TRF (tocotrienol-rich fraction) of palm oil significantly inhibited the growth of ER-positive (MCF-7) and negative (MDA-MB-435) breast cancer cell lines, and combined treatment of γ-tocotrienol with tyrosine kinase inhibitors such as erlotinib and gefitinib induces synergistic antiproliferative effects [146]. A clinical trial to test the effectiveness of adjuvant TRF therapy in combination with tamoxifen for 5 years in women with early breast cancer has shown a trend of 60% in lowering mortality, although this result was not statistically significant [147].

Another mechanism exploited by tocotrienols for reducing cell proliferation is to reduce the post-translational modification of proteins involved in mitogenic signaling pathways. Especially protein farnesylation and isoprenylation are affected by tocotrienols, because it has been shown that these compounds can inhibit HMG-CoA reductase activity by post-transcriptional downregulation and metabolic degradation. HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis in the mevalonate pathway. This pathway produces various farnesyl and isoprenoid intermediates that are involved in post-translational modifications of proteins, such as small G proteins and αβγ-G protein subunits, that allow them to be anchorated to the plasma membrane. For example, Ras is linked to the plasma membrane and this is crucial for G protein-coupled receptor mitogenic signaling. That's the reason why statins, potent inhibitors of HMG-CoA reductase, are efficient in reducing cancer cell proliferation; however, their conventional use is limited because of their high dose-

related toxicity that is associated with several side effects such as muscle pain and damage that can ultimately lead to rhabdomyolysis, liver damage, kidney failure, and even death. Combined treatment of γ-tocotrienol with statins is very effective in inhibiting mammary tumor cell growth [148], with decreased phosphorylation of MAPK, JNK, p38 and Akt and inducing cell cycle arrest in the G1 phase. Moreover, γ-tocotrienol not only reduces the activity, but also lowers cellular levels of HMG-CoA reductase, and when used in combination with statins it prevents the compensatory statin-induced upregulation of this enzyme.

The antiproliferative activity of tocotrienols has also been investigated on prostate cancer. The growth inhibitory effect of TRF has been tested on normal human prostate epithelial cells and human prostate cancer cells (LNCaP, DU145 and PC3 cell lines): TRF selectively resulted in potent growth inhibition in cancer cells but not normal cells, through G1 arrest and sub-G1 accumulation. Moreover, colony formation is arrested by TRF [149]. However, the TRF is a mixture of various vitamin E isomers (it is rich in tocotrienols but also contains some α-tocopherol), so subsequent studies have demonstrated the prevalent role of tocotrienols, in particular γ- and δ-, in the antiproliferative effect on prostate cancer cells. A reduction of cyclin D1 and E, as well as an increment of the cell cycle inhibitors $p27^{Kip1}$, $p21^{cip1}$ and $p16^{INK4a}$, have been observed in human prostate cells in culture [150], together with the suppression of NFκB, EGF-R and Id family proteins. The suppression of mesenchymal markers and the restoration of E-cadherin and γ-catenin expression have also been observed, and this is associated with the suppression of cell invasion capability [151]. As an additional result, γ-tocotrienol eliminates prostate cancer stem cells, and downregulates the expression of prostate CSC markers (CD133, CD44), in androgen-independent prostate cancer cell lines [152]. Furthermore, a synergistic effect has been observed when cells are cotreated with γ-tocotrienol and docetaxel; moreover, the radiotherapy efficacy on prostate cancer can be increased with the treatment with γ-tocotrienol *in vivo* [153], demonstrating a sensitizing effect of tocotrienols with respect to standard therapies.

Similar observations on cell proliferation and cell cycle blockade have been observed in other type of cancers, such as colorectal, liver, pancreatic and lung cancer.

Several reports demonstrate that tocotrienols induce **apoptosis** in tumor cells, whereas tocopherols have no such effect. In particular, it has been reported that γ- and δ-tocotrienols have more apoptosis-inducing potency than α-tocotrienol, and this effect can be mediated by induction of both the intrinsic or extrinsic apoptotic pathway. Takahashi and Loo [154] have demonstrated that apoptosis induced by γ-tocotrienol in human breast cancer cells involves the mitochondria-mediated apoptotic pathway, by induction of cytochrome *c* release, activation of initiator caspase-9 and effector caspase-3 and cleavage of the caspase-3 target poly-ADP-ribose polymerase (PARP). Tocotrienol-induced activation of mitochondrial apoptosis pathway rely on downregulation of antiapoptotic factors, such as Bcl-2, Bcl-xL, nuclear NFκB and the caspase inhibitor XIAP, and the upregulation of proapoptotic factors such as Bax. It has also been demonstrated that TRF induces the expression of $p21^{Waf1}$, independently of cell cycle regulation but dependently of p53, in human colon carcinoma RKO cells [155].

Tocotrienols have also been shown to initiate the extrinsic apoptosis pathway, depending on the individual cancer cell type. Activation of the death receptors leads to the cleavage of the initiator caspase-8, that then activates effector caspase-3. Studies on mammary tumors have shown that the activation of caspase-8 is not mediated through death receptor activation, but through a decrease in the levels of FLICE-inhibitory protein (FLIP), an apoptosis inhibitory protein that inhibits caspase-8 activation [156].

In some cases, both the intrinsic and the extrinsic apoptosis pathway can be activated: for example, one study has been reported that γ-tocotrienol induces apoptosis in human T-cell lymphoma through ROS production, calcium release, changes in Bax/Bcl-2 ratio and loss of mitochondrial membrane potential. Moreover, it also upregulates surface expression of Fas and FasL and triggers caspase-8 activation [157].

Tocotrienols-induced apoptosis might be mediated through the ER stress apoptotic way. Studies on breast cancer cells show a wide interconnection between the three apoptosis mechanisms upon treatment with γ-tocotrienol. The activation of the PERK/eIF2α/ATF4/CHOP signaling is accompanied by activation of caspase-4 and PARP

cleavage, as well as caspase-8 and -9 activation [158]. Furthermore, this same treatment activates JNK and p38 MAPK, and upregulates death receptor 5 (DR5), and knockdown of either JNK or p38 MAPK results in reduced DR5 and CHOP levels and partially blocks tocotrienol-induced apoptosis.

Despite most of the studies have been conducted on breast cancer, the proapoptotic activity of tocotrienols has been demonstrated in different type of tumors, such as prostate cancer, colorectal adenocarcinoma, T-cell leukemia and lymphoma, gastric adenocarcinoma, hepatoma and glioma.

The third anticancer mechanism of tocotrienols is related to their **antiangiogenic** effects. Tumor-induced angiogenesis results from aberrant blood vessel formation. It is important for the delivery of nutrients and oxygen to tumor cells and then for tumor growth in hypoxic conditions, as well as for the spread of metastatic disease. One of the major drivers to tumor angiogenesis is hypoxia, a common feature of many solid tumors: hypoxia-inducible factors (HIFs) represent a family of transcription factors whose activation is regulated by oxygen levels. Once activated, these factors transactivate genes involved in tumor cell adaptation under hypoxic condition, such as genes involved in glycolysis and angiogenesis [159]. Some of the known angiogenic activators or growth factors include vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor α (TNFα). These factors induce proliferation, migration and differentiation of endothelial cells and stimulate them to tube formation. Several studies show that tocotrienols reduce the activation of angiogenic signaling pathways on both tumor and endothelial cells. It has been observed that tocotrienols induce a decreased HIFs activation and the consequent VEGF downregulation in malignant mesothelioma cells [160], lower levels of IL-8 and IL-6 in HUVEC (human umbilical vein endothelial cells) and of VEGR receptors (VEGF-R1 and VEGF-R2) in HUVEC and in mammary tumor cells [161], the induction of apoptosis in HUVEC cells, and the inhibition of the intracellular pathway downstream the VEGF-R (PI3K/PDK/Akt), both *in vitro* and *in vivo* [162]. Furthermore, *in vivo* experiments show that tumor size

inversely corresponds with VEGF levels. Among tocotrienols, the δ isomer shows the better antiangiogenic activity, while tocopherols do not possess this property at all.

Modulation of nuclear receptors

Different studies suggest that tocotrienols might be effective in modulating some nuclear receptors in various disease models. For example, apart their antioxidant actions, tocotrienols have been found to prevent atherosclerosis through modulating the activities of peroxisome-proliferators activated receptors (PPARs). In particular, TRF from palm oil transactivates the isoforms PPARα, PPARγ1, PPARγ2 and PPARδ, and their antiatherogenic effect is due to the PPAR modulation of the expression of another nuclear receptor, liver X receptor α (LXRα) [163]. Other reports show that PPARs represent a molecular target of tocotrienols: α- and γ-tocotrienols have been found to transactivate PPARα, while δ-tocotrienol modulates PPARα, γ and δ in reporter based assays. In this study, TRF improved whole body glucose utilization and insulin sensitivity in diabetic Db/Db mice by selectively regulating PPARs target genes [164].

PPARγ is known to be important for inhibition of cell proliferation and induction of apoptosis in breast cancer. It has been found that both γ- and δ-tocotrienols activate PPARγ transcription in estrogen receptor-positive breast cancer cell lines, with δ-tocotrienol having a better activity than γ-tocotrienol. The transcriptional activity of this receptor is also activated by tocotrienols in SW480 colon cancer cells, exerting an antitumor effect also in this type of cancer.

Pregnan X receptor (PXR) is a nuclear receptor that recognizes xenobiotics, and it mediates the induction of genes involved in oxidation, conjugation and transportation of these compounds. In HepG2 cells, it has been found that PXR is strongly transactivated by α- and γ-tocotrienols, suggesting a potential role of individual form of vitamin E on the metabolism of certain drugs and environmental chemicals [165].

The activity of tocotrienols through estrogen receptors is still a matter of debate, and most of research has been focused on estrogen receptor in breast cancer. Among the first studies, Nesaretnam *et al* conducted experiments on estrogen receptor-positive and -negative breast cancer cell lines, where they show that TRF can inhibit the growth

of both cell lines, and furthermore that ER-positive cell growth can be inhibited irrespective of the presence or absence of estradiol. This might have important therapeutic implications, but fails to demonstrate a direct action of tocotrienols on estrogen receptors [166]. Accordingly to these results, other studies have been published, showing the inhibition of proliferation of both MDA-MB-435 (ER-negative breast cancer cell line) and MCF-7 (ER-positive) cells by tocotrienols and tamoxifen, alone or in combination treatment [167].

On the other hand, more recent findings by Comitato *et al* seem to demonstrate a direct interaction between tocotrienols and the estrogen receptor β, in reducing breast cancer cell proliferation. They performed docking simulation through a software-based approach, and revealed a high likehood of the binding of δ- and γ-tocotrienols to ERβ, rather than α-tocotrienol. This difference in tocotrienols binding to ERβ follows the number of substitutions in the phenolic ring, a common feature of other estrogens and estrogen-like molecules. Moreover, in silico simulations also suggest a low affinity for the tocotrienol binding to ERα. Treatment of MDA-MB-231 breast cancer cells (ERβ-positive, ERα-negative) with TRF or the individual δ- and γ-tocotrienols has been associated with apoptosis activation, ERβ cytoplasmic-to-nuclear translocation and enhanced expression of ERE-containing genes such as *MIC-1*, *EGR-1* and *cathepsin D*, while ERE-devoid gene expression was not affected. However, cotreatment with the ER antagonist ICI-182,780 totally or partially prevented the upregulation of ERE-responsive genes, suggesting that the effect of tocotrienols is probably only partially mediated by ERβ [168]. Similar results have been obtained with the MCF-7 breast cancer cell line, expressing both ERα and ERβ [169].

Another recent study also confirms the molecular interaction between tocotrienols (especially δ-tocotrienol) and ERβ. In particular, the neuroprotective role against Parkinson's disease has been explored, exploiting human neuroblastoma SH-SY5Y cells as a cellular model for studying tocotrienols-induced cytoprotection. The tamoxifen-dependent inhibition of γ- and δ-tocotrienols activity indicates that estrogen receptor signaling is associated with their cytoprotective effect: the binding activity between the natural compounds and estrogen receptors has been measured through

radiometric competitive assay, showing the direct and pronounced binding of tocotrienols to ERβ, but a weak binding to ERα. Treatment with tocotrienols induces the ERβ translocation in the perinuclear space, but does not induce upregulation of ERE-responsive genes. Indeed, in this disease model, ERβ mediates the cytoprotective effects of tocotrienols through a non-genomic mechanism, that involves the PI3K/Akt pathway [170].

AIMS

Aims

Malignant melanoma represents the deadliest form of skin cancer. Cutaneous melanoma *in situ* is characterized by a good prognosis: in this context, time of diagnosis is crucial because the treatment of choice is represented by surgical resection. On the contrary, invasive and metastatic melanomas are almost incurable: in these cases the surgical therapy is quite difficult and no effective systemic therapeutic strategies are actually available to warrant a significant prolonging in survival. The available therapies are also characterized by toxic side effects, tumor easily develops resistance and patients often relapse. This is true for chemotherapies, immunotherapies and the more recently developed targeted therapies. Moreover, malignant melanomas are highly heterogeneous, since tumors are characterized by a wide spectrum of different mutations, even if most melanomas show the hyperactivation of the Ras/Raf/MEK pathway. For these reasons, efforts are needed to identify new molecular targets and more effective therapeutic approaches for the management of malignant melanoma.

Some epidemiological and histopathological evidences support the notion that melanoma could be classified among the “hormone-dependent tumors”. Especially, a tumor suppressive relationship with estrogens can be hypothesized, despite no studies have been conducted to clarify the role of estrogens in this type of tumor. The first aim of this work was then to characterize the estrogenic system on different human melanoma cell lines, focusing on the role of the estrogen receptor β. Indeed, this receptor subtype has been demonstrated to have a tumor suppressive function in different tumors, both related and unrelated to the reproductive system. For this reason, experiments were conducted in order to assess the role of ERβ on melanoma cell proliferation, and to investigate the molecular mechanisms associated with its activity, in terms of transcriptional and epigenetic regulation. Moreover, the relationship between ERβ activity and different mutational status of human melanoma cell lines was investigated.

The second task of the project was directed towards the study of the possible ERβ activity modulation through the vitamin E derivative δ-tocotrienol. This compound has been demonstrated to interact with the receptor and to transactivate it, inducing apoptosis in breast cancer cell lines. Experiments were then performed in order to verify the molecular mechanisms of the antitumor activity of δ-tocotrienol on human melanoma cell lines, and to investigate whether its effects could be mediated by the estrogen receptor β.

Several nutraceutical compounds exert an antitumor activity on different types of cancers, and it has been demonstrated that such effects can be due to their proapoptotic role. The molecular mechanisms, other than the modulation of ERβ, associated with the antitumor activity of δ-tocotrienol were investigated in this project, with a particular attention on the possible induction of the endoplasmic reticulum stress-related apoptosis. The efficacy of the compound was assessed on melanoma cell lines and on melanoma xenografts in nude mice.

The development of resistance to standard therapies and tumor recurrence have been associated with the existence of a population of cells in the tumor bulk named “cancer stem cells”. These cells are endowed of higher malignancy and possess the capacity of cancer initiation. In the third task of the project, melanoma stem cells were characterized *in vitro*, in term of stemness markers expression and ability to grow as spheroids. Finally, the antitumor effectiveness of δ-tocotrienol was assessed on this aggressive subpopulation of cells.

MATERIALS AND METHODS

Materials

Cell cultures

The human BLM melanoma cell line was provided by Dr. G.N. van Muijen (Department of Pathology, Radbound University Nijmegen Medical Center, Nijmegen, The Netherlands). This cell line is a subline of BRO melanoma cells isolated from lung metastases after subcutaneous inoculation of nude mice with BRO cells; mutation status: BRAF wild-type, NRAS mutated [171].

The human MCF-7 breast cancer and A375 melanoma cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). A375 mutation status: $BRAF^{V600E}$ mutated NRAS wild-type.

The human WM1552 ($BRAF^{V600E}$ mutated, NRAS wild-type) and WM115 ($BRAF^{V600D}$ mutated, NRAS wild-type) melanoma cell lines, derived from patient melanomas, were provided by Dr. R. Giavazzi (Department of Oncology, Mario Negri Institute for Pharmacological Research, Milano, Italy) and were from Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) [172, 173]. The human IGR-39 melanoma cell line ($BRAF^{V600E}$ mutated, NRAS wild-type) was from Leibniz-Institut DSMZ-Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (38124 Braunschweig, Germany) and kindly provided by Dr. C.A. La Porta (Biosciences Department, University of Milano).

Human melanocytes were provided by Dr. F. Crovato (Regional Reference Centre for Human Epidermis *in vitro* Culture and Bank for Tissue Cryopreservation, Niguarda Hospital, Milano, Italy). Stocks of cells were stored frozen in liquid nitrogen and kept in culture for no more than 10-12 weeks.

Animals

Female CD1 hairless mice, at 6 weeks of age, were purchased from Charles River, under the guidelines established by the University of L'Aquila regulations officially authorized by the Italian Ministry of Health. The official authorization to perform these experiments has been requested by the University of L'Aquila (Dr. C. Festuccia) to the

Italian Ministry of Health, according to the Directive 2010/63/EU incorporated into the Italian law with the D.Lgs. 26/2014.

Developed from the transfer of the nude gene from Crl:NU-*Foxn1*^{nu} to a CD-1 mouse through a series of crosses and backcrosses beginning in 1979, the animal lacks a thymus, is unable to produce T-cells, and is therefore immunodeficient.

In vivo experiments were performed in collaboration with Dr C. Festuccia, Department of Clinical and Applied Sciences and Biotechnologies, University of L'Aquila.

Reagents

17β-Estradiol (E_2) was purchased from Sigma-Aldrich; the ER antagonist ICI-182,780 and the ERβ agonist DPN (diarylpropionitrile) were from Tocris Biosciences. The selective ERβ agonists KB1, KB2, and KB4 were kindly provided by Dr. S. Nilsson (Karo Bio AB). The chemotherapeutic agent Dacarbazine, the selective eIF2α dephosphorylation inhibitor Salubrinal and the SERCA inhibitor Thapsigargin were purchased from Sigma-Aldrich.

A commercial palm oil fraction enriched in tocotrienols/tocopherols (Gold Tri E 70% w/w) was obtained from Golden Hope Biorganic, and a commercial "DeltaGold" extract of Annatto fruits (*B. orellana*) was obtained from American River Nutrition Inc.

Methods

Cell cultures

Adherent cells

BLM, A375, WM1552, IGR-39 cells and human melanocytes were routinely cultured in DMEM medium supplemented with 7,5-10% FBS, glutamine (1 mmol/l) and antibiotics (100 IU/ml, penicillin G sodium and 100 µg/ml streptomycin sulfate).

MCF-7 and WM115 cells were routinely grown in RPMI-1640 medium supplemented with 10% FBS, glutamine (1 mmol/l) and antibiotics (100 IU/ml, penicillin G sodium and 100 µg/ml streptomycin sulfate). Cells were cultured in humidified atmosphere of 5% CO_2/95% air at 37 °C, were detached through a trypsin-EDTA solution and passaged once a week.

Melanospheres culture enrichment

A375 and WM115 cell lines were grown in Euromed-N medium, supplemented with 10 ng/ml EGF, 10 ng/ml FGF2 and 1% N2 in normal (A375) or low adherence (WM115) flasks. Cells were initially seeded at 10^6 cells per 25-cm^2 flask. Floating tumor spheres were formed within 5-7 days, while cells without stem cells features were adherent. To enrich melanoma cell culture of initiating-tumor cells, floating spheres were harvested and passaged every ten days, or once reached 100 to 150 µm diameter size, otherwise, cells in the center of the sphere will undergo apoptosis. The supernatant in the flask was recovered, and centrifuged for 5 minutes at low speed (100xg), because spheres are quite fragile. Moreover, since their volume is higher than single cells, centrifugation at low speed allows to separate voluminous melanospheres from single cell-suspension. Spheres were then resuspended in 3 ml Euromed-N, mechanically dissociated, and splitted in new 25-cm^2 flasks. 1 ml Euromed-N was added every 48 hours to the culture, to provide fresh medium to the cells.

Conditioned medium derived from culturing spheres was centrifuged to eliminate single cells and collected to be used for subsequent assays, mixed with 70% fresh medium.

Cell proliferation assays

BLM melanoma cells were plated ($1.5x10^4$ cells/dish) in 6-cm dishes in DMEM complete medium. After 48 hours, the medium was replaced with phenol red free medium supplemented with 10% charcoal stripped FBS. Cells were then treated as follows: 1- DPN (10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} M); E_2 (10^{-9},10^{-8}, 10^{-7} M); KB1, KB2, or KB4 (10^{-9}, 10^{-8}, 10^{-7} M); KB1 (10^{-9}, $5x10^{-9}$, 10^{-8}, $5x10^{-8}$, 10^{-7} M) every 48 hours, for three times; 2- ICI- 182,780 (10^{-6} M) for 1 hour, followed by DPN, E_2, or K1 (10^{-8} M) every 48 hours, for three times. Cells were then harvested and counted by hemocytometer.

Experiments were also performed on melanoma A375, WM115, WM1552 and IGR-39 cells, that were treated with DPN (10^{-9}, 10^{-8}, 10^{-7} M), every 48 hours, for three times.

ERβ overexpression

The plasmid pCMV5-hERbeta, expressing human ERβ, was kindly provided by Dr. A. Maggi (Department of Pharmacological and Biomolecular Sciences, University of Milano, Milano, Italy).

BLM cells were plated ($8x10^4$ cells/well) in 6-well plates in DMEM complete medium. After 48 hours, the medium was replaced with DMEM medium without FBS and the cells were transiently transfected using Lipofectamine 2000 reagent (Invitrogen), mixing 1 µg DNA plasmid with 0,5 µl Lipofectamine 2000 per well (2:1 w/v), final volume 500 µl/well, according to the manufacturer's protocol. After 4 hours, 1 ml of complete medium was added to each well, and at 24 hours transfection medium was replaced with 2 ml of fresh, complete medium. After 24-72 hours of transfection the cells were harvested and lysed in RIPA buffer for protein extraction. Protein concentration in lysates was determined using the BCA method.

ERβ transcriptional activity assay

BLM cells, seeded in 24-well plates ($5x10^4$ cells/well) in phenol red free DMEM medium supplemented with 10% charcoal stripped FBS, were transfected using Lipofectamine 2000 reagent (Life Technologies). The following constructs were cotransfected: pVERE-tk-Luc (1µg), the reporter plasmid encoding the firefly luciferase reporter gene under the control of the estrogen response element (ERE; kindly provided by Dr. A. Maggi**,** Department of Pharmacological and Biomolecular Sciences, University of Milano, Milano, Italy), to evaluate the transcriptional activity of ERβ, and pCMVβ (0.4 µg), the reporter plasmid encoding the β-galactosidase (Clontech), as the internal control plasmid. Efficiency of transfection was evaluated by fluorescent microscopy by transfecting the plasmid vector pCMV-pEGFP-N1 (Clontech).

ERβ transcriptional activity was measured using the neolite system (PerkinElmer), according to the manufacturer's protocols. 6 hours after transfection, BLM cells were treated with DPN (10^{-8} M), E_2 (10^{-8} M), δ-tocotrienol (10 or 20 µg/ml) or vehicles for 24 hours. Cell medium was removed and 175 µl of fresh medium were added to each well. Neolite luciferase reagent was added to each sample (1:1) and firefly luminescence was read. At the end of this step, 100 µl of the lysate were added to ortho-Nitrophenyl-β-galactoside (ONPG). If β-galactosidase is present, it hydrolyses the ONPG molecule into galactose and ortho-nitrophenol (yellow colour). The samples were then analysed in an EnSpire Multimode Plate reader (PerkinElmer) at 420 nm. Data are expressed as the mean of the ratio (± SE) between luminescence of the experimental reporter (firefly luciferase activity) and that of the control reporter (β-galactosidase activity).

Methylation analysis of GC-rich regions

DNA from human melanocytes and BLM cells was extracted using Qiagen column methods, according to the manufacturer's protocol. DNA quality and concentration was evaluated by measuring the 260/280 nm optical ratio using a Nanodrop 2000 spectrophotometer (Thermo Scientific).

Digestion of genomic DNA with restriction enzymes RsaI, MspI and HpaII (Euroclone) was performed. For each DNA sample, 2 restriction digests were performed: one with RsaI and MspI, and one with RsaI and HpaII. RsaI is methylation insensitive, while MspI and HpaII are sensitive to DNA methylation and are able to cut only unmethylated restriction sites. These enzymes recognize the same tetranucleotide sequence (5'-CCGG-3') but display different sensitivity to DNA methylation: in general, MspI will not cut if the external cytosine is methylated, and HpaII will not cut if any of the two cytosines is methylated. Global DNA digestion was performed o/n at 37 °C. Restriction digests were performed with 1 µg of DNA and 5 units of RsaI in Roche buffer L. After 1 hour incubation at 37 °C, 2.5 unit aliquots of MspI or HpaII were added, 2 hours apart. Total incubation time was 18 hours. The enzymes were inactivated by 10 min incubation at 65 °C, and the digests were used for PCR using a single primer (5'-AACCCTCACCCTAACCCCGG-3') that arbitrarily binds within GC-rich regions of DNA. Samples were resolved on 1% agarose gel. The intensity of the band was determined using ImageLab (Chemidoc Imager, Bio-Rad). Data are expressed as the MspI/RsaI or HpaII/RsaI ratios relative to the intensity of the bands.

To investigate the effects of ERβ activation on the global DNA methylation profile of melanoma cells, BLM cells, seeded in 6-well plates ($3x10^4$ cell/well), in phenol red free DMEM medium supplemented with 10% charcoal stripped FBS, were treated with DPN (10^{-8}M), E_2 (10^{-8}M), or vehicle for 24 or 48 hours. The analysis of DNA methylation profile was performed as described above.

ERβ isoform expression by quantitative PCR

Total RNA from BLM, A375 and WM115 cells was isolated by RNeasy MINI kit (Qiagen) for the evaluation of the expression of ERβ isoforms; RNA pellets were dissolved in sterile distilled water and their concentrations were assessed using Nanodrop 2000 (OD260/280). Specific set of primers for each ERβ isoform were synthesized (Sigma-Aldrich) and utilized, as previously described by Collins and coworkers [174]. Real time-DNA amplification for ERβ was performed in CFX96 Bio-Rad using 20 µl of total volume. The efficiency of each set of primer was evaluated in preliminary experiment and it

was found close to 100% for target genes and for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). 600 ng of total RNA were retrotranscribed using the IScript Supermix kit (Bio-Rad), according to the manufacturer's protocol. The reaction was carried out on 40 ng of total cDNA using SYBR chemistry (iTAQ Universal SYBR green supermix, Bio-Rad) according to the manufacturer's protocol. Real-time PCR was run according to the following protocol: an initial step of 30 sec at 95°C followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. A dissociation stage with a melt curve analysis was also performed. Gene expression was quantified using the comparative threshold-cycle (Ct) method considering that the targets and the reference genes have the same amplification efficiency (near to 100%). In each cell line, ΔCt values (difference between target and reference gene Ct) for ERβ2 or ERβ5 were compared with ERβ1 ΔCt value in the same cell line.

Tocotrienol extraction

HPLC analysis and isolation of δ-tocotrienol were done using a 940-LC Liquid Chromatography Varian (Varian SPA) equipped with a binary pump system (pump head volume V=10 ml, eluent flow range: 0.3-25 ml/min), an autosampler (5 ml vials, injection 5-4000 mL), a scale up module, a photodiode array (operative wavelengths range: λ=200-400 nm, monitoring: λ=290 nm), and an automatic fraction collector.

Chromatographic separation of tocotrienols was conducted using an analytical reversed phase Kinetex column (5 µm C18 100 Å, 100x4.6 mm) and setting the eluent flow at 2 ml/min (injection volume 5 µl). For the isolation of δ-tocotrienol at semipreparative scale, a Kinetex AXIA column (5 µm C18 100Å, 100x21.5 mm) with eluent running at 20 ml/min and injection volume of 4000 µl were used. Mobile phase composition was always acetonitrile 100%. Single run time: 10 min.

δ-tocotrienol was isolated from either: 1- a commercial palm oil fraction enriched in tocotrienols/tocopherols (Gold Tri E 70% w/w, Golden Hope Biorganic); 2- a commercial extract of Annatto (*B. orellana*) fruits (DeltaGold, American River Nutrition Inc.).

1) A suitable amount of palm oil fraction was first extracted with acetonitrile (1:1 vol/vol). After vigorous shaking, the solvent phase was separated from the insoluble material by centrifugation (6000 rpm, d=15 cm, t=5 min), and solvent evaporated under reduced pressure (pressure endpoint 1 mbar) at room temperature. Aliquots of the oily extract (100 mg, OE) were dissolved in HPLC grade methanol (5 ml) and injected first in analytical mode and then in semipreparative conditions. The automatic fraction collector was set to collect in time slice mode the δ-tocotrienol peak in the RT window 2.3-2.5 min. To eliminate the contamination from the adjacent and partially overlapping peak due to γ-tocotrienol, all fractions of interest were pooled together, acetonitrile evaporated, the resulting residue diluted again in methanol and reinjected. These last steps were repeated (once or twice, depending from the chromatographic system stability) until a purity of at least >95% was achieved. The average recovery yield was around 10 mg per 100 mg of OE. Aliquots of 50 mg were diluted in dimethyl sulfoxide at the concentration of 50 mg/ml and stored at -20°C until further use.
2) Annatto extract was processed as in (1) with minor modifications. Around 200 mg of the rough extract were extracted directly with methanol (5 ml), the mixture centrifuged as above described, and the supernatant injected in the HPLC apparatus and further processed under the same conditions as for (1). Aliquots of purified δ-tocotrienol were pooled together until the needed amount was obtained, and diluted in olive oil.

The extractions were performed in collaboration with Dr G. Beretta, Department of Pharmaceutical Sciences, University of Milano.

MTT assay

BLM, A375, WM115 and IGR-39 cells were seeded at a density of $3x10^4$ cells/well in 24-well plate and human melanocytes at a density of 10^4 cells/well, in DMEM complete medium. After 48 hours, cells were treated with different doses of δ-tocotrienol (5, 10, 15 or 20 μg/ml) or dacarbazine (10, 20, 50, or 100 μM) for 24 or 48 hours.

In order to assess the ER-stress involvement in the δ-tocotrienol-induced cell death, BLM and A375 cells were seeded at a density of $3x10^4$ cells/well in 24-well plate, in DMEM complete medium. After 48 hours, cells were treated with Salubrinal (25 µM for BLM cells and 10 µM for A375 cells) or vehicle. After 1 hour treatment, 20 µg/ml of δ-tocotrienol or vehicle were added to each well.

At the end of the treatments, the medium was changed with a sterile MTT solution, in DMEM without phenol red, without FBS (0,5 mg/ml) and cells were incubated at 37°C for 15-45 minutes, depending on the cell type. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazolium that is reduced to purple formazan crystals in living cells, because of its conversion by mitochondrial NAD(P)H-dependent oxidoreductase enzymes. Following MTT incubation, violet precipitate was dissolved with isopropanol. Absorbance at 550 nm was measured through an EnSpire Multimode Plate reader (PerkinElmer).

Trypan blue exclusion assay

BLM and A375 cells were plated ($5x10^4$ cells/dish) in 6-cm dishes in DMEM complete medium. After 48 hours, the medium was replaced and cells were treated with different doses of δ-tocotrienol (5, 10, 15 or 20 µg/ml) for 24 or 48 hours. Adherent and floating cells were then harvested, stained with Trypan blue 0.4% (1:1 v/v) and counted by Luna automated cell counter (Logos Biosystems), discriminating between total, viable and dead cells.

Colony Formation Assay

BLM, A375 and WM115 cells were seeded (100-250 cells/well, depending on the cell type) in 6-well plate in DMEM complete medium. After 48 hours, cells were treated with δ-tocotrienol (20 µg/ml) or vehicle, for 72 hours, then the treatment was removed and cells were cultured for 7-10 days in DMEM complete medium. Colonies were then fixed with 70% methanol and stained with Crystal Violet 0.15%. Images of stained colonies were captured by a Nikon photocamera. The number of cell-forming

colonies and the dimension of colonies were compared between controls and treated cells, and these reflect the cytotoxic or cytostatic effects of δ-tocotrienol, respectively.

Melanospheres formation assays

1- Assessment of melanospheres morphology. Floating A375 melanospheres were collected, centrifuged at low speed (100xg) and gently resuspended in fresh serum-free Euromed-N medium supplemented with 10 ng/ml EGF, 10 ng/ml FGF2 and 1% N2. Spheres were then plated in a 12-multiwell plate, diluted to obtain about 1-2 spheres/well. After 48 hours, melanospheres were treated with δ-tocotrienol (20 μg/ml) for 5 days. During treatment, spheres in each well were monitored, and at the end of the treatment they were photographed under a Zeiss Axiovert 200 microscope with a 10x objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments).

2- Assessment of melanospheres number. Floating A375 melanospheres were collected, centrifuged at low speed (100xg) and rinsed in PBS. Spheres were then disaggregated in single cells through accutase, cells were counted and seeded at $3x10^4$ cells per well in a 12-multiwell plate, in complete Euromed-N composed by 70% fresh medium and 30% conditioned medium from melanosphere cultures, to provide sphere-forming cells of paracrine factors that warrant new spheres formation, even if plating cells in low density. After 48 hours, and during melanospheres formation, cells were treated with δ-tocotrienol (40 μg/ml) or vehicle, and the treatment was repeated after 7 days. Melanospheres for each well were then counted and photographed under a Zeiss Axiovert 200 microscope with a 4x objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments). The dimension and number of spheres per well were compared between control and treated cells, reflecting the ability of δ-tocotrienol to exert a cytostatic or a cytotoxic effect on melanoma-initiating cells, respectively.

Western blot analysis

Estrogen receptors

To assess the expression of estrogen receptors in human melanoma cell lines, cells were harvested and lysed in RIPA buffer (0.05 M Tris-HCl pH 7.7, 0.15 M NaCl, 0.8% SDS, 10 mM EDTA, 100 µM $NaVO_4$, 50 mmol/L NaF, 0.3 mM PMSF, 5 mM iodoacetic acid) containing leupeptin (50 µg/ml), aprotinin (5µl/ml) and pepstatin (50 µg/ml). Protein concentration in lysates was determined using the BCA method.

Protein extracts (20-30 µg) were resuspended in reducing Sample buffer (Bio-Rad) and heated at 95°C for 5 min. Following electrophoretic separation by 10% acrylamide SDS-PAGE, proteins were transferred onto nitrocellulose membranes. Membranes were blocked prior to incubation at 4 °C overnight with the primary antibodies: rabbit polyclonal antibody (1:1000; clone H-150, Santa Cruz Biotechnology) and mouse monoclonal antibody (1:500; clone 14C8, Abcam) for ERβ; rat monoclonal antibody (1:1000; clone H222, Thermo Scientific) for ERα. Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents (GE Healthcare, Life Sciences).

Cell cycle-related proteins

To investigate the effects of ERβ activation on the expression of cell cycle and apoptosis-related proteins, BLM cells were plated ($5x10^5$ cells/dish) in 10-cm dishes, in standard culture conditions, for 48 hours. Medium was then changed to phenol red free medium supplemented with 10% charcoal stripped FBS and treated with DPN (10^{-8} M) or vehicle for 24, 48, or 72 hours.

Adherent and floating cells were harvested and lysed in RIPA buffer (0.05 M Tris-HCl pH 7.7, 0.15 M NaCl, 0.8% SDS, 10 mM EDTA, 100 µM $NaVO_4$, 50 mmol/L NaF, 0.3 mM PMSF, 5 mM iodoacetic acid) containing leupeptin (50 µg/ml), aprotinin (5µl/ml) and pepstatin (50 µg/ml). Protein concentration in lysates was determined using the BCA method.

Protein preparations were then processed for Western blotting, as described above, and following electrophoretic separation by 10-15% acrylamide SDS-PAGE proteins

were transferred onto nitrocellulose membrane. After blocking, membranes were incubated with the following primary antibodies: cyclin D1 mouse monoclonal antibody (1:2000; clone DCS-6), cyclin D3mouse monoclonal antibody (1:1000; clone DCS22), $p21^{Waf1/Cip1}$ rabbit monoclonal antibody (1:1000; clone 12D1), $p27^{Kip1}$ rabbit monoclonal antibody (1:1000; clone D69C12), CDK4 rabbit monoclonal antibody (1:1000; clone D9G3E), CDK6 mouse monoclonal antibody (1:2000, clone DCS83), caspase-3 rabbit monoclonal antibody (1:500, clone 8G10), and cleaved caspase-3 rabbit monoclonal antibody (Asp175; 1:500, clone 5A1E). All these primary antibodies were from Cell Signaling Technology. Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents (GE Healthcare, Life Sciences).

ER stress markers and apoptosis-related proteins

To investigate the effects of δ-tocotrienol on the expression of ER stress and apoptosis-related proteins, BLM and A375 cells were plated ($5x10^5$ cells/dish) in 10-cm dishes, in standard culture conditions, for 48 hours. Cells were then treated with δ-tocotrienol (20 μg/ml) or vehicle for 1, 6, 18, 24 or 48 hours. Positive control of ER stress induction was obtained treating cells with 1 μM Thapsigargin, a selective inhibitor of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA).

Adherent and floating cells were harvested and lysed in RIPA buffer as previously described, and protein concentration in lysates was determined using the BCA method.

Protein preparations were then processed for Western blotting, as described above, and following electrophoretic separation by 7.5, 10, 12 or 15% acrylamide SDS-PAGE proteins were transferred onto nitrocellulose membrane. After blocking, membranes were incubated with the following primary antibodies: BiP rabbit monoclonal antibody (1:500; clone C50B12), PERK rabbit monoclonal antibody (1:1500; clone D11A8), IRE1α rabbit monoclonal antibody (1:1000; clone 14C10), PDI rabbit monoclonal antibody (1:1000; clone C81H6), ATF4 rabbit monoclonal antibody (1:1000; clone D4B8), CHOP mouse monoclonal antibody (1:1000; clone L63F7), ERO1-Lα rabbit antibody (1:1500), caspase-4 rabbit antibody (1:1000), cleaved caspase-3rabbit monoclonal antibody

(Asp175; 1:500, clone 5A1E), PARP rabbit antibody (1:1000). All these primary antibodies were from Cell Signaling Technology. Moreover, the activation of the intrinsic apoptosis pathway was investigated by incubating membranes with Bcl-2 mouse monoclonal antibody (1:1000; clone C-2, Santa Cruz Biotechnology) and Bax mouse monoclonal antibody (1:500; clone 6A7, Santa Cruz Biotechnology).
Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents (GE Healthcare, Life Sciences).

Cancer stem cell markers

To investigate the expression of cancer stem cell markers in the whole cell population, adherent cells were harvested and lysed in RIPA buffer as previously described. Protein extracts from A375 melanospheres were obtained collecting spheres through culture medium centrifugation (100xg), and melanospheres were then lysed in RIPA buffer. Protein concentration in lysates was determined using the BCA method.
Protein preparations were then processed for Western blotting, as described above, and following electrophoretic separation by 7.5-10% acrylamide SDS-PAGE proteins were transferred onto nitrocellulose membrane. After blocking, membranes were incubated with the following primary antibodies: CD271 rabbit monoclonal antibody (1:1000, clone D4B3), CD44 mouse monoclonal antibody (1:1000, clone 156-3C11). Both antibodies were from Cell Signaling Technology. Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents (GE Healthcare, Life Sciences).

In each experiment, actin, β-tubulin or GAPDH expression were evaluated as loading control, using the actin goat polyclonal antibody (1:1000; I-19, Santa Cruz Biotechnology), β-tubulin mouse monoclonal antibody (1:1000, clone TUB2.1, Sigma-Aldrich), and GAPDH rabbit monoclonal antibody (1:2000, Santa Cruz Biotechnology). Detection was done using horseradish peroxidase-conjugated secondary antibodies, and enhanced chemiluminescence ECL-Prime reagents (GE Healthcare, Life Sciences).

Immunofluorescence assay

To assess the localization of ERβ in human melanoma cells after activation with E_2 or DPN, BLM cells ($2x10^4$ cells) were seeded on 13-mm diameter coverslips in DMEM complete medium. After 48 hours, the medium was replaced with phenol red free medium supplemented with 10% charcoal stripped FBS. Cells were then treated with either DPN or E_2 (10^{-8} M) for 24 hours, fixed with 3% paraformaldehyde in 2% sucrose-PBS for 15 min and permeabilized with 0.2% PBS/Triton buffer (1 mM PBS, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 20 min at room temperature and stained with the primary rabbit anti-human ERβ polyclonal antibody (1:50; clone H-150, Santa Cruz Biotechnology), followed by FITC-conjugated goat anti-rabbit secondary antibody Alexa Fluor 488 (Molecular Probes Inc.). DAPI nuclear staining was also performed (Sigma-Aldrich), to verify the nuclear translocation of ERβ.

To assess the localization of ER stress markers after treatment with δ-tocotrienol, BLM and A375 cells were seeded at $2x10^4$ cells per well on 13-mm diameter coverslips in DMEM complete medium. After 48 hours, cells were treated with δ-tocotrienol (20 μg/ml) for 6, 18 or 24 hours, fixed with 3% paraformaldehyde in 2% sucrose-PBS for 15 min and permeabilized with 0.2% PBS/Tryton buffer (1 mM PBS, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 20 min at room temperature and stained with ATF4 rabbit monoclonal antibody (1:100; clone D4B8) or CHOP mouse monoclonal antibody (1:1000; clone L63F7), followed by FITC- or TRITC-conjugated secondary antibody Alexa Fluor 488 or 594 (Molecular Probes Inc.) and DAPI.

To assess the release of cytochrome *c* from mitochondria after treatment with δ-tocotrienol, A375 and BLM cells were seeded as previously described, followed by 24 hours treatment with δ-tocotrienol. Before fixation, cells were incubates for 30 minutes with Mitotracker (Molecular Probes Inc.), a mitochondrial-specific marker conjugates with TRITC, then cells were fixed and stained with mouse monoclonal antibody against cytochrome *c* (1:50; clone 7H8, Santa Cruz Biotechnology), followed by FITC-conjugated secondary antibody.

To assess the expression and localization of melanoma stem cell markers in adherent cells, A375, WM115 and BLM cells were seeded at $3x10^4$ cells per well on 13-mm

diameter coverslips in DMEM complete medium. After 72-96 hours cells were fixed with 4% paraformaldehyde in 2% sucrose-PBS for 15 min, permeabilized with 0.2% PBS/Triton buffer (1 mM PBS, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100) for 20 min at room temperature and stained with CD271 rabbit monoclonal antibody (1:1600; clone D4B3, Cell Signaling Technology), CD44 mouse monoclonal antibody (1:500; clone 156-3C11, Cell Signaling Technology), ABCB5 mouse monoclonal antibody (1:1000, clone 5H3C6, LifeSpan BioSciences, Inc.) and CD133 mouse monoclonal antibody against (Prominin-1, 1:1000, clone 17A6.1, EMD Millipore Corporation), followed by FITC- or TRITC-conjugated secondary antibody Alexa Fluor 488 or 594 (Molecular Probes Inc.) and DAPI.

To assess the expression and localization of melanoma stem cells markers in melanospheres, A375 melanospheres were collected through recovery and low speed centrifugation of culture medium. Spheres were then rinsed and fixed with 4% paraformaldehyde, maintaining them in suspension with gentle pipetting. Fixed melanospheres were centrifuged and rinsed with PBS. After 20 minutes block with FBS, melanospheres were incubated with primary antibodies: CD271 rabbit monoclonal antibody (1:1600; clone D4B3, Cell Signaling Technology), CD44 mouse monoclonal antibody (1:500; clone 156-3C11, Cell Signaling Technology) and Oct-4 rabbit antibody (1:100, EMD Millipore Corporation), followed by FITC- or TRITC-conjugated secondary antibody Alexa Fluor 488 or 594 (Molecular Probes) and DAPI. All the incubations were performed keeping spheres in gentle agitation.

Labelled cells were examined under a Zeiss Axiovert 200 microscope with a 63x/1.4 objective lens linked to a Coolsnap Es CCD camera (Roper Scientific-Crisel Instruments).

In vivo experiments

Mouse melanoma xenograft model

Female immunodeficient CD1-nu/nu mice, at 6 weeks of age, were purchased from Charles River, under the guidelines established by the University of L'Aquila regulations officially authorized by the Italian Ministry of Health. The official authorization to

perform these experiments has been requested by the University of L'Aquila (Dr. C. Festuccia) to the Italian Ministry of Health, according to the Directive 2010/63/EU incorporated into the Italian law with the D.Lgs. 26/2014.

All mice were anesthetized with a mixture of ketamine (25 mg/ml) and xylazine (5 mg/ml) and received subcutaneous flank injections (2 each) of 10^6 A375 or BLM cells. Tumor growth was assessed twice a week by measuring tumor diameters with a Vernier caliper (length x width). The volume of the tumor is expressed in mm^3 according to the formula $4/3\ \pi r^3$. At about 10 days after tumor injection, mice with tumor volume 0.5-0.8 cm^3 were retained and randomly divided into two groups receiving placebo (control, olive oil) and tocotrienols (in olive oil), and were orally treated with δ-tocotrienol (100 mg/kg, 5 days/week;) for up to 4 weeks. At the end of the experiments (35 days after the start of treatments) animals were sacrificed by carbon dioxide inhalation, tumors were subsequently surgically removed.

Evaluation of treatment response

The following parameters were used to quantify the antitumor effects upon different treatments [175]:

1- Tumor volume measured during and at the end of experiments;
2- Tumor weight measured at the end of the experiments;
3- Time to tumor progression (TTP).

Statistical analysis

When appropriate, data were analysed by Dunnet's or Bonferroni's test, after one-way analysis of variance. A *P* value <0.05 was considered statistically significant.

RESULTS

Results

Expression of ERs in human melanoma cell lines

The expression of ERβ in human melanoma cell lines was analyzed by Western blot assay. Since no validated antibodies exist for the detection of this receptor, we utilized two primary antibodies, that recognize the N-terminal domain of the protein: H-150 (Santa Cruz Biotechnology) and 14C8 (Abcam). First, in order to obtain an appropriate positive control, ERβ was evaluated in BLM melanoma cells engineered to overexpress the receptor protein. Fig. 10A shows that a protein band, corresponding to the molecular weight of 59 kDa, is expressed in BLM cells either in normal culture conditions (C) or in the presence of Lipofectamine (Lipo), at 24-72 hours, indicating the basal expression of ERβ in BLM cells. More importantly, Fig. 10A also shows that the expression levels of this protein band are sharply increased in BLM cells overexpressing the receptor (ERβ) at 24 hours after transfection, and slightly decrease at 48 and 72 hours. These data, confirmed by the two different antibodies, evidenced the specificity of the antibodies used in recognizing the 59 kDa ERβ1 protein.

ERβ expression was then analyzed, utilizing the two primary ERβ antibodies (H-150 and 14C8), in a panel of human melanoma cell lines. Fig. 10B shows that a specific band, corresponding to the molecular weight of 59 kDa, is expressed in BLM (lane 3; confirming the data reported in Fig. 10A), A375 (lane 4), WM115 (lane 5) and WM1552 (lane 7) human melanoma cells. Fig. 10B also shows that in the human IGR-39 melanoma cell line (lane 6) the receptor is expressed at almost undetectable levels. The molecular weight of the protein band detected in the melanoma cell lines corresponds to that found in human MCF-7 breast cancer cells (positive control; lane 1). As expected, no band of this size could be detected in the HEK293 cells (negative control; lane 2), confirming previous observations [176]. It should be underlined that, when evaluated with the 14C8 primary antibody, the level of expression of the receptor in MCF-7 cells was found to be low, and this agrees with previous data in the literature [177, 178]. On the other hand, in these cells the receptor seems to be

expressed at higher levels when evaluated with the H-150 antibody. At present, the reason for this discrepancy is unclear; however, it might be due to a different degree of specificity of the two antibodies.

No band corresponding to ERα (67 kDa) could be detected in any of the melanoma cell lines analyzed (Fig. 10C, lanes 2-6), confirming previous observations [124]; as expected, this estrogen receptor subtype was expressed at high levels in human MCF-7 breast cancer cells (positive control; Fig. 1, lane 1). These data indicate that ERβ, but not ERα, is the estrogen receptor subtype expressed in most human melanoma cells.

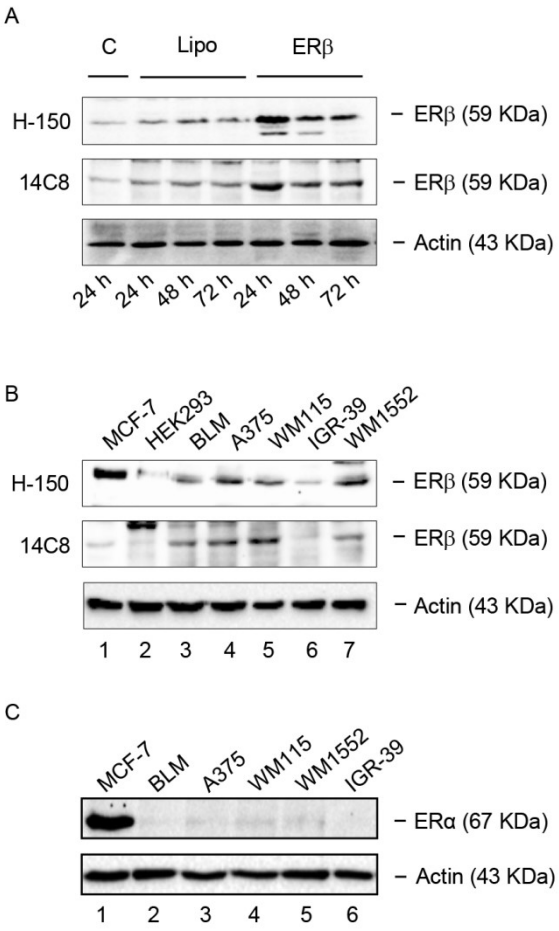

Fig. 10. ERβ, but not ERα, is expressed in human melanoma cells. (A) As a positive control, the expression of ERβ was evaluated by Western blot analysis in human BLM melanoma cells engineered to overexpress the receptor subtype protein, utilizing two primary antibodies: H-150 (Santa Cruz) and 14C8 (Abcam). A band corresponding to the receptor protein (59 kDa) was detected in basal conditions, both in control (C) and in Lipofectamine (Lipo) treated BLM cells. As expected, the intensity of this band was found to be significantly increased after ERβ overexpression (24-72 h), with the highest level of expression at 24 h. **(B)** By Western blot analysis, utilizing the two primary antibodies H-150 and 14C8, ERβ was found to be expressed at high levels in human BLM, A375, WM115, WM1552 melanoma cell lines (lanes 3, 4, 5, 7), while the human IGR-39 melanoma cell line expressed the receptor at almost undetectable levels (lane 6). ERβ was also expressed in human MCF-7 breast cancer cells, utilized as a positive control (lane 1), but it was not expressed in the human HEK293, utilized as a negative control. **(C)** On the other hand, all the human melanoma cells lines tested (lanes 2-6) did not express the ERα receptor isoform, which was expressed only in the control cell line (MCF-7, lane 1). β-actin was utilized as a loading control.

ERβ agonists inhibit the proliferation of BLM melanoma cells

Experiments were first performed to investigate the effects of ERβ activation on the growth of BLM melanoma cells, a well characterized cell line, previously utilized in Limonta's laboratory to study the antitumor activity of gonadotropin-releasing hormone receptors in melanoma [179, 180]. The selective ERβ agonist DPN decreased BLM cell proliferation at the concentrations of 10^{-9} and $5x10^{-9}$ M, being significantly effective at the dose of 10^{-8} M (Fig. 11A). This significant effect was followed by a decline at concentrations of $5x10^{-8}$ and 10^{-7} M. Accordingly, the endogenous estrogenic ligand E_2 exerted a significant antiproliferative effect on BLM cell proliferation at the concentration of 10^{-8} M (Fig. 11B). The antiproliferative activity of both DPN and E_2 (10^{-8} M) was completely counteracted by cotreatment of the cells with the ER antagonist ICI-182,780 (10^{-6} M) (Fig. 11C).

Experiments were also performed with different ERβ agonists (KB1, KB2, KB4). We found that all these compounds significantly inhibit BLM cell proliferation at the concentration of 10^{-8} M (Fig. 11D); the dose-response curve obtained after treating the cells with different concentrations of KB1 was similar to that obtained with DPN (Fig. 11E *vs.* Fig. 11A). This effect was completely counteracted by the ER antagonist ICI-182,780 (10^{-6} M) (Fig. 11F).

These data demonstrate that ERβ activation is associated with antiproliferative activity in BLM melanoma cells, with 10^{-8} M being the most effective dose, as previously reported for different tumor cells [181-184]. A curve of the dose-response effect of ERβ agonists on cancer cell proliferation, similar to that here shown, has been previously reported for cholangiocarcinoma and mesothelioma cells [182, 183]. On the basis of these results, the concentration of 10^{-8} M was selected for the subsequent studies.

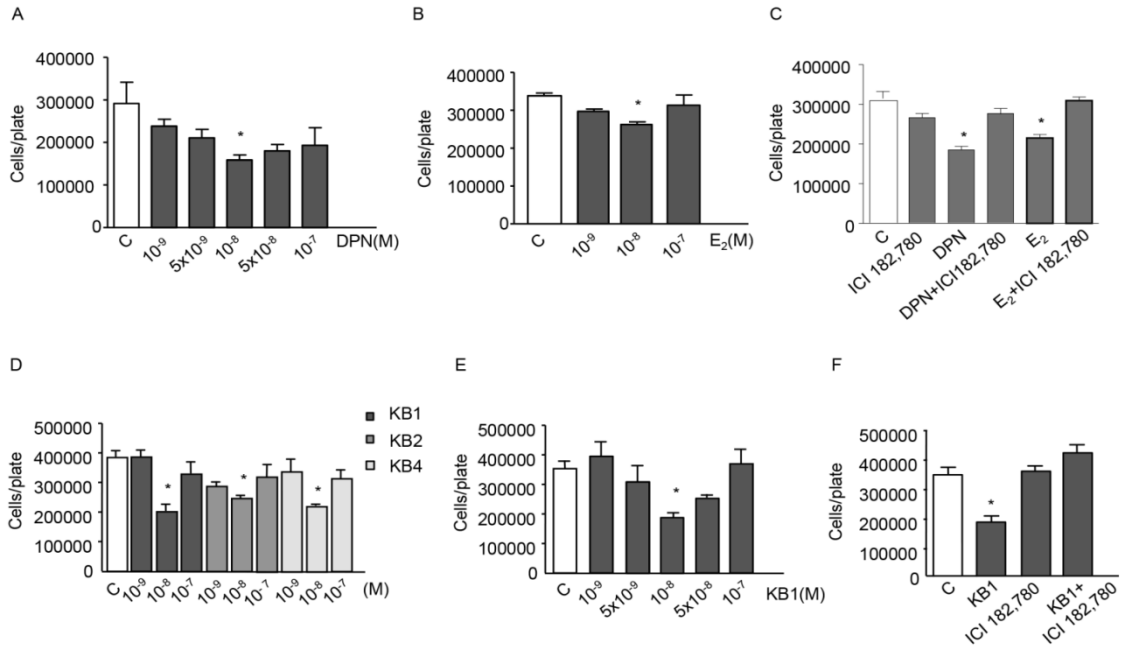

Fig. 11. ERβ agonists significantly and specifically inhibit the proliferation of BLM melanoma cells. (A) BLM cells were treated with different doses of the classical ERβ agonist DPN every 48 h for three times. DPN significantly decreased cell proliferation at the dose of 10^{-8} M. **(B)** Similar results were obtained when the cells were treated with E_2. **(C)** The antiproliferative effect of both ERβ ligands (10^{-8} M) was found to be specific since it was completely abrogated by cotreatment of the cells with the ER antagonist ICI 182,780 (10^{-6} M). **(D)** BLM cells were treated with KB1, KB2, or KB4 (10^{-9}, 10^{-8}, 10^{-7} M) every 48 hours for three times. All three ERβ ligands significantly reduced cell proliferation at the dose of 10^{-8} M. **(E)** BLM cells were treated with KB1, at different doses (10^{-9}-10^{-7} M). The ERβ agonists decreased cell growth, being significantly effective at the dose of 10^{-8} M. **(F)** The antiproliferative activity of KB1 (10^{-8} M) was found to be specific since it was completely abrogated by cotreatment of the cells with the ER antagonist ICI 182,780. Data represent mean values ± SEM. **P*<0.05. C, controls.

Activation of ERβ induces its cytoplasmic-to-nuclear translocation and transcriptional activity in BLM cells

Experiments were performed to verify ERβ functionality in BLM melanoma cells, and in particular to verify if ERβ might function according to the classical model of estrogen action [88].

Immunofluorescence analyses were then performed in order to firstly assess the ERβ localization in BLM cells: we could show that, in basal conditions, most of the ERβ staining was confined in the cytoplasm (Fig. 12A). Treatment of melanoma cells (24 hours) with both DPN and E_2 (10^{-8} M) induced its nuclear translocation (Fig. 12A).

Then, we analyzed the effects of ERβ ligands on the transcriptional activity of the receptor in melanoma cells. BLM cells were transfected with the reporter plasmid encoding the firefly luciferase reporter gene under the control of the ERE (pVERE-tk-Luc), to evaluate the transcriptional activity of ERβ, and the reporter plasmid encoding the β-galactosidase (pCMVβ), as the internal control plasmid. Fig. 12B shows that treatment of BLM cells with either DPN or E_2 for 24 hours significantly increased the transcriptional activation of the ERE-Luc reporter plasmid (normalized for β-galactosidase), as measured by luciferase activity induction, indicating that ERβ is associated with the classical transcriptional activity at the nuclear level.

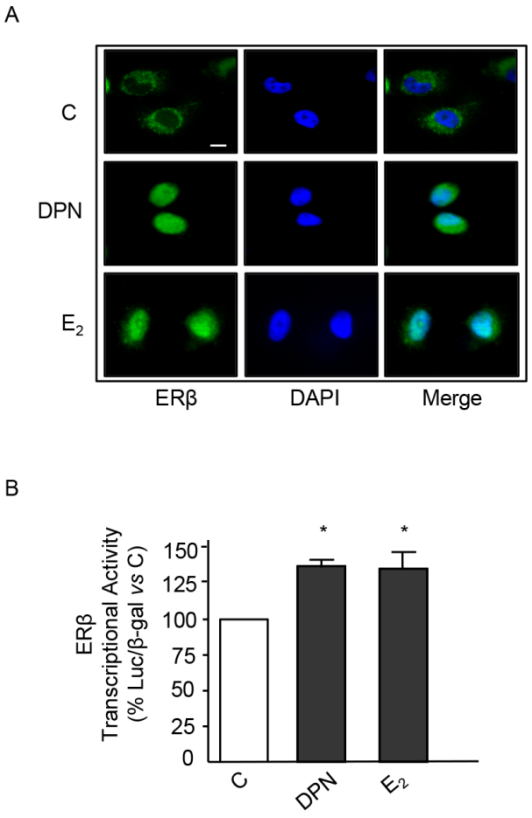

Fig. 12. ERβ ligands trigger cytoplasmic-to-nuclear translocation of ERβ and induce its transcriptional activity in BLM melanoma cells. (A) Immunofluorescence assay of ERβ intracellular localization. In control BLM melanoma cells, ERβ is mainly localized at the cytoplasmic level. Treatment of the cells with either DPN or E_2 (10^{-8} M, for 24 h) induces ERβ translocation into the nucleus. Scale bar, 5 μm. **(B)** The transcriptional activity of the ERβ protein in BLM cells was analyzed using the pVERE-tk-LUC plasmid (cotransfected with pCMVβ). The results were normalized for β-galactosidase activity. Treatment of the cells with either DPN or E_2 (10^{-8} M, for 24 h) significantly increased ERβ transcriptional activity. Data represent mean values ± SEM. **P*<0.05. C, controls.

ERβ agonists affect the expression of cell cycle-related proteins in BLM cells

Estrogens have been shown to affect cancer cell growth through the regulation of proteins involved in cell cycle progression [185]. Experiments were performed to investigate whether ERβ agonists might affect melanoma cell proliferation through alteration of the expression of cell cycle-related proteins. BLM cells were treated with the ERβ agonist DPN (10^{-8} M) for different time intervals (24-72 hours). By Western blot assay, we could demonstrate that treatment with DPN induced a significant reduction in the expression of G1 cyclins, such as cyclin D1 and D3 (at 72 hours of treatment), and a significant increase in the expression of the CDK inhibitor p27 (at 48 hours of treatment) (Fig. 13A). On the other hand, the expression of the cyclin D partners CDK4 and CDK6 was not modified by the treatment; the CDK inhibitor p21 was found to be expressed at undetectable levels in basal condition in BLM cells and its expression was not affected by DPN treatment (Fig. 13A).

Experiments were then performed to investigate whether the activation of ERβ might induce cell death through the apoptosis process. Interestingly, DPN did not modify the expression of procaspase-3 as well as that of the cleaved (active) form of caspase-3 (Fig. 13B). Taken together, these results indicate that ERβ activation in melanoma cells decreases cell proliferation, through the modulation of the expression of proteins involved in the G1-S progression of the cell cycle, and that the apoptosis pathway is not involved in this activity.

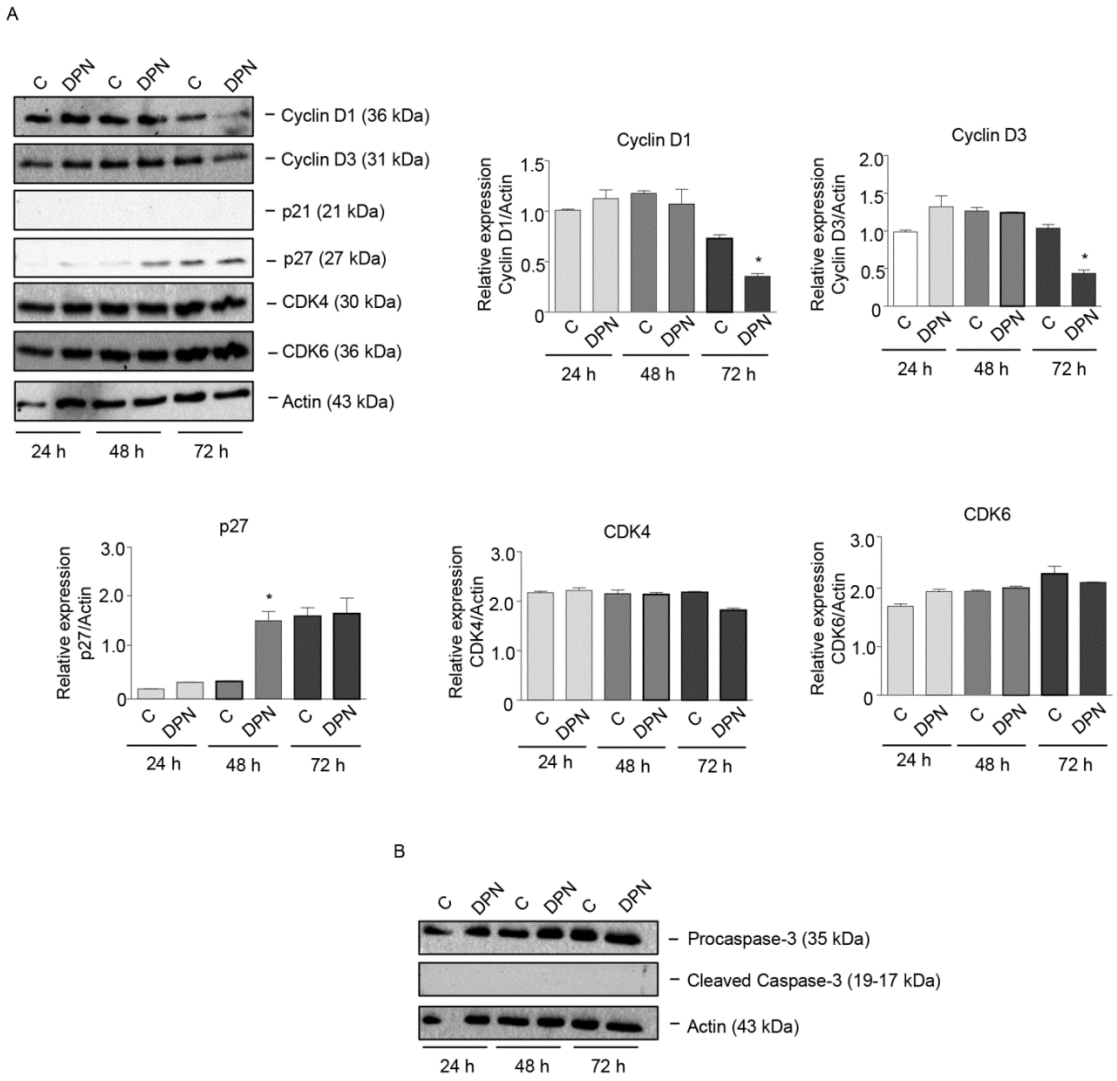

Fig. 13. The specific ERβ ligand DPN affects the expression of cell cycle-related proteins in BLM melanoma cells. BLM cells were treated with DPN (10^{-8} M) for 24, 48, or 72 h. Western blot analysis was performed on whole cell extracts by using specific antibodies against cell cycle-related proteins, such as cyclin D1, cyclin D3, p21, p27, CDK4, CDK6 **(A)**, procaspase-3 and cleaved caspase-3 **(B)**. Actin expression was evaluated as a loading control. The treatment with DPN reduced the expression of cyclin D1 and cyclin D3 and increased that of p27, while the levels of cleaved (active) caspase-3 were not affected by the treatment. A statistical evaluation has been performed on the densitometric analysis of the results obtained from the three Western blot experiments performed on cell cycle-related proteins **(A)**.

ERβ activation induces global DNA methylation reprogramming in BLM cells

Experiments were first carried out to analyze the global DNA methylation status of human BLM melanoma cells when compared to that of human melanocytes. To this purpose, a restriction enzymatic assay, utilizing the two methylation sensitive restriction enzymes MspI and HpaII, was performed. These enzymes recognize the same tetranucleotide sequence (5'-CCGG-3') but display different sensitivity to DNA methylation. In particular, MspI does not cut when the external cytosine is methylated while HpaII does not cut when any of the two cytosines is methylated [186, 187], so that differential methylation patterns of DNA can be evidenced. Following DNA digestion, a CG site-directed PCR was performed, and only when CG sites are intact, primers can bind to DNA and allow the amplification of the sequence. In summary, bands in agarose gel electrophoresis indicate amplification, that means a higher methylation of DNA. Fig. 14A shows that BLM cells are globally hypomethylated when compared to human melanocytes (hMEL), when both MspI and HpaII restriction enzymes are utilized. These data confirm that melanoma cells are characterized by an aberrant global DNA hypomethylation, which is known to be associated with genome instability.

We then evaluated whether activation of ERβ might affect the DNA methylation status of melanoma cells. BLM cells were treated with either DPN or E_2 (10^{-8} M) for 24 or 48 hours; the DNA methylation status was analyzed as described above. Fig. 14B shows that DPN significantly increased DNA methylation at 24 and 48 hours of treatment, when the MspI restriction enzyme was utilized. On the other hand, E_2 significantly increased the methylation degree of CG-rich regions at 24 hours of treatment, when both restriction enzymes were utilized. These data indicate that ERβ activation reverts the DNA hypomethylation status in melanoma cells and suggest that different ERβ ligands might increase the methylation of the different cytosines of the CG-rich regions (internal *vs.* external) in a specific way.

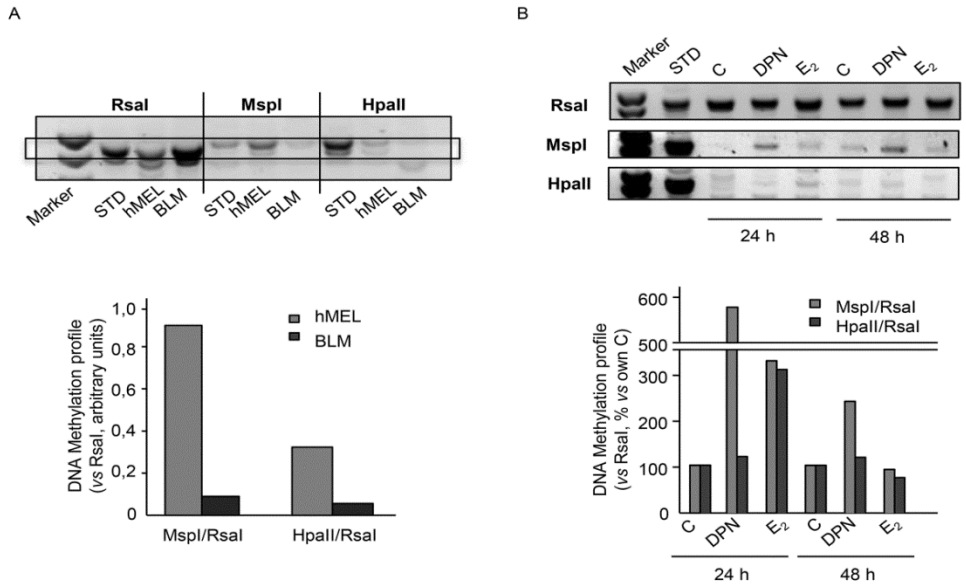

Fig. 14. ERβ activation induces global DNA methylation reprogramming in BLM melanoma cells. (A) Preliminary experiments were carried out to analyze the global DNA methylation status of BLM cells when compared to that of human normal melanocytes (hMEL). To this purpose, a restriction enzymatic assay was employed. For each DNA sample, two restriction digests were performed: one with RsaI and MspI, and one with RsaI and HpaII. RsaI is methylation insensitive, while MspI and HpaII are sensitive to DNA methylation and are able to cut only unmethylated restriction sites. The digests were then amplified by PCR. Data are expressed as the MspI/RsaI or HpaII/RsaI ratios relative to the intensity of the bands. BLM melanoma cells were found to be globally hypomethylated when compared to normal melanocytes, when both MspI and HpaII restriction enzymes were utilized. One representative of three different experiments, which gave similar results, is reported. **(B)** Experiments were performed to evaluate whether activation of ERβ might affect the global DNA hypomethylation status observed in melanoma cells. BLM cells were treated with either DPN or E_2 (10^{-8}M) for 24 or 48 h; the DNA methylation status was then evaluated as described above. Both DPN (at 24 and 48 h) and E_2 (at 24 h) increased the DNA methylation profile of BLM cells, indicating that ERβ activation reverts the DNA hypomethylation status in melanoma cells. One representative of three different experiments, which gave similar results, is reported. C, controls.

ERβ agonists differentially affect the proliferation of melanoma cell lines

Based on the results obtained in BLM melanoma cells (expressing the ERβ receptor subtype and harboring the NRAS mutation), further experiments were performed to assess the effects of ERβ activation on the proliferation of different melanoma cell lines, either lacking the expression of ERβ or expressing ERβ while harboring different oncogenic mutations (*e.g.*, BRAF). Specifically, the effects of ERβ agonists were assessed on the proliferation of the following human melanoma cell lines: IGR-39 cells (expressing almost undetectable levels of ERβ), A375 and WM1552 cells (expressing ERβ and harboring the BRAF V600E mutation), and WM115 cells (expressing ERβ and harboring the rare BRAF V600D mutation). IGR-39, A375 and WM1552 cells were treated with DPN (10^{-9}-10^{-7} M) while WM115 cells were treated with DPN, E_2 and KB1 (10^{-10}-10^{-7} M), as described for BLM cells. As expected, we found that DPN does not affect the proliferation of IGR-39 cells, lacking ERβ expression (Fig. 15A). Unexpectedly, and interestingly, the ERβ agonist also failed to affect the growth of A375 and WM1552 melanoma cells, expressing the receptor subtype (Fig. 15B and C). On the other hand, the proliferation of WM115 cells was reduced by the treatment with DPN, E_2 and KB1, with a dose-response curve similar to that observed in BLM cells (Fig. 15D). Taken together, these results indicate that ERβ activation differentially affects the proliferation of melanoma cell lines. The reasons for these observations are still unclear; however, we might speculate that the efficacy of ERβ agonists in reducing melanoma growth might depend not only on the presence of the receptor but also on other particular features of each melanoma, such as the oncogenic mutation status (NRAS, BRAF) of the tumor.

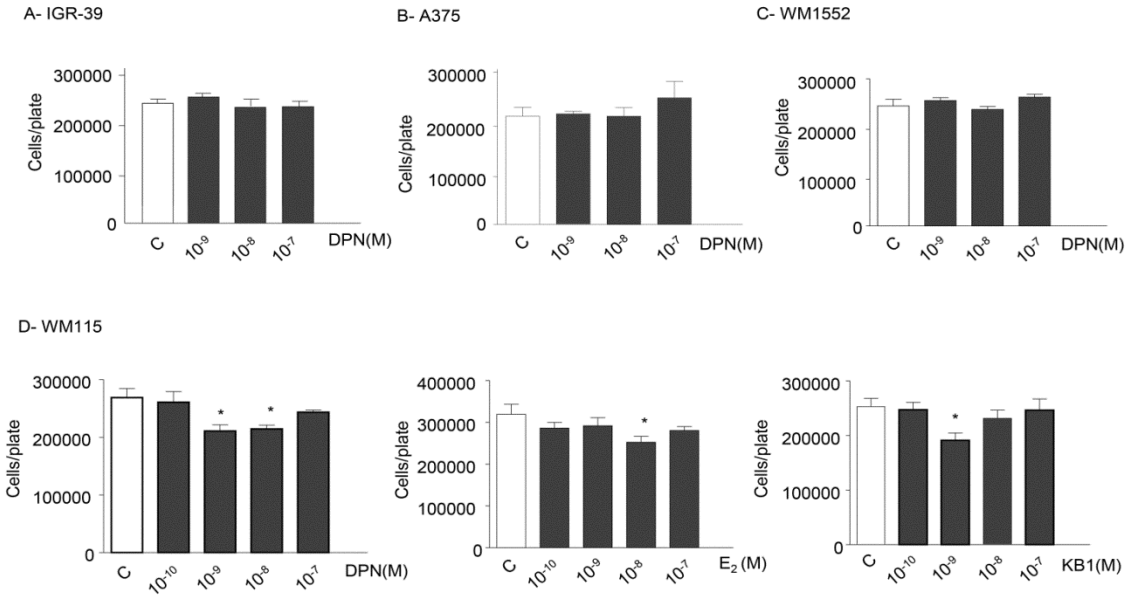

Fig. 15. ERβ agonists differentially affect the proliferation of melanoma cell lines. (A) IGR-39, A375 and WM1552 melanoma cells were treated with DPN (10^{-9}-10^{-7} M) every 48 h for three times. No effect on cell proliferation could be observed in any cell line tested. **(B)** WM115 cells were treated with DPN, E_2, or KB1 (10^{-10}-10^{-7} M) every 48 h for three times. DPN was significantly effective in decreasing cell proliferation at the doses of 10^{-9} and 10^{-8} M. On the other hand, both E_2 and KB1 significantly reduced cell proliferation at the dose of 10^{-8} M. Data represent mean values ± SEM. **P*<0.05. C, controls.

Expression of ERβ isoforms in melanoma cell lines

So far, five alternatively spliced transcript variants of the ERβ gene have been described (ERβ1-5) in humans [188]. ERβ wild type, also referred to as ERβ1, is the main variant and ERβ2 and ERβ5 are the most studied splice variants [189]. The expression of these variants has been shown to be tissue-specific and to differentially modulate E_2 signaling [190].

As discussed above, the reasons for the differential effects of ERβ agonists on the proliferation of melanoma cell lines are still unclear. In addition to the proposed correlation with specific oncogenic mutations, these effects might also be related to the differential expression of ERβ isoforms in the various melanoma cell lines. Based on these observations, by quantitative RT-PCR we analyzed the expression of ERβ1, ERβ2, and ERβ5 in BLM, A375 and WM115 melanoma cell lines.

Fig. 16 shows that the pattern of expression of the ERβ isoforms is similar in BLM and in WM115 cells, with ERβ1 and ERβ5 being expressed at similar levels and ERβ2 showing a higher level of expression. On the other hand, in A375 cells, both ERβ2 and 5 are expressed at higher levels than the ERβ1 isoform.

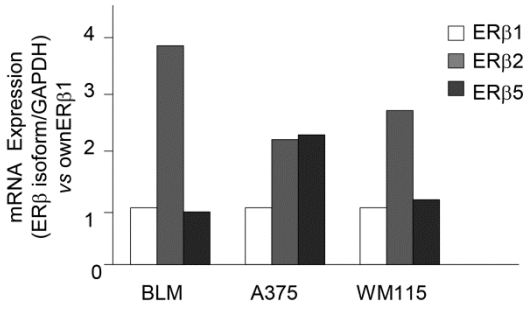

Fig. 16. ERβ isoforms (1, 2, and 5) are differentially expressed in melanoma cell lines. The relative expression of ERβ1, 2, and 5 isoforms was evaluated by quantitative PCR, utilizing specific sets of primers. BLM and WM115 cells show a similar expression of ERβ1 and 5, while expressing higher levels of ERβ2. On the other hand, a high expression of both ERβ2 and 5 isoforms (when compared to ERβ 1) is observed in A375 cells.
One representative of three different experiments, which gave similar results, is shown.

Analysis of ERβ transactivation upon δ-tocotrienol treatment

Several natural compounds have been reported to exert estrogenic effects, preferentially through the activation of the estrogen receptor β subtype [129], these compounds have been defined as "phytoestrogens". Despite tocotrienols are not ascribed to this class of natural molecules, different reports demonstrated a direct interaction between ERβ and δ-tocotrienol, through which this compound may exert its benefits for health, independently from its antioxidant activity [168-170].

First of all, the antitumor effect of δ-tocotrienol was assessed through MTT assay on BLM cells, where ERβ exerts a significant antiproliferative activity, as previously described. Cells were treated with different doses (5, 10, 15 or 20 μg/ml) of this compound, for 24 or 48 hours, and a significant, dose-dependent, reduction in cell viability was observed (Fig. 17A).

To investigate whether such effect of δ-tocotrienol could be mediated by ERβ transcriptional activation, a luciferase-reporter assay was performed.

Fig. 17B shows that treatment of BLM cells with 10 or 20 μg/ml of δ-tocotrienol for 24 hours did not increase the transcriptional activation of the ERE-Luc reporter plasmid (normalized for β-galactosidase), in every given dose, indicating that, in BLM melanoma cells, δ-tocotrienol does likely not interact with the receptor nor it induces its genomic activity.

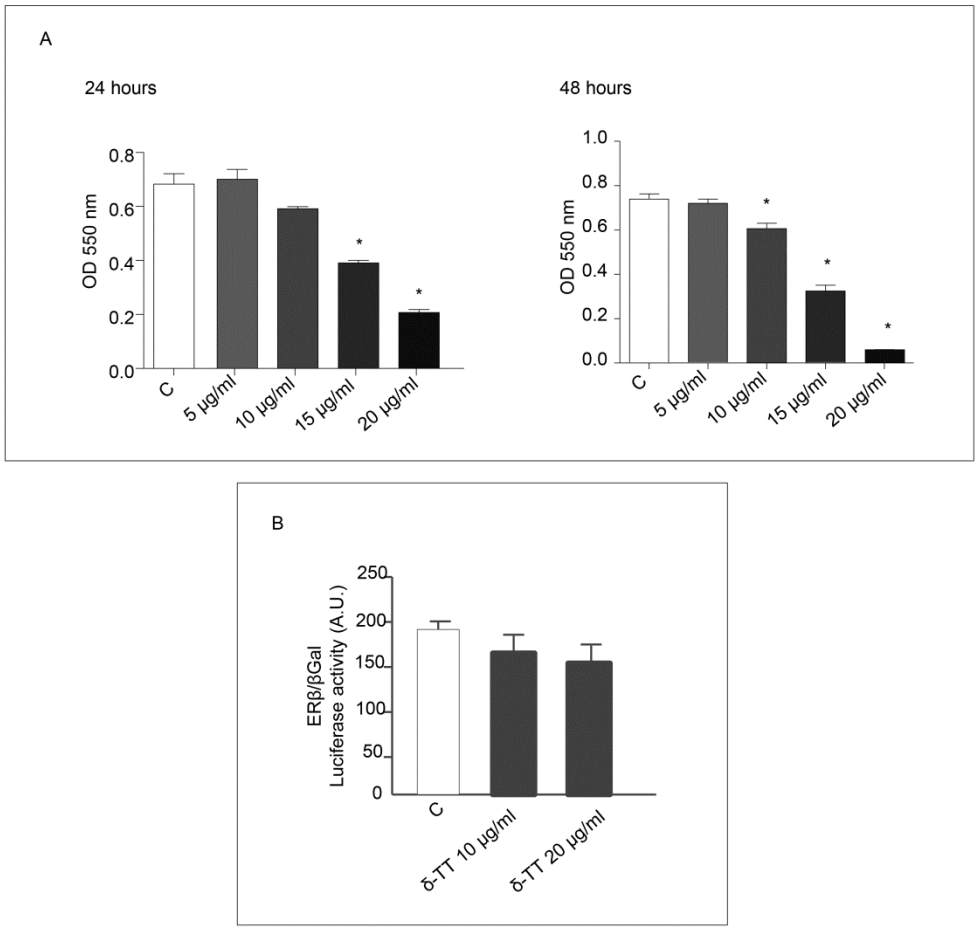

Fig. 17. δ-tocotrienol exerts an antitumor activity without inducing ERβ transactivation. (A) BLM cells were treated with different doses (5, 10, 15 or 20 µg/ml) of δ-tocotrienol or vehicle, and MTT assay was performed at 24 or 48 hours of treatment. A significant dose-dependent reduction of BLM cell viability was observed. Data represent mean values ± SEM. **P*<0.05. C, controls. **(B)** In order to verify if the antitumor effect of δ-tocotrienol is mediated by ERβ, BLM cells were transfected with the reporter plasmid encoding the firefly luciferase reporter gene under the control of the ERE (pVERE-tk-Luc), to evaluate the transcriptional activity of ERβ, and the reporter plasmid encoding the β-galactosidase (pCMVβ), as the internal control plasmid. Cells were then treated with 10 or 20 µg/ml of δ-tocotrienol for 24 hours, in the same experimental setting of the DPN-induced ERβ transactivation. Luciferase activity, normalized for β-galactosidase activity, revealed no significantly differences between treated and untreated cells, so δ-tocotrienol is not able to modify the transcriptional activation of the receptor, both at low and high doses. Data represent mean values ± SEM. C, controls

δ-tocotrienol induces a reduction of cell viability in different melanoma cell lines

It has been reported that tocotrienols possess anticancer activities in different type of tumors [143], through antiproliferative and proapoptotic effects. Moreover, our preliminary viability assay indicates that δ-tocotrienol exerts a significant antitumor effect on BLM melanoma cells. We then wanted to verify if this compound could be effective on different human melanoma cell lines. To this purpose, both ERβ-responders and -non responders cells were treated with different doses (5, 10, 15 or 20 μg/ml) of δ-tocotrienol for 24 hours. As shown in Fig. 18A, a significant, dose-dependent reduction in cell viability was observed in all melanoma cell lines considered (BLM, A375, WM115 and IGR-39), independently from the expression of ERβ and from its antiproliferative activity.

The effect of δ-tocotrienol was then compared with the standard chemotherapeutic agent dacarbazine, in cells that express ERβ, but harbor different Ras/Raf mutations. A375, BLM and WM115 cells were treated with different doses (10, 20, 50 or 100 μM) of dacarbazine for 48 hours, then MTT assay was performed. Except for WM115 cells, the chemotherapeutic agent induced a significant dose-response reduction in cell viability, even if in a lesser extent with respect to δ-tocotrienol treatment (Fig. 18B), considering that the dose of 20 μg/ml of δ-tocotrienol corresponds to a molarity of 50.4 μM.

BLM (N-Ras^{mut}, B-Raf^{wt}) and A375 (N-Ras^{wt}, B-Raf^{mut}) cells were then selected for subsequent studies, as models for the two most frequent mutations found in melanomas.

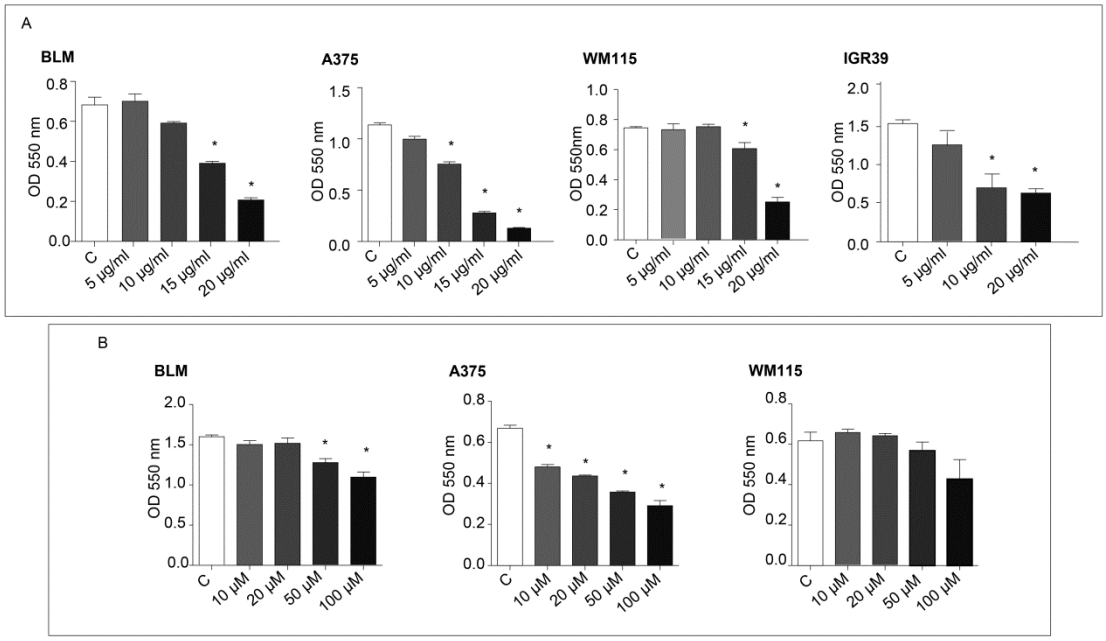

Fig. 18. δ-tocotrienol is more effective than dacarbazine in reducing melanoma cell viability. (A) BLM, A375, WM115 and IGR-39 cells were treated with vehicle or different doses (5, 10, 15 or 20 µg/ml) of δ-tocotrienol, for 24 hours. Then, MTT assay was performed in order to assess cell viability: a significant dose-dependent reduction in cell viability was observed for all melanoma cell lines, independently from the ERβ-expression and ERβ-responsiveness status. **(B)** BLM, A375 and WM115 cells were treated with vehicle or different doses (10, 20, 50 or 100 µM) of the standard chemotherapeutic agent dacarbazine, for 48 hours. Then, MTT assay was performed in order to assess cell viability. The chemotherapeutic drug induced a dose-dependent reduction in cell viability in BLM and A375 cells, even if in a lesser extent with respect to δ-tocotrienol. Conversely, WM115 cells were resistant to the drug, despite a non-significant trend in reducing cell viability was observed. Data represent mean values ± SEM. **P*<0.05. C, controls.

Human melanocytes viability is not affected by δ-tocotrienol treatment

Conventional chemotherapeutic agents are often associated with significant systemic toxicities, due to their aspecific activity on non-cancerous cells. Dacarbazine has been reported to induce mild nausea and vomiting, myelosuppression, flu-like syndrome, hepatotoxicity, alopecia, facial flushing, neurotoxicity and dermatological reactions.

In order to investigate if δ-tocotrienol exerts a cytotoxic effect on normal cells, and, more importantly, to assess its specific antitumor activity on melanoma cells, human primary melanocytes were seeded and treated with vehicle or with different doses (5, 10, 15 or 20 µg/ml) of the natural compound. MTT assay was then performed after 24 hours of treatment, and the effect on cell viability was compared with that of tumor cells, treated with the same doses of δ-tocotrienol (see Fig. 18A).

Fig. 19 shows that, in contrast to melanoma cells, melanocytes growth was not affected by δ-tocotrienol treatment, even at high doses, suggesting that it may selectively target tumor cells.

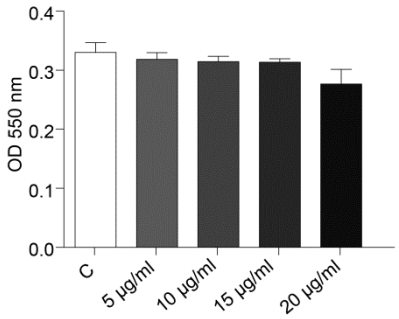

Fig. 19. δ-tocotrienol does not reduce melanocytes viability. Human normal melanocytes were treated with vehicle or different doses (5, 10, 15 or 20 µg/ml) of δ-tocotrienol, for 24 hours. Then, MTT assay was performed in order to assess cell viability: conversely to tumor cells, melanocytes viability was not affected by the treatment with the natural compound. Data represent mean values ± SEM. **P*<0.05. C, controls.

δ-tocotrienol exerts a cytotoxic/apoptotic activity on melanoma cells

Data in the literature supports the notion that tocotrienols might exert their antitumor effects both by inhibiting cell proliferation or by inducing cell death [144]-[146], [154]-[156].

Given the reduction in cell viability observed in melanoma cell lines, experiments were performed in order to investigate whether δ-tocotrienol might exert a cytostatic or a cytotoxic effect.

To this purpose, a colony formation assay was firstly conducted. This assay is based on the ability of single cells to form distinct colonies. BLM and A375 cells were treated with δ-tocotrienol (20 μg/ml) for 72 hours and then, after withdrawal of the treatment, cells were left to grow for 7-10 days, dependently of the cell line-specific proliferation rate, to assess: 1- the ability of the cells to proliferate forming colonies (dimensions of colonies); and 2- the survival of colony-forming cells (number of colonies).

Crystal violet staining evidenced that control cells were able to grow and to proliferate to form colonies, as shown in Fig. 20A. On the other hand, none of the treated cells survived upon δ-tocotrienol treatment, so that colony formation was completely prevented. This drastic reduction in the number of colonies indicated that δ-tocotrienol exerts a cytotoxic, rather than a cytostatic, effect on melanoma cells, inducing cancer cell death.

In order to confirm the δ-tocotrienol-induced cell death in BLM and A375 cells, a Trypan blue exclusion assay was performed. Trypan blue dye is a vital stain that is excluded by the intact membrane of living cells whereas it is included in dying cells, because of their higher cell membrane permeability.

Cells were treated with different doses of δ-tocotrienol (5, 10, 15 or 20 μg/ml) for 24-48 hours, dependently of the cell line-specific proliferation rate, then floating (dying) and adherent (living) cells were harvested, stained with 0.4% of Trypan blue (1:1) and counted with automated cell counter, differentiating from total, living and dead cells.

Fig. 20B shows that in both A375 and BLM cells, δ-tocotrienol induced a dose-response effect with the reduction of total and viable cells, and a parallel increase of dead cells, stained with Trypan blue.

In order to verify if δ-tocotrienol might induce programmed or non-apoptotic cell death, Western blot analyses were performed, to assess the cleavage of procaspase-3, both in a dose-dependent and in a time-dependent setting. Cells were treated with different doses (5, 10, 15 or 20 μg/ml) of δ-tocotrienol for 48 hours or with 20 μg/ml for 18, 24 or 48 hours. Floating and adherent cells were harvested and protein extracts were obtained and used for Western blot analyses. The cleavage, that means the activation, of procaspase-3 occurred at 15 and 20 μg/ml of treatment in both BLM and A375 cells. A time-course activation of procaspase-3 was also observed at the dose of 20 μg/ml, with a mild cleavage at 18 hours treatment in BLM cells, that rised at 24 and 48 hours, and a strong activation at all time points in A375 cells (Fig. 20C).

Moreover, Western blot analyses were conducted to assess the functional activation of the apoptosis process, verifying the cleavage, that means the degradation, of the caspase-3 direct target PARP (Poly (ADP-ribose) polymerase). PARP is involved in DNA repair: when apoptotic pathways are triggered, activated caspase-3 cleaves this enzyme, preventing DNA damages repair and contributing to cell death. Accordingly to the time-course cleavage of procaspase-3, cleavage of PARP occurred when A375 and BLM cells were treated with 20 μg/ml of δ-tocotrienol for 18, 24 or 48 hours, clearly indicating the effectiveness of the apoptosis process (Fig. 20C).

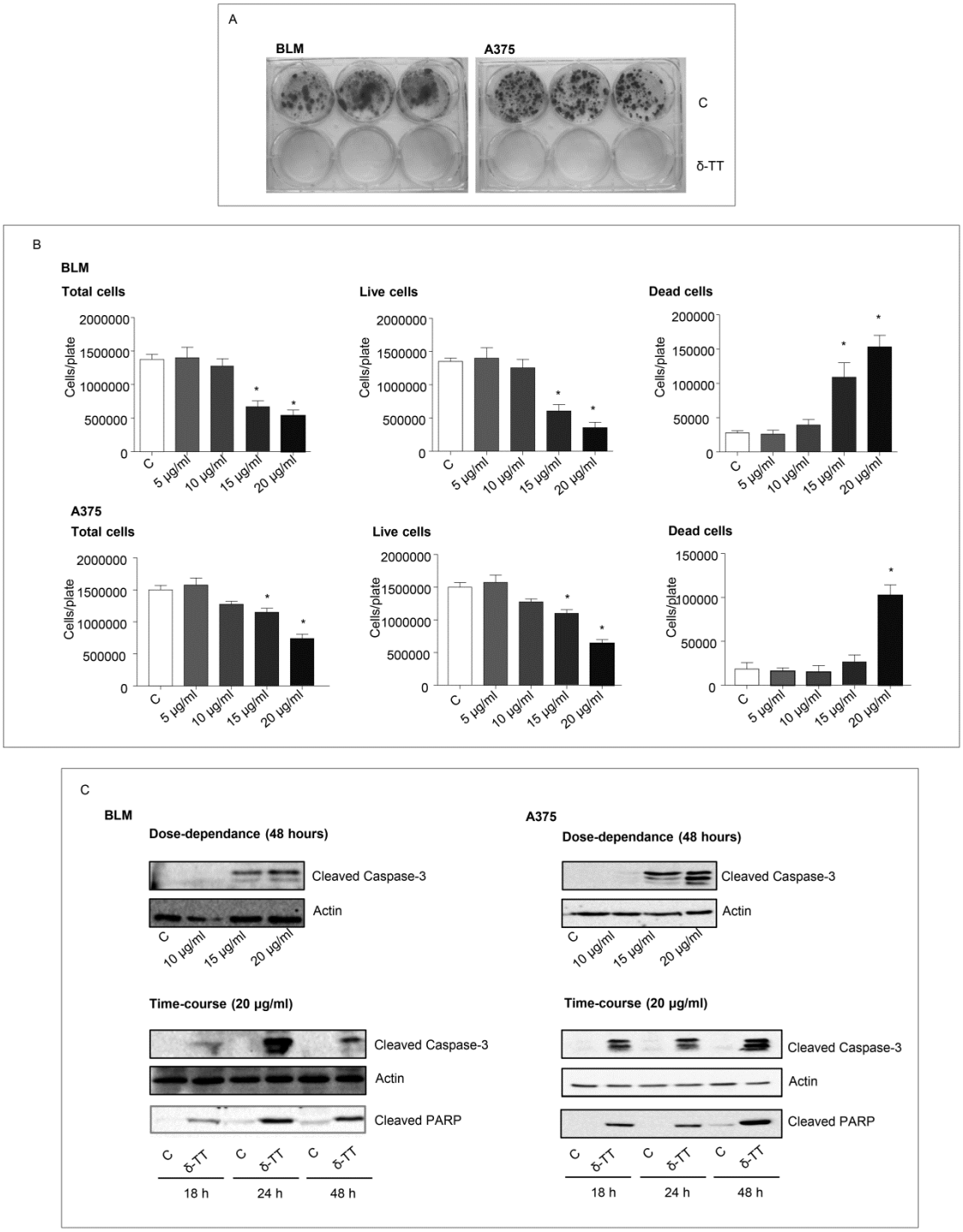

Fig. 20. δ-tocotrienol exerts a cytotoxic/apoptotic effect on melanoma cells. (A) A clonogenic assay was performed in order to verify the cytostatic *vs* cytotoxic activity of δ-tocotrienol. BLM and A375 cells were seeded at low density and treated with 20 μg/ml of δ-tocotrienol for 72 hours, then the treatment was removed and cells were left to grow until significant colony formation and enlargement were observed in controls. In both cell lines, δ-tocotrienol induced a drastic reduction in the number of colonies, since the colony formation capability was completely abrogated. **(B)** A Trypan blue-exclusion assay was conducted to confirm the death-inducing effect of δ-tocotrienol on BLM and A375 cells. Cells were treated for 24-48 hours, then adherent and floating cells were harvested and stained with Trypan blue. A significant reduction of total and living (not stained) cells was observed, with a parallel dose-

dependent increase in the number of dead (stained) cells, for both melanoma cell lines. Data represent mean values ± SEM. **P*<0.05. **(C)** In order to discriminate between apoptotic *vs* non-apoptotic cell death, the dose- and time-dependent cleavage of procaspase-3 was analyzed by Western blot. Caspase-3 activation was seen starting from 15 μg/ml of δ-tocotrienol treatment, accordingly with the significant dose-dependent reduction in cell viability, and the cleavage was already observed at 18 hours treatment (20 μg/ml), for BLM and A375 cells. Cleavage of PARP, as the direct target of caspase-3, was also observed in a time-dependent way, accordingly to the cleavage of procaspase-3. Actin expression was evaluated as a loading control. One representative of three different experiments, which gave similar results, is shown. C, controls

δ-tocotrienol increases the expression of ER stress markers

Several natural compounds exert their effects through the induction of the endoplasmic reticulum stress [130]. Among these molecules, thapsigargin is a well characterized irreversible SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) inhibitor, that is known to disrupt Ca^{2+} homeostasis.

In order to investigate the apoptotic mechanisms on the basis of δ-tocotrienol-induced cell death, the expression of ER stress markers was evaluated. Thapsigargin was used as a positive control, so BLM cells were treated with the SERCA inhibitor (1 μM) in the same setting of δ-tocotrienol (20 μg/ml), for 1, 6, 18 or 24 hours. Adherent and floating cells were then harvested and protein extracts were used for Western blot analyses. Four ER stress markers (1-4) and three ER stress-related apoptosis markers (5-7) were evaluated:

1. BiP: a Ca^{2+}-dependent chaperon protein located in the endoplasmic reticulum lumen;
2. PERK: one of the three sensors of protein overload and endoplasmic reticulum stress; it is located in the ER membrane;
3. IRE1α: beside PERK, it is a sensor of ER stress and it's also located in the ER membrane;
4. PDI: protein disulfide isomerase, a Ca^{2+}-dependent chaperon protein located in the ER lumen;
5. ATF4: a transcription factor induced by the activation of the PERK-eIF2α pathway in response to ER stress; it's one of the first steps involved in the ER stress-induced apoptosis;
6. CHOP: a key transcription factor induced by ATF4, that changes the balance between prosurvival and proapoptotic significance of the ER stress, in favor of the induction of programmed cell death;
7. ERO1α: a target of CHOP, involved in the hyperoxidation of the ER environment through generation of ROS.

As shown in Fig. 21A, thapsigargin induced a significant, time-dependent, increase in the expression of all these markers. δ-tocotrienol induced an increase in the expression of the two ER sensors PERK and IRE1α, beside an increment of the levels of the chaperon protein BiP, and of the transcription factors ATF4 and CHOP, in both BLM and A375 cell lines. The expression of ERO1α was unaffected in BLM cells, while its increment was observed in A375 cells. On the other hand, the protein expression of the chaperon protein PDI seemed to be unaffected by the treatment with δ-tocotrienol in both melanoma cell lines.

Immunofluorescence analyses were also performed on BLM and A375 cells, in order to investigate the localization of the key transcription factors involved in the ER stress process.

Since ATF4 and CHOP protein expression rised at 6-18 hours of treatment, BLM and A375 cells were treated with 20 μg/ml of δ-tocotrienol for 18 hours, then cells were fixed and stained with specific antibodies. TRITC-conjugated secondary antibodies were utilized to localize the proteins, and DAPI staining was also conducted. Fig. 21B shows that cells in basal conditions expressed ATF4 and CHOP at almost undetectable levels, since red fluorescence is almost absent. δ-tocotrienol treatment induced: 1- the expression of these transcription factors, as evidenced by the appearance of the red fluorescence; and 2- their nuclear localization, as shown by the overlapping staining between TRITC-conjugated antibodies and DAPI.

All together, these findings suggest that δ-tocotrienol might induce endoplasmic reticulum stress in melanoma cells, and this effect is likely accompanied by the induction of programmed cell death.

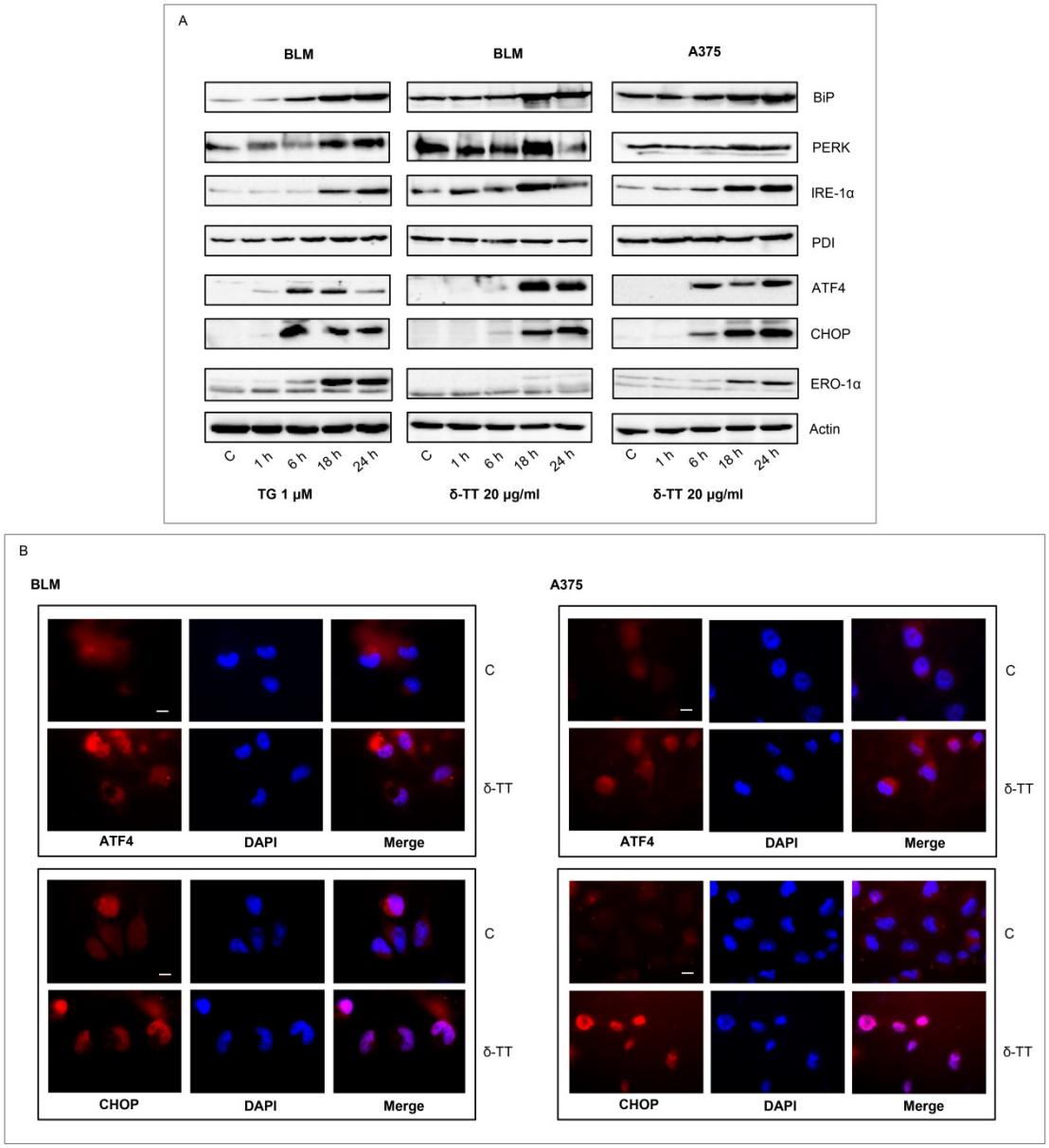

Fig. 21. δ-tocotrienol induces endoplasmic reticulum stress and UPR in melanoma cells. (A) δ-tocotrienol ability in inducing ER stress was evaluated by Western blot analysis. Induction of ER stress markers can be observed in thapsigargin-treated BLM cells, used as a positive control. These markers were also induced by δ-tocotrienol treatment, both in BLM and A375 cell lines, despite few differences. Actin protein expression was evaluated as a loading control. One representative of three different experiments, which gave similar results, is shown. C, controls. **(B)** The expression and localization of the two key apoptosis-related transcription factors ATF4 and CHOP were analyzed by immunofluorescence studies. In both BLM and A375 control cells, the basal ATF4 and CHOP-related fluorescence was almost undetectable. Conversely, δ-tocotrienol treatment induced the nuclear expression of these transcription factors. Scale bar, 5 μm.

δ-tocotrienol-induced ER stress is associated with apoptotic cell death

After ER stress induction, cells might enter two different destinies. When the environmental or endogenous stressful conditions are mild, the ER stress mechanisms are directed at improving cell survival: the attenuation of protein translation, the degradation of misfolded proteins and the folding of unfolded proteins are processes that contribute to overcome the ER protein overload. If stressful conditions are too severe, these mechanisms are not sufficient to improve cell survival: the balance between prosurvival and prodeath mechanisms is then disrupted in favor of the activation of the apoptosis process [134].

In order to relate the ER stress induction with the induction of the apoptosis process, BLM and A375 cells were pretreated with 10-25 μM salubrinal (an ER stress-induced apoptosis inhibitor, due to its inhibitory activity on eIF2α dephosphorylation), 1 hour before δ-tocotrienol.

Cell viability and caspase-3 activation were investigated, by means of MTT assay and Western blot analysis, respectively. Fig. 22 shows that salubrinal alone did not significantly reduce cell viability, while δ-tocotrienol alone did it. Pretreatment with salubrinal only partially reverted the cytotoxic effect of δ-tocotrienol, both in BLM and in A375 cells. These results were further confirmed by caspase-3 cleavage: Western blot analysis evidenced that cotreatment with salubrinal inhibited caspase-3 activation; however, this inhibition was partial, suggesting that other apoptotic mechanisms, beside the PERK/eIF2α/CHOP pathway, are likely involved in the antitumor activity of δ-tocotrienol.

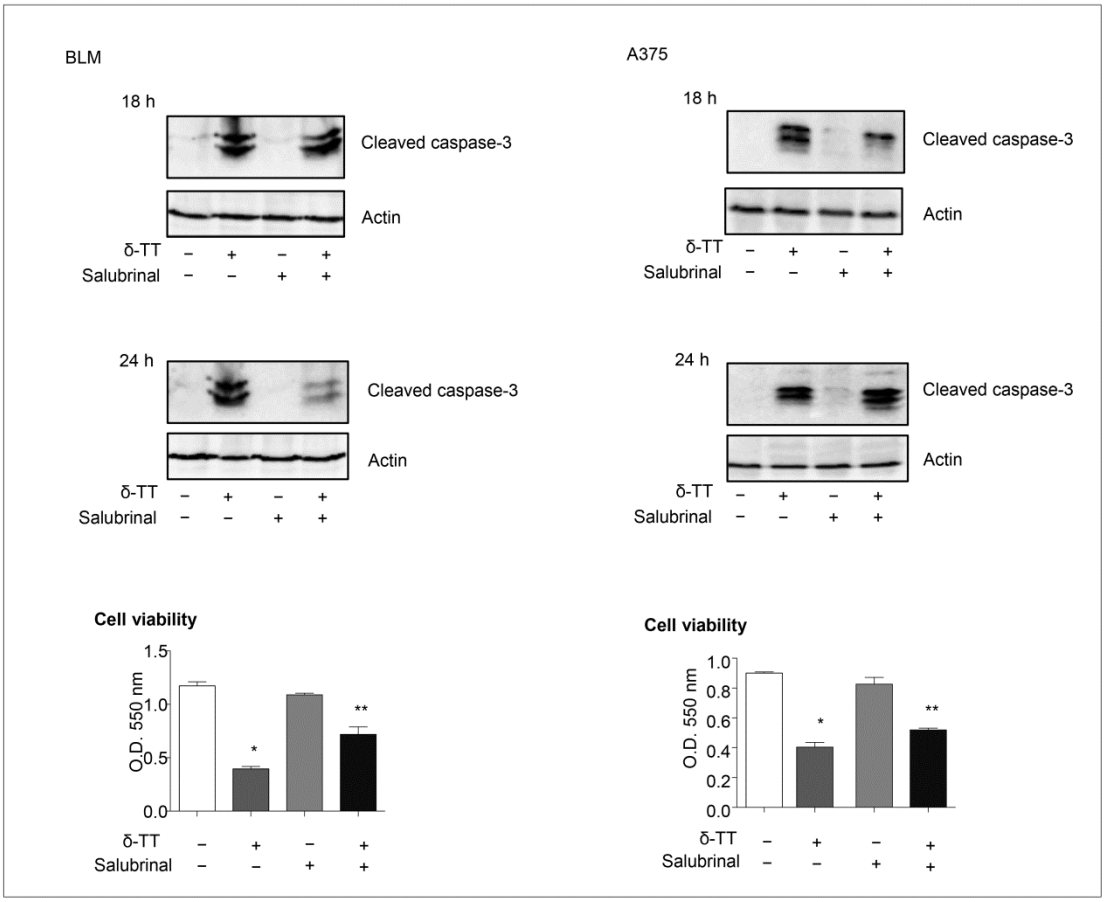

Fig. 22. δ-tocotrienol induces the ER-stress mediated apoptosis. In order to correlate the activation of the apoptosis process with the ER stress, treatment with salubrinal was conducted 1 hour prior to δ-tocotrienol addiction. Western blot analysis evidenced that salubrinal only did not activate caspase-3, both at 18 and 24 hours treatment, and the cotreatment with the two compounds only partially blocked its cleavage (at 24 hours treatment on BLM and 18 hours treatment on A375 cells). Accordingly to these results, salubrinal alone did not reduce melanoma cell viability, and cotreatment with δ-tocotrienol only partially reverted the cytotoxic effect of the natural compound. Data represent mean values ± SEM. **P*<0.05.

δ-tocotrienol induced-apoptosis is associated with caspase-4 activation

Caspase-4 is the initiator caspase directly involved in the ER stress-induced apoptosis. It is bound to the ER membrane where it is normally kept in an inactive state, however, procaspase-4 is cleaved, that means activated, following ER stress [130, 191, 192]. Removal of the caspase-4 prodomain can be achieved through a number of mechanisms, one of which is cleavage by calpains. A number of ER stressors lead to the release of ER Ca^{2+} stores, and thereby to elevation of cytosolic calcium levels and calpains activation [193]. Once activated, caspase-4 cleaves the effector caspase-3, thus leading lo mitochondrial outer membrane permeabilization (MOMP)-independent cell death.

Since we demonstrated the activation of the ER stress-related apoptosis upon treatment of melanoma cells with δ-tocotrienol, Western blot analysis was conducted to verify the cleavage of procaspase-4. A375 and BLM melanoma cells were treated with 20 μg/ml of this compound for 1, 6, 18 or 24 hours: in both cell lines the cleavage, that means the activation, of caspase-4 is observed, and this is demonstrated by the 40 and 32 KDa fragments on Western blot (Fig. 23). Its activation occurs at shorter time intervals than caspase-3 cleavage (see Fig 20C), supporting its role in caspase-3 activation.

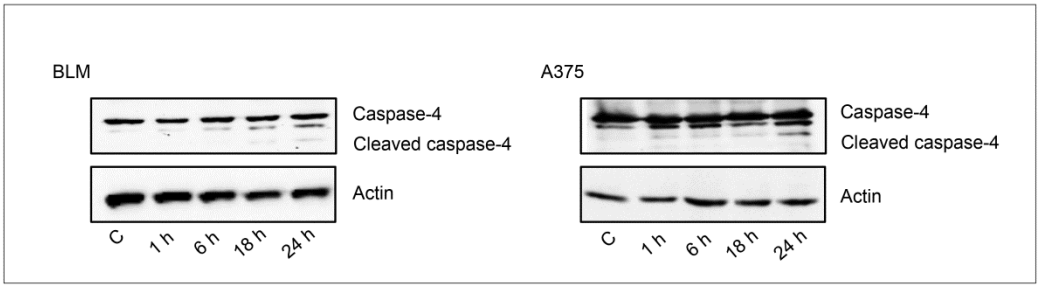

Fig. 23. δ-tocotrienol induces the cleavage of procaspase-4. (A) Western blot analysis shows the time-dependent procaspase-4 cleavage, induced by the treatment of BLM and A375 cells with 20 μg/ml of δ-tocotrienol. Actin protein expression was evaluated as a loading control. One representative of three different experiments, which gave similar results, is shown. C, controls.

Analysis of the activation of the intrinsic apoptosis pathway

The intrinsic apoptosis pathway is triggered by molecular events that lead to the disruption of MOMP (mitochondrial outer membrane potential) and, subsequently, to the cytosolic release of mitochondrial proteins such as cytochrome *c*. MOMP alterations are due to the formation of pores through the outer mitochondrial membrane, by proapoptotic proteins belonging to the Bax family. In physiologic conditions, the maintenance of MOMP is warranted by the equilibrium between proapoptotic (Bax family) and antiapoptotic (Bcl-2 family) proteins: for example, heterodimers Bax/Bcl-2 are crucial for keeping Bax proapoptotic proteins in an inactive state. Changes in this balance imply an improved survival (excess of antiapoptotic factors) or an increased cell death through the apoptosis process (excess of proapoptotic factors).

In order to assess if δ-tocotrienol might induce an alteration of MOMP, with consequent triggering of the intrinsic apoptosis pathway, Western blot and immunofluorescence analyses were performed to investigate the expression levels of Bcl-2 and Bax, and cytochrome *c* localization, respectively.

Fig. 24A shows that, in BLM cells, a significant induction of Bax expression occurred since 1 hour treatment of BLM cells with 20 μg/ml of δ-tocotrienol, and the rise of its expression was seen until at least 24 hours treatment. On the other hand, Bcl-2 expression was unaffected. Opposite results were obtained for A375 cells: in this case, δ-tocotrienol treatment induced a reduction in Bcl-2 expression at 18-24 hours, but no changes in the expression levels of Bax were observed. However, in both cell lines, a change in the Bax/Bcl-2 ratio occurs, so it might be indicative of alterations in MOMP.

Modifications in the permeabilization of mitochondrial membrane lead to the release of cytochrome *c* from mitochondria to the cytosol. Immunofluorescence localization of cytochrome *c* was performed with a FITC-conjugated antibody, together with mitochondrial staining with Mitotracker (in red) (Fig. 24B). In control (untreated) cells, the fluorescence overlapping (yellow) clearly indicates a basal co-localization of the protein with mitochondria. δ-tocotrienol treatment (24 hours) induced a different

localization of green fluorescence, that is diffused in the cytosol and does not overlap with Mitotracker staining. This finding demonstrates that δ-tocotrienol is able to induce cytochrome *c* release both in A375 and BLM cells, and this is in line with the alteration in the proapoptotic *vs* antiapoptotic protein expression, suggesting the activation of the intrinsic apoptosis pathway.

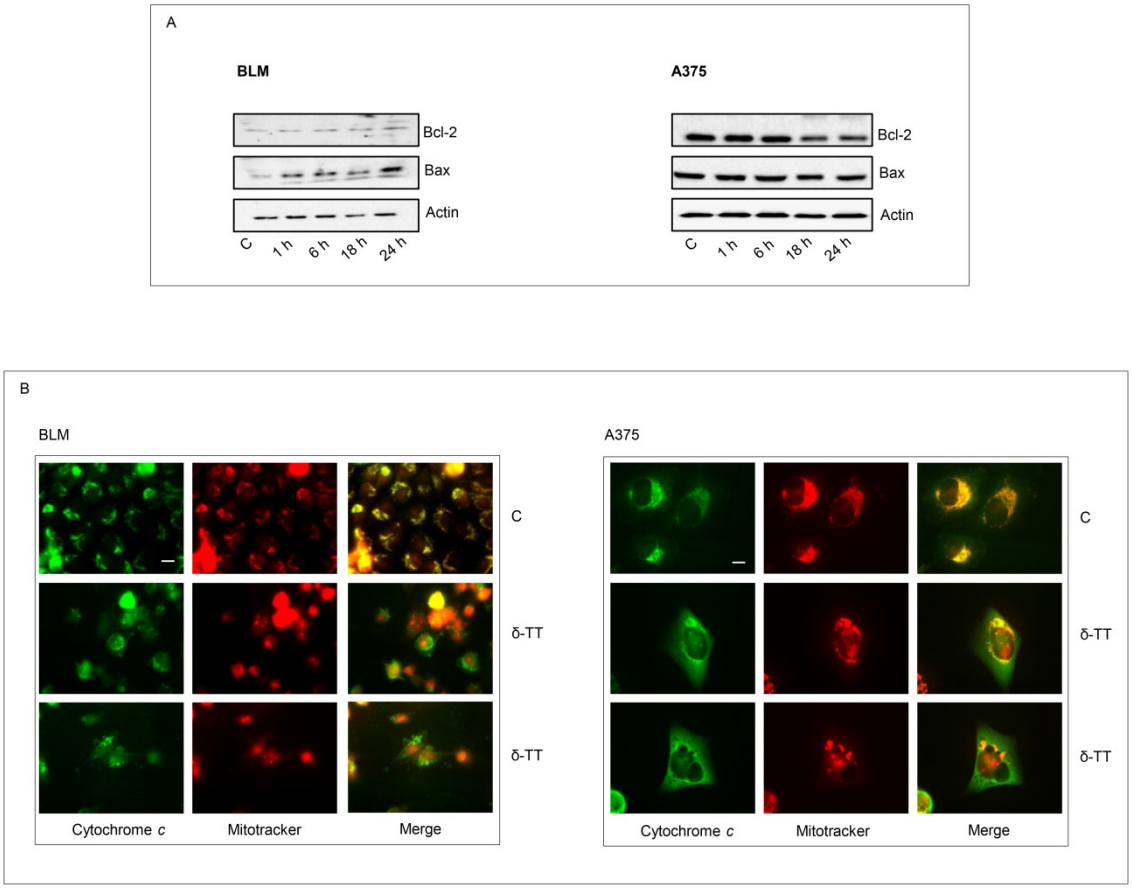

Fig. 24. δ-tocotrienol induces the activation of the intrinsic apoptosis pathway. (A) Western blot analysis was conducted to verify whether δ-tocotrienol might affect the expression of proapoptotic *vs* antiapoptotic proteins. A significant induction of Bax expression occurred since 1 hour treatment of BLM cells, and the rise of its expression is seen until at least 24 hours treatment. On the other hand, Bcl-2 expression was unaffected. Opposite results were obtained with A375 cells: in this case, δ-tocotrienol treatment induced a reduction in Bcl-2 expression at 18-24 hours, but no changes in the expression levels of Bax are observed. Actin protein expression was evaluated as a loading control. One representative of three different experiments, which gave similar results, is shown. **(B)** Immunofluorescence staining of mitochondria and cytochrome *c* was performed in order to assess the alteration of MOMP. In control cells, staining overlap is observed, while, in treated cells, the green fluorescence (cytochrome c) is not overlapped with the red fluorescence (Mitotracker), and is more diffuse in the cytoplasm: this is indicative of the release of cytochrome *c* from mitochondria. Scale bar, 5 μm.

Growth-inhibitory activity of δ-tocotrienol on melanoma xenografts -in nude mice

Given the significant activity of δ-tocotrienol *in vitro*, preclinical experiments were conducted on animal models. Since δ-TT exerted a similar proapoptotic activity on both melanoma cell lines, we selected the A375 cell line to perform preclinical experiments. A375 were subcutaneously inoculated in 6 months-old female immunodeficient CD1-nu/nu mice. At about 10 days after tumor injection, mice were orally treated with δ-tocotrienol extract in olive oil (100 mg/kg, 5 days/week), up to 35 days, while controls received olive oil only. Tumor volumes were calculated at the beginning of the treatment, at 3, 6, 12, 18, 24, 30 days, and at the end of the treatment: a significant reduction was observed at all time points, comparing the treated group with control group (Fig. 25A).

At the end of the treatment, tumors were also weighted, observing that δ-tocotrienol treatment reduced not only tumor volumes, but also tumor mass, (Fig. 25B). Moreover, time to progression probability (TTP) was also determined by Kaplan-Meier analysis, that evidenced a delay of tumor progression in δ-tocotrienol-treated group (Fig. 25C).

- Importantly, no systemic toxic side effects were observed during all the experiments, confirming *in vitro* studies on normal melanocytes and underlying the significance of the usefulness of δ-tocotrienol against this type of cancer.

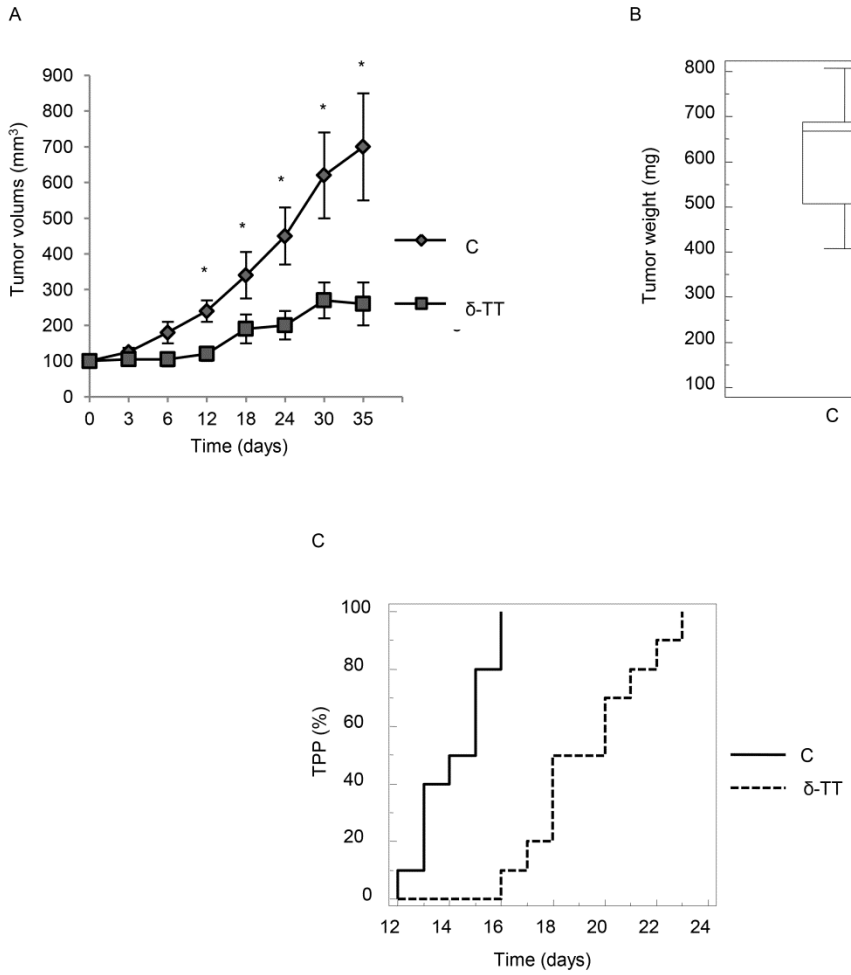

Fig. 25. Growth-inhibitory activity of δ-tocotrienol on melanoma xenografts in nude mice. I*n vivo* experiments were conducted using A375-bearing nude mice receiving δ-tocotrienol (100 mg/kg, 5 days/week) or olive oil, administered by oral gavage when tumor reached 0.5-0.8 cm^3. **(A)** Tumors were measured over time with a Vernier caliper and tumor volumes were calculated: δ-tocotrienol induced a significant reduction of tumor volumes at all time points, compared to control mice. Data represent mean values ± SEM. **P*<0.05. C, controls. **(B)** At the end of the experiments, tumors were harvested and weighted. A significant reduction in tumor mass was observed for A375 xenografts. Data represent mean values ± SEM. **P*<0.05. C, controls. **(C)** Kaplan-Meier analysis was conducted in order to determine time to tumor progression probability (TTP): δ-tocotrienol delayed tumor progression with respect to control.

Melanoma cancer stem cells (CSCs) characterization

Cancer stem cells represent a population of tumor cells also known as "cancer initiating cells", due to their tumor-initiating capability. They are considered to be the most aggressive population of cells in a tumor mass, responsible for resistance to treatment, tumor recurrence and metastases dissemination. At least two distinct properties contribute to define CSCs *in vitro*: 1- the specific and differential expression of surface markers with respect to their non-stem counterpart [62]; and 2- the ability to grow as spheres in suspension in appropriate stem cell medium [63].

In order to assess the ability of δ-tocotrienol to kill this high malignant population of cells, CSCs in our *in vitro* melanoma models were characterized.

Expression of melanoma stem cell markers in melanoma cell lines

CD271, also known as low-affinity nerve growth factor receptor (NGFR) or p75NTR, has been identified as a marker of neural crest cells, so it represents a de-differentiation marker for the melanocyte lineage and melanoma cells. For this reason, it is a well-accepted marker of melanoma cell stemness [194].

CD44 is an ubiquitous cell surface glycoprotein involved in cell migration, cell-cell and cell-matrix interactions. It has been associated with self-renewal, drug resistance and apoptosis resistance, and it has been identified as one of the most consistent markers of CSCs for a variety of malignancy including leukemia, breast, colon, ovarian, prostate, pancreatic, head and neck cancer and also melanoma [195].

ABCB5 is a member of the ATP-binding cassette (ABC) transporter family that has known roles in multidrug resistance to cancer therapeutics, since it is thought to play a role in drug efflux. Firstly detected in tissues derived from the neuroectodermal lineage, including melanocyte progenitors, ABCB5 has been also found in melanoma cell lines and patient specimens, and it seems to be restricted to a subpopulation of cells endowed with self-renewal, differentiation and tumorigenicity abilities [196].

First of all, in order to characterize cancer cell stemness in our melanoma models, Western blot analyses were performed to verify the basal expression of these markers in BLM, A375 and WM115 melanoma cell lines, routinely grown in standard conditions. Fig. 26 shows that CD271 is expressed in A375 and WM115 cells, while no protein

bands were detected for BLM cells. Conversely, CD44 and ABCB5 were found to be expressed in all melanoma cell lines considered.

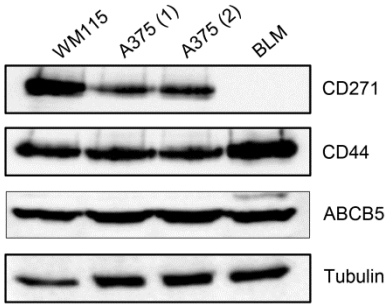

Fig. 26. Protein expression of cancer stem cell markers on different melanoma cell lines. Western blot analysis was conducted to assess the expression of cancer stem cell markers on WM115, A375 and BLM melanoma cells. The neural crest marker CD271 was found to be expressed in WM115 and A375 cells, while BLM cells do not express it. On the contrary, the cell-cell and cell-matrix interactions marker CD44 and the drug resistance marker ABCB5 were found to be expressed in all the three melanoma cell lines. Tubulin expression was evaluated as a loading control.

Differential expression of cancer stem cells markers on A375 cells

Since in A375 cells all these markers were found to be expressed, and we conducted all the previous studies on this cell line, we selected this melanoma model to verify the presence of a stem cell population, and to characterize it.

Immunofluorescence analyses were conducted on A375 cells, in order to assess the expression and colocalization patterns of CD271, CD44 and ABCB5: if one of them will be differentially expressed in the same cell line, then it would be a good candidate marker to identify a subpopulation of cells. Fig 27A shows that in A375 cells, CD271 is expressed in some cells, while in some others it does not; on the contrary, CD44 and ABCB5 are evidenced in all of them.

Colocalization analysis revealed that the relative pattern of expression of CD271/CD44 and CD271/ABCB5 is not mutually exclusive: cells that show CD271 expression are $CD44^{+}$ and $ABCB5^{+}$, but not all the CD44- and ABCB5-expressing cells show CD271 positivity (Fig 27B).

These findings suggest that A375 cells might be composed of at least two distinct populations of cells, and that CD271 might be assumed as a putative melanoma stem cell marker in our *in vitro* model, given its differential pattern of expression in different cells of the same cell line.

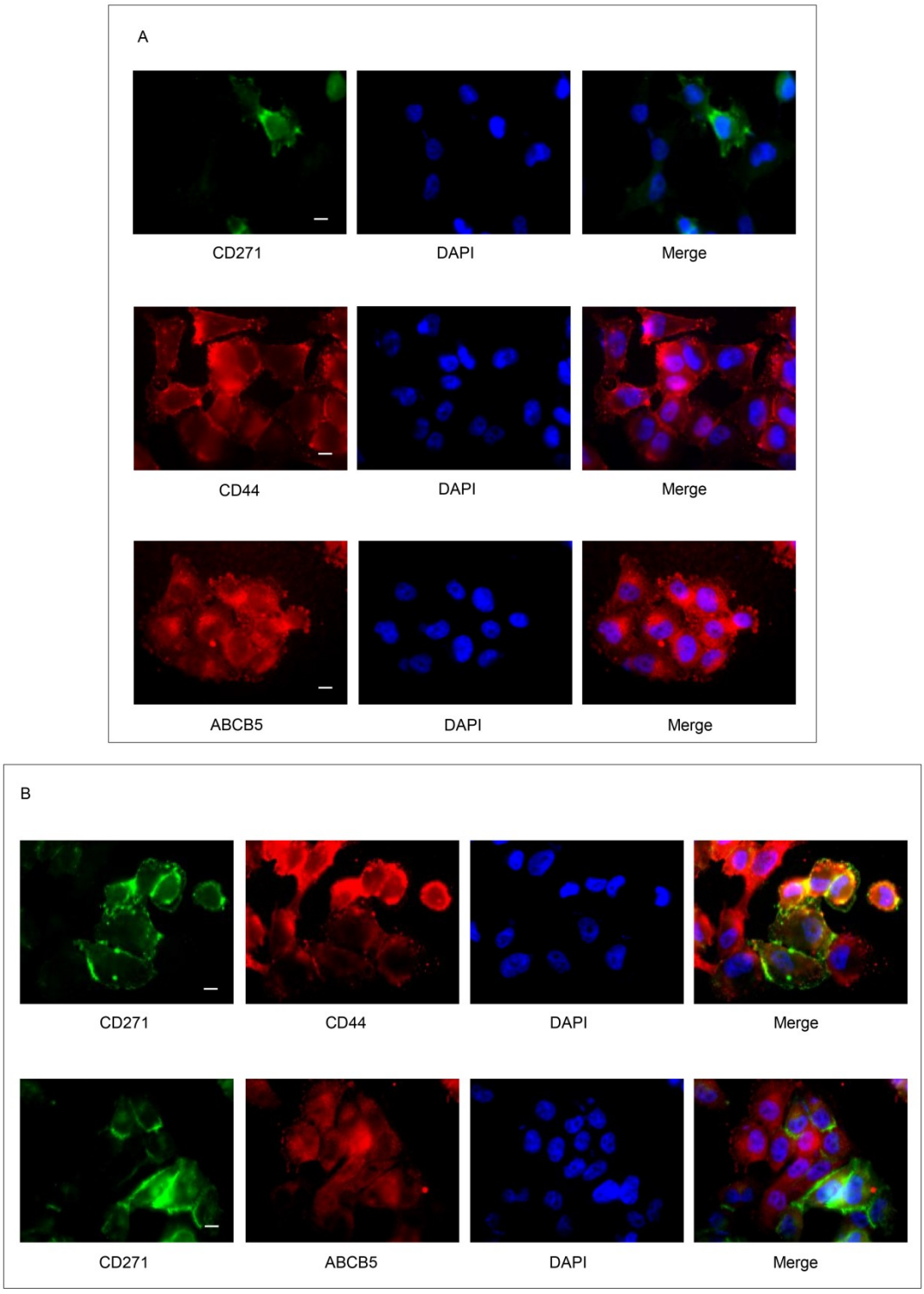

Fig. 27. Immunolocalization of the pattern of expression of cancer stem cell markers on A375 cells. (A) Immunofluorescence analyses were performed in order to verify the expression pattern of the putative melanoma stem cell markers CD271, CD44 and ABCB5 on the whole A375 cell population. CD44 and ABCB5 were found to be expressed in all of the cells, CD271 was found to be expressed only in some of them. **(B)** The colocalization pattern of CD271/CD44 and CD271/ABCB5 showed that all the CD271-expressing A375 cells showed positivity for CD44 and ABCB5; on the contrary, not all the $CD44^{+}$ and $ABCB5^{+}$ cells showed positivity for CD271. Scale bar, 5 µm.

Melanospheres growth

Cancer stem cells are characterized by the ability to grow arranged in spheres, floating in suspension when cultured in appropriate stem cell medium [63].

Given the results obtained when analyzing stem cell markers expression, we wanted to assess the ability of A375 cells to grow in melanospheres: they were plated as single cell-suspension, in Euromed-N medium, without FBS, plus 10 ng/ml EGF, 10 ng/ml FGF2 and 1% N2, and were cultured since spheres were formed. We found that A375 cells were able to proliferate forming spheres, despite a certain amount of cells were adherent. To enrich the culture of cancer stem cells, and to obtain a pure culture, only floating melanospheres were serially collected and replated in new culture flasks. Melanospheres were harvested and passaged once reached 100 to 150 µm diameter size, otherwise, cells in the center of the sphere would undergo apoptosis.

Images of melanospheres were taken, after 5 and 15 days of culture, under a Zeiss Axiovert 200 microscope with a 10x objective lenses linked to a Coolsnap Es CCD camera (Fig. 28).

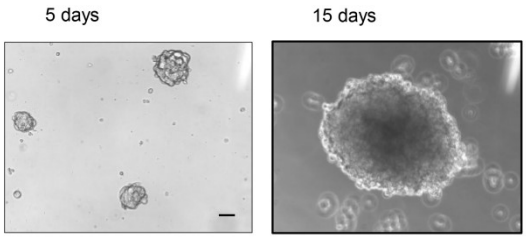

Fig. 28. Melanoma-initiating cells formed floating sphere-like structures *in vitro*. A375 cells were plated in Euromed-N serum-free medium containing 10 ng/ml EGF, 10 ng/ml FGF2 and 1% N2, and grown since 100-150 µm diameter size was reached. Melanospheres were photographed during formation, at 5 and 15 days of culture. Scale bar, 30 µm.

Cancer and embryonic stem cell markers expression on A375 melanospheres

The last step of characterization of melanoma stem cells was directed at analyzing the expression and localization of: 1- the above characterized melanoma stem cell marker CD271; and 2- the embryonic stem cell marker Oct4, in A375 melanospheres. Indeed, cancer stem cells share similar features with embryonic stem cells, in term of self-renewal and plasticity [67]: analyzing the expression of embryonic stem cell markers in cancer cells enforces the stemness characterization of this cell population.

Oct4 is a transcription factor that, together with others such as Nanog and Sox2, maintains pluripotency and self-renewal in embryonic stem cells. In the adult, its expression is normally restricted to the ovary and testis, and its ectopic expression in somatic cells results in dedifferentiation and malignant transformation. Moreover, ectopic expression in tumor cells results in enhanced CSC-like properties such as sphere formation, drug resistance and increased tumorigenicity [197].

CD271 and Oct4 immunofluorescence analyses were performed on suspension-fixed melanospheres (Fig. 29). CD271 was found to be expressed in all melanospheres-forming cells, in contrast with findings on the whole A375 cell culture (see Fig. 27A), indicating that only the $CD271^{+}$ population is able to grow in cancer stem cell culture conditions.

Moreover, all melanospheres-forming cells express the pluripotency and malignancy marker Oct4, suggesting that only cells with stem cell-like features are able to grown arranged in melanospheres, and show positivity for the melanoma stem cell marker CD271.

This finding confirms that CD271 is a good candidate marker to identify cancer stem cells in A375 melanoma cell line.

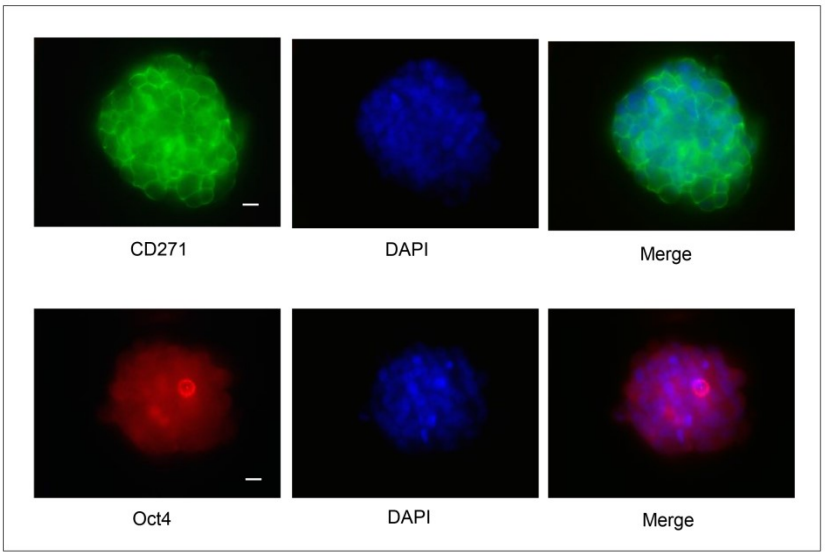

Fig. 29. Immunolocalization of stemness markers in A375 melanospheres. Immunofluorescence analyses were conducted in order to assess the expression and localization of stemness markers on whole melanospheres. The neural crest marker CD271 was found to be expressed in all the cell-forming spheres (upper panel). A375 melanospheres also express the embryonic stem cell marker Oct4. Scale bar, 15 µm.

δ-tocotrienol treatment affects the growth of A375 melanospheres

Cancer stem cells represent the most aggressive population of tumor cells, that might be responsible for drug resistance. Despite the effectiveness of surgical therapy for melanomas *in situ*, chemotherapies or targeted therapies against metastatic melanomas are characterized by initial response, followed by relapse within few months, so that new more efficient therapeutic strategies should be developed.

Given the above demonstrated antitumor activity of δ-tocotrienol, both *in vitro* and *in vivo*, experiments were performed in order to verify the efficacy of this natural compound on melanoma stem cells.

First of all, in order to observe any morphological changes, A375 melanospheres were seeded at a very low density (about 1-2 melanospheres/well), and after 48 hours they were treated with 20 µg/ml of δ-tocotrienol for 5 days. Melanospheres in every well were monitored during treatment, and were photographed under a Zeiss Axiovert 200 microscope linked to a Coolsnap Es CCD camera. Fig. 30A shows that treatment of well-formed spheres, with this dosage, makes melanospheres prone to disaggregate, as observed from the border irregularity of every single sphere, and the lack of the compactness, in contrast with the same spheres before treatment. No changes in the number of melanospheres for each well was observed, even if this experimental setting is not optimal for a statistic evaluation. For this reason, another study was conducted.

A375 melanospheres were disaggregated through accutase, and the obtained single cells were plated in 70% fresh medium and 30% conditioned medium, to warrant new melanospheres formation. Assuming that all the single stem cells have the same melanospheres-formation potential, after 48 hours, during formation, melanospheres were treated with δ-tocotrienol (40 µg/ml) or vehicle for 7 days, in order to assess the spherical colony-forming ability of cells. At the end of the treatment, melanospheres for each well were counted and photographed under a Zeiss Axiovert 200 microscope with a 4x objective lens, linked to a Coolsnap Es CCD camera. Fig. 30B shows that:

1- The dimension of treated spheres is more variable compared with non-treated melanospheres, suggesting an impairment in the proliferation of these cells.
2- The number of spheres for each well is significantly reduced in δ-tocotrienol-treated cells.

This reflects the ability of δ-tocotrienol to exert a cytotoxic, other than antiproliferative, activity on melanosphere-forming cells, even if at a higher dose than that of the global A375 culture.

In order to confirm the effectiveness of δ-tocotrienol against A375 stem cells, Western blot analyses were performed to verify the expression levels of CD271 after treatment of melanospheres with δ-tocotrienol (40 μg/ml). Fig. 30C shows that a significant reduction in the expression of CD271 occurs in A375-treated melanospheres, supporting previous results on the ability of this compound in reducing melanoma stem cells growth.

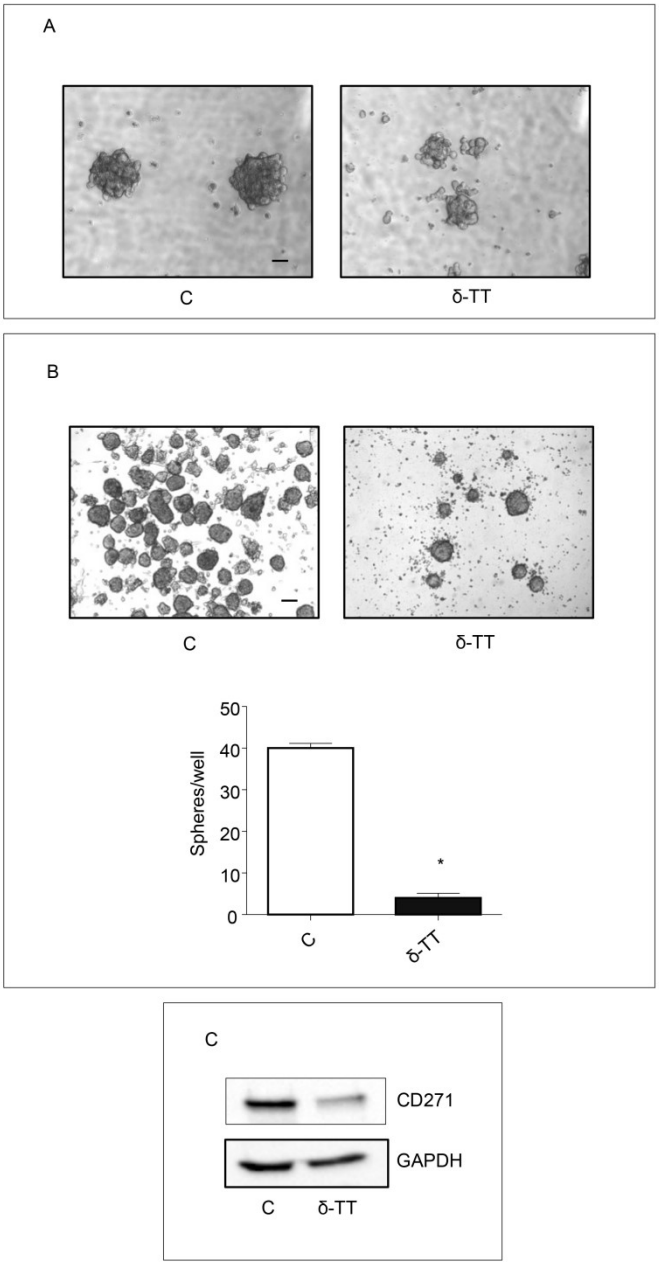

Fig. 30. δ-tocotrienol has an antitumor activity on A375 melanospheres. (A) A375 melanospheres were plated at a very low density and after 48 hours were treated with 20 µg/ml of δ-tocotrienol. At the end of the treatment, spheres were photographed: a trend in disaggregation of the spheres was observed. Scale bar, 30 µm. **(B)** Spheres-forming A375 cells were plated as single cells and, during melanospheres formation, they were treated with δ-tocotrienol (40 µg/ml). At the end of the treatment, spheres for each well were photographed and counted: a significant reduction in the number of spheres was observed in the treated cells. Data represent mean values ± SEM. **P*<0.05. Scale bar, 75 µm. **(C)** The protein expression levels of the melanoma stem cell marker CD271 was evaluated in A375 melanospheres after treatment with 40 µg/ml of δ-tocotrienol. A significant reduction in the amount of this protein was detected. Evaluation of GAPDH expression was used as a loading control. C, controls

DISCUSSION

Discussion

In the early 1970's, Sadoff first proposed that melanoma should be grouped among the "estrogen-dependent" tumors [198], due to several clinical and epidemiological observations, such as the peak of melanomagenesis at menopause and the significant disparity in the prognosis of malignant melanoma between males and females: although males comprise the minority of the melanoma cases, they comprise the majority of melanoma specific deaths [199, 200].

In this context, increasing evidence strongly suggests that the estrogen receptor β plays a fundamental role in the development and progression of melanoma. In particular, the expression of ERβ was shown to inversely correlate with melanoma progression, being significantly lower in thick melanoma compared with thin melanoma tissues [123, 201-203]. These observations point toward a potential role of ERβ as a protein associated with suppressive function in this tumor, as it was found for other type of malignancies.

In the first tasks of this project, we wanted to characterize the estrogenic system in human melanoma, so we first investigated the expression of the two estrogen receptor subtypes, ERα and ERβ, in a panel of melanoma cell lines. We demonstrated that ERβ is expressed in most of these cell lines, whereas ERα does not, suggesting that, at least in our models, all the possible estrogenic actions are mediated through the β receptor subtype. Since the commercially available antibodies against ERβ are not validated, and some of them gave controversial results in immunohistochemical and Western blot analyses [204, 205], we conducted the ERβ protein expression analysis using two different antibodies. Moreover, as an appropriate positive control, BLM cells were transfected with the receptor, and its overexpression was confirmed by both the antibodies.

Then, we analyzed the effects and the mechanisms of action of ERβ activation in BLM cells, that were selected for conducing subsequent experiments due to their characterization in Limonta's lab [179, 180]. We could demonstrate that activation of ERβ; achieved by treating the cells with E_2 or ERβ subtype-selective agonists (the

classical ERβ agonist, DPN, or more recently synthesized agonists, KB1, KB2, and KB4) significantly decreased BLM melanoma cell proliferation. This tumor cell inhibitory activity was found to be specifically mediated by ERβ, since it was completely abrogated by cotreatment of the cells with the ER antagonist ICI-182,780.

In these experiments, the ERβ agonists displayed bell-shaped responses with growth inhibition at low doses, being significantly effective at the dose of 10^{-8}M, and opposite effect at high doses, as previously reported for cholangiocarcinoma and mesothelioma cells [182, 183]. As underlined by Pinton and coworkers, this kind of response is not unusual for hormones. The term "hormesis" has been widely used to describe a biphasic dose-response phenomenon characterized by a low-dose stressful stimulation, following the classical dose-response curve, and a high-dose adaptive compensatory response that increases the resistance of the cell to evoked stress [206, 207]. A possible explanation could be that cells may increase the production of cytoprotective and restorative proteins which can mediate their adaptive response to the stress induced by ERβ agonists.

In BLM cells, the activation of ERβ induced its translocation from the cytoplasm into the nucleus and triggered its transcriptional activity. These data demonstrate that, in these melanoma cells, ERβ is functional, and this receptor subtype exerts its repressive activity through the classical genomic action of steroid receptors at the nuclear level.

We could also show that, in BLM melanoma cells, ERβ agonists exert their antiproliferative activity through the modulation of cell cycle progressing factors (cyclin D1, cyclin D3, p27), without triggering the apoptosis pathway. These data suggest that ERβ activation may inhibit melanoma growth by blocking the cell cycle in the G1-S transition phase, accordingly with other studies in the literature [79, 88, 100, 208]. Specifically, we could observe that the ERβ agonist DPN significantly reduces cyclin D1 and cyclin D3 protein expression at 72 hours of treatment, while increasing the expression of p27 at 48 hours of treatment. Since ERβ nuclear translocation and transcriptional activity occur 24 hours after treatment of the cells with DPN, we hypothesize that these cell cycle-related proteins might not be directly regulated by ERβ but, more likely, they might be the target of the activity of other direct ERβ

downstream proteins. For instance, Wu and coworkers [209] have recently reported that, in bladder cancer cells, the ERβ ligand resveratrol inhibits cell growth through decreased phosphorylation, nuclear translocation and transcription of STAT3, resulting in the downregulation of the expression of STAT3 downstream genes (cyclin D1, survivin, c-Myc and VEGF). Nakamura and coworkers demonstrated that, in prostate cancer cells, activation of ERβ influences cyclin D1 expression through increased expression of FOS and JUN; however, according to the data reported, the authors conclude that the interaction of ERβ with the two transcription factors is not direct and likely involves early responsive genes which still need to be identified [210]. Moreover, Shanle et al showed that, in triple negative breast cancer cell lines, ERβ induced G1-S cell cycle blockade through upregulation of p21 expression, *via* interaction with another transcription factor, Id1 (Inhibitor of differentiation-1), and without triggering apoptosis [211]. In our experiments, ERβ activation failed to induce the expression of this cell cycle inhibitor, that was found not to be expressed also in basal conditions. *CDKN1A,* the gene encoding p21, was found to be frequently silenced in human melanomas. In particular, Prasad et al have recently demonstrated that this gene is a direct target of miRNA 106-b, that is 3-6 fold overexpressed in human melanoma cell lines compared with normal cells [212].

Taken together, our results obtained in BLM cells agree with the concept that the antitumor effect of ERβ is associated with altered expression of proteins involved in cell cycle progression [78, 79]. In agreement with the data here reported, ERβ agonists have been shown to inhibit breast cancer cell growth by causing a cell cycle arrest, through the regulation of cell cycle-related proteins, such as cyclin D1 and the CDK inhibitors p21 and p27. Moreover, it has been demonstrated that ERβ activation might enhance the activity of other anticancer agents, or sensitize cancer cells to conventional therapies, through cell cycle regulation. Nair and coworkers demonstrated that DPN can overcome the breast cancer acquired resistance to letrozole (an aromatase inhibitor) in xenograft models [213]; Tang and colleagues found that ERβ1, but not ERβ2, expression enhanced the antiproliferative effect of

lapatinib, a dual EGFR/HER2 tyrosine kinase inhibitor, through p27 upregulation and consequent cycle arrest in human breast cancer [214].

Several studies reported the ability of ERβ to induce the activation of the apoptosis process: for example, Dey et al demonstrated that ERβ causes apoptosis by increasing the expression of the proapoptotic factor PUMA, independently of p53, but dependently of FOXO3a in prostate cancer cells, with caspase-9 and -3 activation [88]. Other studies reported a decrease in breast cancer cell survival in response to endoplasmic reticulum (ER) stress-inducers, suggesting that the overexpression of ERβ sensitizes breast cancer cells to ER stress-induced apoptosis [215]. However, in BLM cells we didn't observe the activation of caspase-3 in response to the treatment with DPN, indicating that in our melanoma cell line ERβ only exerts an antiproliferative effect, without inducing apoptosis.

The tumor-suppressive action of ERβ here demonstrated enforces data in the literature that supports a role of this receptor on the growth of tumor cells classically unrelated to the reproductive system, such as colon [184], malignant pleural mesothelioma [103, 182, 216], lymphoma [181], glioma [110], and cholangiocarcinoma [183]. More recently, ERβ have been reported to play a role in nonmelanoma skin cancer in mice [217]: in this study, Erb-041, an ERβ selective agonist, significantly reduced UVB-induced photocarcinogenesis through downregulation of cyclin D1, VEGF and UVB-inflammatory responses such as IL1β, IL6 and IL10, and activation of the apoptosis process. Furthermore, tumors were less invasive and showed a reduced epithelial-to-mesenchymal transition, and WNT/β-catenin signaling, which underlies the pathogenesis of skin cancer, was found to be downregulated. Given the UVB-cancerogenesis prevention in nonmelanoma skin cancer, it is not to be excluded a role of ERβ also in the prevention of the UVB-induced melanomas, and this point should be explored further.

It is now well accepted that epigenetic mechanisms play a central role in tumor development. In particular, melanoma cells have been reported to present global DNA hypomethylation, contributing to the genomic instability of tumor cells, when compared to normal cells [45, 48]. Mologni et al demonstrated that the epigenetic

reprogramming is a key event in melanocyte malignant transformation. In this study, different cell lines were developed starting from melan-a melanocytes, that were progressively cultured in sequential cycles of anchorage blockade: different melanoma lineages were then obtained, with different degrees of aggressiveness. Global DNA methylation analysis revealed that, progressing from premalignant melanocytes to the more aggressive melanoma cells, a reduction in the relative amount of DNA methylation occurred, suggesting that global DNA aberrant methylation pattern towards an hypomethylation state is crucial for melanomagenesis [218]. Thus, reversibility of these epigenetic modifications might represent an effective strategy of treatment and prevention for this aggressive form of cancer. In our study, we first confirmed that DNA is globally hypomethylated in human BLM melanoma cells when compared to normal human melanocytes, accordingly to the assumption of a reduction in global DNA methylation in cancer cells compared to normal cells. Then, we could show that treatment of BLM cells with both DPN and E_2 significantly increased global DNA methylation, as to restore the normal pattern of methylation found in non-tumor cells. The finding that ERβ can affect epigenetic mechanisms in tumor cells is in agreement with data in the literature that show its ability to epigenetically regulate the expression of oncogenes and tumor suppressor genes, with a resulting antitumor effect. Bosviel and coworkers demonstrated that S-equol, a soy phytoestrogen derived from daidzein metabolism, is able to induce the reversal of *BRCA1* and *BRCA2* promoter hypermethylation in breast cancer cell lines, allowing the reexpression of these silenced oncosuppressor genes. Specifically, this effect was evident in MCF-7 and MDA-MB-231 cells, expressing low and high ERβ levels, respectively, but it wasn't in MCF-10a cells, that do not express this receptor [219]. Conversely, in other studies, ERβ was also found to be associated with a more aggressive phenotype on prostate cancer, due to its interaction with eNOS and the epigenetic silencing of the cytoprotective GSTP1 [220].

Taken together, our data demonstrate that, in BLM melanoma cells, ERβ activation reduces cell growth, through the modulation of cell cycle related proteins, and that, given the ERβ ability in influencing epigenetic events, this antitumor activity is

associated with the reversal of the global DNA hypomethylation status of these cancer cells.

As expected, we could show that ERβ agonists did not affect the proliferation of melanoma cells expressing almost undetectable levels of ERβ (IGR-39).

On the other hand, surprisingly, we found that ERβ agonists were also ineffective in reducing the proliferation of A375 and WM1552 melanoma cells, shown to express the estrogen receptor isoform. At present, the reason for these unexpected results is unclear. However, a possible explanation is that ERβ agonists differentially affect the proliferation of various cell lines, expressing ERβ, according to the cell line-specific oncogenic mutation status. Actually, NRAS and BRAF mutations are very frequently found in melanoma tumors; in particular, BLM cells are NRAS-mutant (a mutation present in about 30% of patients), while both A375 and WM1552 cells harbor the BRAF V600E mutation (the predominant BRAF mutation, occurring in about 50% of cases) [221, 222](**228**). In melanoma cells, NRAS mutations have been shown to be associated with increased activation of two main downstream signaling pathways: the PI3K/Akt and the MEK/ERK cascades [221, 223]. On the other hand, in melanoma cells harboring BRAF mutations, only the MEK/ERK pathway results to be overactivated. Interestingly, ERβ agonists have been shown to exert their significant antitumor/proapoptotic effect through RAS inactivation and specific inhibition of its downstream PI3K/Akt pathway in different cancer cells [79, 224]. Wang and coworkers [225] reported that ERβ expression inversely correlate with PTEN/PI3K/Akt pathway in tissue samples of triple-negative breast cancer, and that this status predicts a more favorable prognosis. Moreover, in breast cancer cells, calycosin, a natural phytoestrogen, activated ERβ leading to a decreased activity of the PI3K/Akt pathway, while the ERK1/2 cascade was not affected [226]. A recent study by Nikolos and colleagues investigated the role of ERβ in mediating an antitumor activity on non-small cell lung cancer [100]. It was demonstrated that ERβ exerts an antitumor activity through G1-S cell cycle blockade and intrinsic apoptosis induction, especially on NSCLC cell lines that harbor the NRAS or KRAS mutation, with an overactivation of both the MAPK and PI3K/Akt pathways, so that the receptor might specifically exert its effect on the oncogenic Ras signaling.

Accordingly to Nikolos et al, the activation of ERβ inhibited the proliferation of BLM melanoma cells, harboring the NRAS mutation that leads to the iperactivation of both the proliferative pathways. Based on our results, as well as on these recently reported observations, we hypothesize that ERβ agonists might effectively reduce the proliferation of melanoma cells harboring RAS mutations, through the specific inhibition of the activity of one of the two downstream signaling pathways: the PI3K/Akt cascade. On the other hand, ERβ agonists did not reduce the growth of A375 and WM1552 melanoma cell lines, harboring the BRAF (V600E) mutation, which is associated with the overactivation of the MEK/ERK signaling pathway only. Studies are ongoing in our laboratory to confirm this hypothesis.

Taken together, these data would suggest that, in melanoma patients harboring the NRAS mutation, ERβ might represent a novel molecular target for personalized therapeutic or adjuvant strategies, based on ERβ agonists, either alone or in combination with a specific inhibitor of the MEK pathway (*i.e.,* trametinib). Moreover, these results support the notion that not only the expression of ERβ, but also the genetic analysis of the concurrent oncogenic mutations should be considered to predict the possible response of melanomas to ERβ targeted therapeutic approaches.

We could also show that ERβ agonists are able to decrease the proliferation of WM115 melanoma cells, harboring the BRAF V600D mutation. However, no hypothesis can be suggested in this case, since this is considered a very rare BRAF mutation and very little is known about its associated intracellular signaling alterations; it has actually been reported that, in melanoma cells, BRAF mutations can be associated to different intracellular pathways, in addition to the MEK/ERK cascade, including E2F1, BCAT, IGF1, ALK and MYC signaling pathways [227]. Moreover, rare BRAF V600 mutations were shown to be associated with distinct clinicopathological features, so a different response to specific inhibitors is not to be excluded: whether BRAF inhibitors might have the same effectiveness in patients with this rare BRAF mutation still has to be evaluated [228].

The differential effect of ERβ agonists on the proliferation of the various melanoma cell lines here reported might also be associated with the relative expression of the

ERβ isoforms in each cell line. We found that BLM and WM115 cells show a similar pattern of expression of the isoforms with similar levels of ERβ1 and ERβ5, but higher expression of ERβ2. On the other hand, in A375 cells both ERβ2 and ERβ5 are expressed at higher levels than ERβ1. The possible correlation between the expression of the ERβ isoforms and the differential effects of ERβ agonists on melanoma cells is at present unclear. ERβ isoforms have been shown to be co-expressed in various types of tumors, (including breast, ovarian, endometrial, prostate, colon and lung cancers); however, conflicting results have been so far reported on the potential collective effect of their co-existence. The most studied ERβ isoforms in cancers are ERβ2 and ERβ5. These two isoforms are truncated in the N-terminal region of the protein, lacking or having weak functionality in ligand binding and transactivation, so that they are still considered to be inactive *per* se. However, they are generally associated with a negative modulation of ERβ1 and ERα, through heterodimerization, and their expression pattern and amount has a differential prognostic value depending on the tumor and on the cell context. In breast cancer, conflicting results were obtained about ERβ5: protein expression analysis on tumor samples failed to demonstrate its prognostic significance, despite a trend in association with a worse prognosis [229]. On the contrary, *in vitro* studies evidenced an apoptosis-sensitizing effect of ERβ5 in breast cancer cell lines, through direct interaction and inhibition of Bcl2L12 [87]. Dey et al showed that, in prostate cancer cell lines, ERβ1 and ERβ2 have opposing roles in regulating proliferation and metastatization, with the first being tumor-suppressive and the second being associated with pro-metastasizing effects [90]. Other studies reported the immunohistochemical expression of the different isoforms on tumor samples: in ovarian cancer ERβ2 seemed to be related to a good prognosis [230]; in lung cancer the prognosis seemed to be related to the cellular localization of these isoforms, with nuclear ERβ1 and cytoplasmic ERβ2 and 5 in negative correlation with pathological stage and lymph node metastasis [231].

RT-qPCR analysis of ERβ1, 2 and 5 expression on BLM, A375 and WM115 melanoma cells suggests that ERβ2 might not have a negative role in the response to ERβ selective agonists, while the differential expression of ERβ5 in these cell lines might

underline its involvement in inhibiting the antitumor activity of the receptor. However, in agreement with Hapangama and coworkers [232], we believe that the lack of commercially available specific antibodies for the different receptor isoforms represents a major obstacle in the investigation and clarification of their functions.

In summary, our characterization of the estrogenic system in human melanoma demonstrates that the ERβ subtype is expressed in a panel of human melanoma cell lines (BLM, WM115, A375, WM1552). In BLM cells, as well as in WM115 cells, the activation of ERβ is associated with a significant and specific antiproliferative effect. In particular, in BLM cells, this antitumor activity is associated with the modulation of the expression of G1-S cell cycle-related proteins and with the reprogramming of global DNA methylation. On the other hand, ERβ agonists failed to affect the proliferation of A375 and WM1552 cell lines. This differential effect of ERβ agonists on the growth of the different melanoma cell lines might be related either to the specific oncogenic mutational status (NRAS, BRAF) or to the relative expression of receptor isoforms in each cell line.

Given the oncosuppressive function of ERβ in melanoma cells, the receptor could be exploited as a molecular target for the treatment and/or prevention of this type of cancer. The interest in nutraceutics is still increasing, due to the relative safety and abundance of natural compounds possessing pharmaceutical activities. Phytoestrogens, such as soy isoflavons, flavonoids and stilbenes, are among these molecules, that have been reported to bind preferentially to ERβ with respect to ERα [129]. Tocotrienols belong to the vitamin E family, and have been extensively studied for their antioxidant properties. They also possess antitumor activity in different type of cancers, that is unrelated to their antioxidant action [138, 140].

Despite tocotrienols are not considered as phytoestrogens, some structural similarities with estrogenic molecules motivated researches to study their possible interaction with ERβ and the modulation of its activity. The involvement of ERβ in mediating the biological effects of δ-tocotrienol has been indagated and demonstrated in a Parkinson's disease model [170], as well as in breast cancer. Interestingly, Comitato et al [168, 169] reported that δ-tocotrienol exerts an antiproliferative/proapoptotic

activity on breast cancer cell lines, and that this effect is mediated by the specific transcriptional activation of ERβ. Furthermore, *in silico* docking studies showed the likely interaction between the LBD of the receptor and δ-tocotrienol.

Starting from these observations, and giving the oncosuppressive role of ERβ in our melanoma models, we wanted to verify if δ-tocotrienol might exert an anticancer activity in human melanoma cell lines through direct binding and transactivation of the receptor. We conducted these experiments on BLM cells, since we mainly used this cell line to characterize the oncosuppressive role of ERβ. First of all, we demonstrated that δ-tocotrienol exerts a dose-dependent reduction of cell viability on BLM cells, so it possesses an anticancer activity on melanoma cells. However, δ-tocotrienol was not able to induce the ERβ transcriptional activation on BLM cells. Moreover, when all melanoma cell lines were treated with the natural compound, it was able to reduce cell viability independently of: 1- ERβ protein expression, since IGR-39 cells, that express almost undetectable levels of the receptor, were affected by δ-tocotrienol treatment; and 2- the responsiveness of the cells to ERβ activation. Indeed A375 and WM1552 cells, that are resistant to DPN, were significantly affected by δ-tocotrienol treatment. Furthermore, δ-tocotrienol is able to reduce melanoma cell viability independently of the differential expression of the ERβ isoforms and, more importantly, independently of the Ras/Raf mutational status. This finding is a very important point, since this hyperproliferative pathway is involved in tumorigenesis as well as in resistance to therapies. Even targeted therapies against specific mutations, such as vemurafenib against B-Raf^{V600E}, become ineffective due to development of resistance and to the acquisition of additional mutations in the same proliferative signaling pathway. Then, if δ-tocotrienol exerts an antitumor activity independently of the hyperactivation of this cascade, it should be considered for the development of new, more effective, therapeutic strategies. It has been demonstrated that δ-tocotrienol can act by suppressing tumor cell proliferation through inhibition of the MAPK pathway in pancreatic cancer cells [145, 233], even if, to our knowledge, its role on the mutated Raf has not been studied yet. However, it might also induce cell cycle arrest through alternative mechanisms. A very recent study by Ye et al showed that δ-

tocotrienol inhibited cell proliferation, induced apoptosis and chemosensitization of human bladder cancer cells by inhibition of the STAT3 pathway [234]. Another report demonstrated that the inhibition of cell growth and induction of apoptosis in non-small lung cancer cells was associated with Notch-1 downregulation [235]. Moreover, inhibitory effects were observed on PI3K/Akt and NFκB signaling or on the activity of HMGCoA reductase [148]. In addition to the growth-inhibitory activity, δ-tocotrienol has been reported to induce apoptosis on cancer cells, by stimulating either the intrinsic or the extrinsic apoptosis pathway [154-156].

Another important question is whether δ-tocotrienol is more effective than standard therapies. We selected BLM, A375 and WM115 cells, representatives of the main Ras/Raf mutations found in melanomas, and we treated them with dacarbazine, the conventional chemotherapeutic agent used for the management of metastatic melanoma. Except for WM115, dacarbazine significantly reduced melanoma cell viability. However, this effect was lower than that exerted by δ-tocotrienol, considering that the δ-tocotrienol concentration of 20 μg/ml, the higher dosage we used, corresponds to a molarity of 50.4 μM. Our results then suggest that δ-tocotrienol is more effective than dacarbazine in inducing the reduction of melanoma cell viability, at least *in* vitro. Studies in the literature also report significant synergistic effects of tocotrienols in association with different standard therapies. A potentiation of the antitumor effects of statins was observed on malignant mesothelioma and mammary adenocarcinoma [236, 237]; chemosensitization to chemotherapeutic agents such as docetaxel, cisplatin and gemcitabine has been reported for oral cancer cells, pancreatic and hormone-refractory prostate cancer [238-240], and a combined action with erlotinib/gefitinib (receptor tyrosine kinase inhibitors) in mouse mammary cancer cells [241]. The possible synergism or chemosensitization exerted by δ-tocotrienol with standard therapies, referring to both chemotherapies and targeted therapies, should be investigated also in melanoma models.

In addition to development of resistance, chemotherapies induce several toxic side effects, due to their aspecific cytotoxic activity. In order to assess if δ-tocotrienol might have a specific antitumor activity, without affecting normal cells, we conducted studies

of cell viability on human normal melanocytes: we did not observe a toxic effect of the compound, even at high dosages, suggesting that it may selectively target cancerous cells. Data in the literature support this finding: vitamin E-derived compounds, and specifically tocotrienols, have been reported to exert significant antiproliferative effects in breast and prostate malignant cells, but not in normal mammary or prostate epithelial cells [242]: moreover, McIntyre et al showed that highly malignant mouse mammary epithelial cells were more sensitive to the antiproliferative effects of tocotrienols than were preneoplastic or neoplastic cells [243].

Despite the anticancer properties of tocotrienols have been demonstrated in different tumors, only few studies investigated the antiproliferative and proapoptotic activity of tocotrienols in melanoma. Chang et al reported that γ-tocotrienol induced G1 cell cycle arrest and apoptosis in human melanoma cells, through suppression of NFκB, EGFR and Id family proteins, and induction of JNK signaling. In the same work, it was demonstrated that this vitamin E isoform leads to the suppression of mesenchymal markers and of the invasive behavior of the cells [244]. Beside this study, Fernandes and colleagues reported that δ-tocotrienol was effective in suppressing A375 cell proliferation, through G1 cell cycle arrest, and observed that apoptosis was concomitantly induced. Moreover, δ-tocotrienol was able to potentiate the effect of lovastatin, because of its inhibitory activity on HMGCoA reductase [245].

We demonstrated that δ-tocotrienol exerts a cytotoxic activity on both A375 and BLM melanoma cells, evidenced by the reduction in the viability of colony-forming cells. Moreover, the induction of cell death was demonstrated by Trypan blue-exclusion assay and the activation of the apoptosis process was evidenced by cleavage of procaspase-3 and PARP. Our observations about the proapoptotic effects of δ-tocotrienol are in agreement with data showing the activation of programmed cell death in tumor cells, induced by tocotrienols. Most of these studies were conducted on breast cancer cells, and, in particular, it has been reported that γ- and δ-tocotrienols have more apoptosis-inducing potency than α-tocotrienol, and this effect can be mediated by induction of both the intrinsic or extrinsic apoptotic pathway [154, 156].

A third mechanism for inducing programmed cell death is known, and it's referred as to the endoplasmic reticulum (ER) stress-induced apoptosis. To our knowledge, only two studies reported an association between tocotrienols-induced apoptosis and the activation of ER stress. Both studies were conducted on breast cancer, and were performed treating cells and animal models with γ-tocotrienol [246, 247]: in these investigations, γ-tocotrienol induced cell cycle arrest and apoptosis, that occurred concomitantly to the modulation of ER stress markers such as PERK, ATF6, IRE1α, eIF2α and the ER stress-associated apoptosis marker CHOP.

To shed light on the molecular mechanisms through which δ-tocotrienol induced apoptosis on melanoma cells, we analyzed its ability to induce the unfolded protein response (UPR) and ER stress: we found that treatment of BLM and A375 cells with 20 μg/ml of δ-tocotrienol triggered the overexpression of ER stress markers.

PERK and IRE1α are the "ER stress sensors", located in the ER membrane, that are normally kept inactive by the luminal chaperon protein BiP. Stressful conditions that cause an aberrant protein folding also stimulate the release of BiP in the ER lumen, to allow correct protein folding. This is the event that activates PERK and IRE1α in inducing cytosolic signaling pathways, that in turn activates downstream processes to overcome ER protein overload. The attenuation of global protein translation and the specific overexpression of chaperon proteins are among these mechanisms [130].

The analysis of the expression of ER stress markers in A375 and BLM cells evidenced an increase in the expression of PERK and IRE1α after treatment with δ-tocotrienol, suggesting the triggering of the UPR. Accordingly to this result, an increased expression of the chaperon protein BiP was observed: notably, this protein itself is a transcriptional target of the UPR via ER stress-responsive elements that can bind to ATF6 [248].

Importantly, we observed that δ-tocotrienol induces the expression of ATF4 and its target CHOP, both through Western blot and immunofluorescence analysis. These transcription factors are induced downstream the PERK and IRE1α pathways, and might be indicative of the activation of the ER stress-related apoptosis. Several natural compounds have been reported to induce apoptosis through the activation of ER

stress, in different type of tumors, especially through the activation of the PERK/eIF2α/ATF4/CHOP pathway. All these studies demonstrates the relationship between the induction of these two processes, since the inhibition of the ER stress branches through specific inhibitors, or through silencing of key factors such as ATF4 or CHOP, attenuated cell death [249-253].

In our experiments, the expression of PDI was not affected by δ-tocotrienol. PDI (protein-disulfide isomerase) is crucial for disulfide formation during folding of nascent peptides, and can directly catalyze this reaction in reduced substrates. Disulfide transfer will result in reduction of the active site, which must be reoxidized to carry out further oxidations: the flavoprotein ERO1α can catalyze the reoxidation of reduced PDI, by coupling *de novo* disulfide formation to the reduction of oxygen to hydrogen peroxide (H_2O_2). Accordingly to the induction of CHOP, we found that the expression of ERO1α, a target of this transcription factor, was induced by δ-tocotrienol, but in A375 cells only, while in BLM cells its protein levels was not affected by the treatment. However, both findings may be indicative of an ER imbalance towards cell death, rather than cell survival. On one hand, the induction of ERO1α in A375 cells might reflect a stimulated activity of PDI, that tries to re-establish a correct protein folding. However, if the unfolded/misfolded protein overload is too severe, its action couldn't be enough to rescue cells from the activation of apoptosis. Moreover, an excess of activity of ERO1α could lead to the overproduction of ROS, contributing to cell stress [254]. On the other hand, the lack of ERO1α induction in BLM cells might reflect a reduced activity of PDI in restoring protein folding, so that the ER stressful condition is not attenuated.

Another slight difference was observed between A375 and BLM cells, that is timing of response to δ-tocotrienol. In A375 cells, most of the effects were seen at about 6 hours of treatment, while in BLM cells the same effects were delayed and were seen at least at 18 hours of treatment. This fact could be explained by a different sensitivity to the specific compound, or by a differential proliferation rate of the two cell lines. Indeed, BLM cells have a slower proliferation rate than A375 cells, so that the antitumor activity of δ-tocotrienol may be delayed. Anyway, the final outcome was the

same for the two cell lines, that means ER stress and cell death induction through apoptotic mechanisms.

In order to relate the induction of the ER stress to the activation of apoptosis, cotreatment with salubrinal was performed. Salubrinal is an aspecific inhibitor of the ER stress-induced apoptosis, due to its inhibitory action on the eIF2α dephosphorylation. The mechanisms underlying the cytoprotection conferred by salubrinal are extremely complex: prolonging eIF2α phosphorylation, the inhibition of global protein synthesis alleviates the ER protein overload, leading to cell survival. However, this also results in ATF4 upregulation: since this protein could balance the signals leading to cell survival (by BiP) and those leading to apoptosis (by CHOP), the magnitude or duration of eIF2α phosphorylation appears to be the key factor for determining the net effects of salubrinal on cell fate. Furthermore, mechanisms independent of eIF2α phosphorylation have been suggested to explain the pharmacological actions of salubrinal, such as the preservation of Bcl-2 function [255]. Cotreating A375 and BLM cells with δ-tocotrienol and salubrinal, an inhibition of the cytotoxic effects of δ-tocotrienol was observed. The reduction in the cleavage of caspase-3 and the inhibition of the reduction of cell viability suggest that a relationship exists between the δ-tocotrienol-induced ER stress and the activation of programmed cell death. As for the ER stress markers expression modifications, slightly differences in timing of responses were observed between the two melanoma cell lines. The inhibition of δ-tocotrienol effects occurred at 24 hours for BLM cells, and at 18 hours for A375 cells, accordingly to the data described above. Moreover, different salubrinal dosages were used in different cell lines to allow its inhibitory effects. Indeed, BLM cells were treated with 25 μM of the compound, while A375 were more sensitive to the inhibition (10 μM concentration was used). This is likely due to the quickness of response of A375 cells to δ-tocotrienol treatment: as higher is the amplitude of its cytotoxic effects, as sensitive will be cells to the inhibition of these processes. However, in both melanoma cell lines, the reversal of the apoptotic activity of δ-tocotrienol was only partial. This may be explained, at least partially, by the fact that salubrinal only targets the PERK pathway of the ER stress induction, while it does not

affect the IRE1α and ATF6 branches. Furthermore, we hypothesized that other apoptotic mechanisms might support the ER stress-induced cell death.

Caspase-4 is the caspase associated with the endoplasmic reticulum membrane, that is activated following ER stress, contributing to the triggering of the ER stress-related apoptosis process. Caspase-4 cleavage has been linked to calpain activation, in response to the release of ER Ca^{2+} stores after exposure to ER stressors. Once activated, caspase-4 can, in turn, activate caspase-9 or -3, thereby leading to MOMP-independent cell death [193]. Several studies in the literature show the ability of nutraceutical compounds to stimulate apoptosis through ER stress/caspase-4 activation: the flavonoids wogonin and licochalcone have been demonstrated to exert an antitumor activity on neuroblastoma and hepatocellular carcinoma cells [256, 257], and resveratrol induced ER expansion and ER stress-mediated apoptosis in nasopharyngeal carcinoma cells [258]. In all these studies, the activation of caspase-4 has been observed. We then performed experiments in order to investigate whether δ-tocotrienol could induce the cleavage, that means the activation, of caspase-4. We found that this caspase was activated by δ-tocotrienol treatment, in both cell lines, even if differences in timing of response were observed again: the cleavage occurred at 18 hours treatment for BLM cells, and at 1-6 hours treatment of A375 cells, accordingly to data obtained for the expression of ER stress markers.

Other studies support the notion that δ-tocotrienol is able to induce apoptosis in different tumor cells, by downregulation of antiapoptotic proteins such as Bcl-2, Bcl-xL and Mcl-1, or upregulating proapoptotic proteins such as Bax [234, 259-262].

Given the above discussed results about the ER stress involvement on inducing cell death upon δ-tocotrienol treatment, we wanted to verify if this compound might activate the intrinsic apoptosis pathway on human melanoma cells. In both BLM and A375 cell lines we observed that δ-tocotrienol induced an alteration in the Bax/Bcl-2 ratio, in favor of the triggering of the apoptotic cell death; however, while in BLM cells the variation of this ratio is due to the increased expression of Bax, after 1 hour treatment, in A375 cells the imbalance is due to the reduced expression of Bcl-2, but after 18 hours of treatment. The difference in the timing of response is now inverted,

with a faster reaction in BLM than in A375 cells. Hence, in A375 cells, apoptotic death could be mainly triggered by a CHOP-dependent way. Indeed, it is noteworthy that Bcl-2 is a target of CHOP: if the ER stress signaling precedes the mitochondrial apoptotic activation, then in A375 cells the reduction of the expression of Bcl-2 might be induced by CHOP; i.e., the ER stress may be the inducer of the intrinsic apoptosis in A375 cells. On the other hand, in BLM cells, changes in Bax expression occurred at shorter times than the ER stress markers: in this case the activation of the mitochondrial way may anticipate the ER stress-induced apoptosis. Another possibility is that the increased expression of Bax observed in BLM cells might be indicative of the alteration of Ca^{2+} storage in the endoplasmic reticulum. Indeed, overexpression of Bax/Bak at the level of ER induces a transient Ca^{2+} accumulation, followed by store depletion due to the induction of apoptosis [193]: a major area of functional interaction between the ER and mitochondria is the control of Ca^{2+} signaling. Mitochondria play an important role in shaping the Ca^{2+} signal released from the ER, because they assist in the recovery phase by rapidly sequestering Ca^{2+} and then, later, returning it to the ER. The key process connecting apoptosis to ER-mitochondria interactions is an alteration in Ca^{2+} homeostatic mechanisms that results in massive and/or prolonged mitochondrial Ca^{2+} overload, leading to a breakdown of mitochondrial function [263].

The release of cytochrome *c* from mitochondria was observed through immunofluorescent analysis in both A375 and BLM cell lines, and supports the observed changes in the Bax/Bcl-2 ratio in demonstrating the activation of the intrinsic apoptosis pathway, as well as the mitochondrial breakdown.

Finally, the efficacy of δ-tocotrienol against melanoma was confirmed in preclinical studies on A375 mouse xenografts, treated with δ-tocotrienol purified from Annatto seeds. In particular, we could observe a significant reduction of tumor volume over time and a significant reduction in tumor mass at the end of the treatment with respect to control animals; moreover, δ-tocotrienol was able to delay tumor progression. All these findings were not accompanied by systemic toxicity, suggesting that δ-tocotrienol might act selectively on tumor cells, supporting the above discussed results on human non-cancerous melanocytes. Indeed, normal cells were not affected

by δ-tocotrienol treatment *in vitro*. So far, γ-tocotrienol feeding has been reported to decrease tumor weight and to prolong survival rate of C57BL female mice transplanted with murine B16 melanoma cells [264]. Furthermore, it has been reported that administration of tocotrienol mixture significantly suppresses liver and lung carcinogenesis: Hiura et al [265] showed that, *in vivo*, γ- and δ-tocotrienols significantly delayed tumor growth, without effects on body weight and tissue weight of mice, and in particular they demonstrated that these two isomers accumulated only in tumors. The composition of tocotrienol/tocopherol mixtures plays an important role in their accumulation in tissues. For example, tocotrienols accumulate in more "fatty" tissues, such as adipose tissue and skin; moreover, the compounds are metabolized in a tissue-dependent manner. The same authors also reported that neither γ- nor δ-tocotrienol significantly changed serum Ig levels and Ig productivity of the spleen, suggesting that the immune function may be not involved in the tocotrienol-induced tumor suppressing effects and that the antitumor activity may be due to the direct effects of tocotrienols on tumor cells.

In summary, our findings demonstrate that δ-tocotrienol is endowed with a potent antitumor activity both *in vitro* and in preclinical models of human melanoma. Its antitumor activity *in vitro* has been demonstrated on two distinct human melanoma cell lines, that are representative of the main critical Ras/Raf mutations found in melanoma patients, so that δ-tocotrienol is able to induce melanoma cell death independently of the overactivation of the MAPK and PI3K pathway. This fact could have important implications on the management of malignant melanomas, because the acquired resistance of most melanomas, both to chemotherapies and to the more recent targeted therapies, depends on the acquisition of new mutations on these signaling pathways. Our initial aim was to assess if the anticancer activity of δ-tocotrienol might be, at least in part, due to the interaction with the estrogen receptor β. However, we did not find a relationship between the estrogenic system and δ-tocotrienol on melanoma cells: the natural compound is not able to transactivate the receptor and, moreover, its anticancer effects are independent of the expression and the functionality of ERβ. We then analyzed the molecular mechanisms associated with

such antitumor activity and we demonstrated that δ-tocotrienol induces cell death through a crosstalk between the ER stress-related apoptosis and the intrinsic apoptosis pathway.

The third, that is the last, task of the project was focused on the role of δ-tocotrienol on the most aggressive population of tumor cells, responsible for tumor initiation and recurrence, that means the cancer stem cells. We firstly set up the culture maintenance of this kind of tumor cells, and we concomitantly characterized them, in order to find an appropriate melanoma stemness marker.

Three putative tumor stemness markers were considered: CD271, a melanoma de-differentiation marker because of its physiologic expression on neural crest; CD44, involved in cell migration and in cell-cell and cell-matrix interactions; and ABCB5, an ATP-binding cassette transporter associated with drug efflux and development of drug resistance. The expression of these proteins was analyzed on A375, BLM and WM115 melanoma cell lines. The only protein that was differentially expressed was CD271, that is absent in BLM cells. The others were expressed in all the cell lines considered. All previous studies were performed on A375 and BLM cells: since CD271, CD44 and ABCB5 were expressed on A375 cells, while BLM cells do not express CD271, we conducted the subsequent experiments and stem cells characterization on A375 cells.

Immunofluorescent analyses were then performed in order to assess the pattern of expression of the three putative markers on A375 cells. While CD44 and ABCB5 were found to be expressed in all the cells, CD271 is expressed in a differential way, because some cells show positivity for this protein, some other do not. This fact makes CD271 a good candidate marker for a melanoma subpopulation of cells. Indeed, we found that cells grown in melanospheres were enriched for this marker, and all the cells in a melanosphere do express it. These findings indicate that A375 cells are composed by at least two populations, one showing CD271 positivity and the ability to grow into melanospheres, another without these features. Boiko et al [266] demonstrated that in patient's derived melanomas, CD271 is heterogeneously expressed (from 2.5 to 41% of the total cell population). In this study, the authors demonstrated that tumor stem cells can be isolated as a highly enriched $CD271^{+}$ population, that is able to induce

tumors and re-establish the original CD271 expression heterogeneity of the primary tumor in xenotransplantation assays. Other reports showed the $CD271^+$ cells enrichment *in vitro,* in the subpopulation of cells that grow in spheroids: the WM115 melanoma cell line was found to be composed of two subpopulations of cells. In these experiments cells were analyzed for the expression of two tumor stem cell marker, CD271 and CD20. The parental cell line, propagated as monolayer, had 0.15% of $CD20^+$ and $CD271^+$ cells, whereas the non-adherent, spheroid subpopulation has 2.32% of these double positive cells [267], accordingly to our results and confirming that CD271 can be exploited as melanoma stem cell marker.

Cancer stem cells have been associated with loss of the expression of differentiation markers: for example, dedifferentiated melanoma cells loose pigmentation and the expression of melanocytic markers such as tyrosinase, MITF and S-100 protein. Tumor dedifferentiation is a well-known phenomenon and it has been proposed to be involved in tumor progression; moreover, poorly differentiated tumors show overexpression of genes normally enriched in embryonic stem cells, such as the reprogramming factors Oct4, Nanog, Sox2 and c-Myc. In our studies, we found that melanospheres-forming A375 cells show positivity for the embryonic stem cell marker Oct4. This is a key transcription factor for the reprogramming of somatic cells to induced pluripotent stem cells, since it can mediate this reprogramming as a single factor. It has been demonstrated that Oct4 is detectable in a variety of cancer types, including melanoma, and the cancer stem cell-like phenotype is characterized by Oct4 expression enrichment. Furthermore, Oct4 expression in associated with worse clinical outcome in breast cancer, and its knockdown results in breast CSCs apoptosis. Kumar et al demonstrated that forced expression of Oct4 induces dedifferentiation of melanoma cells, and the dedifferentiated melanoma cells acquire a cancer stem cell-like phenotype, with higher proliferation rates, increased tumorigenicity and metastatic capacity, increased resistance to cisplatin and increased expression of the CSCs markers CD271 and ABCB5 [268]. On the contrary, we did not find a differential expression of the CSCs markers ABCB5 and CD44, possibly suggesting that, rather than

drug resistance and epithelial-to-mesenchymal transition, the dedifferentiation phenotype is crucial for determining cell stemness.

Once characterized the cancer stem cells in our *in vitro* model, we performed experiments in order to verify the effectiveness of δ-tocotrienol on such population of cells. We could observe that the vitamin E derivative significantly reduces the number and dimensions of melanospheres at higher dosage than the reduction in cell viability on adherent culture (40 μg/ml *vs* 20 μg/ml), even if a trend in the antiproliferative activity is seen also with 20 μg/ml. This result could be explained by the fact that cancer stem cells represent the high tumorigenic and resistant population of cells in a tumor bulk, so that their aggressiveness can be counteracted only with a strongest treatment.

Several studies reported the efficacy of nutraceuticals against cancer stem cells. Isothiocyanates, genistein, sulphoraphane and quercetin have been demonstrated to possess the ability to target CSCs self-renewal through the inhibition of crucial proliferative pathways, such as Wnt/β-catenin, Sonic Hedgehog, NFκB signaling. Some key nutrients, as B vitamins, sulfur, iron, magnesium and manganese, together with resveratrol and green tea polyphenols, affects cancer stem cell metabolism, towards an increment of OXPHOS [269]. At the same extent, melanoma stem cells can be targeted through the interference with specific stem cell pathways: for example, Kaushik et al demonstrated that honokiol, a biphenolic compound derived from *Magnolia officinalis*, can affect melanoma stem cells by targeting Notch signaling, with a reduction of stem cell self-renewal and a reduction of the expression of melanoma stem cell markers such as CD271 and ABCB5 [270].

The above discussed results, obtained treating the whole population of adherent A375 melanoma cells, show that δ-tocotrienol induces cell death through the activation of the ER stress-related apoptosis. Whether the ER stress induction might be responsible for the antitumor activity on cancer stem cells remains to be investigated. However, different studies in the literature demonstrate that the induction of the ER stress can be involved in the cancer stem cells impairment. Tseng et al [271] demonstrated that brefeldin A, a lactone antibiotic from the fungus *Eupenicillium brefeldianum* known to

inhibit the transport of proteins from endoplasmic reticulum to Golgi, activates the ER stress process and induces cell death on both adherent cells and spheroid suspension of MDA-MB-231 breast cancer cells. Moreover, these processes are associated with the reduction in the expression of the breast cancer stem cell marker CD44. The same authors demonstrated a similar activity of brefeldin A on colon cancer cells [272]. Other reports show that the activation of the ER stress sensitizes cancer stem cells to chemotherapy. In a study conducted on colon cancer stem cells, it has been demonstrated that the UPR forces these stem cells to differentiate, as it does to induce the differentiation of normal intestinal epithelial stem cells. Indeed, the activation of the ER stress was associated with the loss of expression of intestinal stem cell markers, such as OLFM4 and LGR5, together with the reduction of the expression of the cancer stem cell marker CD133 and the upregulation of enterocyte markers such as CK20, VIL2, FABP2. Furthermore, this kind of differentiation leads to the sensitization of colon cancer stem cells to conventional chemotherapies [273].

In our experiments the effect of δ-tocotrienol on melanospheres was supported by the reduction of the expression of the stemness marker CD271 on melanosphere-forming cells, confirming the antitumor ability of the compound on this high malignant subpopulation of cells. Further studies must be performed in order to analyze the molecular mechanisms leading to such antitumor efficacy.

All these features make δ-tocotrienol a very interesting compound, at least for combinational or adjuvant therapies, since cancer stem cells are responsible for drug resistance and tumor recurrence. Obviously, these results must be confirmed *in vivo*, to test the real highest tumorigenicity of $CD271^{+}$ -A375 cells and the effectiveness of δ-tocotrienol in reducing not only tumor mass, but also the cancer-initiating ability of melanoma stem cells.

CONCLUSIONS

Conclusions

In summary, results obtained from this project demonstrated that:

1- ERβ has a tumor suppressive function on melanoma cells, acting on tumor cell proliferation and epigenetic reprogramming. However, its protective role is influenced by the specific mutational status of the tumor cell. In particular, ERβ activation is effective in reducing cell proliferation in melanoma cells harboring the NRAS (but not the BRAF) mutation.

   Malignant melanoma presents a high mutational heterogeneity, and these results confirm the importance of the genetic profiling of tumor samples in dictating the more appropriate therapeutic strategy. Based on our findings, ERβ could be exploited as a molecular target for melanomas harboring the NRAS mutation.

2- The vitamin E derivative δ-tocotrienol exerts an antitumor activity on melanoma cells, and its effect does not seem to be mediated by the estrogen receptor β. Moreover, its effectiveness is independent of the specific mutational status of the tumor cell. The molecular mechanisms responsible for the antitumor activity of δ-tocotrienol are associated with the activation of the apoptosis process *via* endoplasmic reticulum stress. δ-tocotrienol is also effective on the highly aggressive population of cancer stem cells, that have been characterized in our *in vitro* melanoma model through melanoma stem cell markers expression and ability to grow on spheroid cultures. Accordingly, our preclinical studies show that the natural compound is able to reduce tumor volume and mass, and to delay tumor progression on mice melanoma xenografts.

   These results, obtained from *in vitro* and *in vivo* experiments, strongly support the notion that clinical studies should be performed, in order to assess if δ-tocotrienol could be considered as a possible novel therapeutic strategy, at least as adjuvant therapy, in the management of metastatic melanoma.

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