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Tetraspanins and Melanoma: Role of CD63 in the control of cell motility, invasiveness and proliferation

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Abstract

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Premises:

Melanoma is one of the most aggressive tumors with high metastatic potential and rate of mortality. Over the last decades, the increase in incidence have mainly been reported for thin melanomas (≤1mm) associated with a good prognosis. Whereas the rate for thick melanomas (>1mm), with unfavourable prognosis and associated with a higher rate of distant metastases and worse survival, seems to be fairly stable. Hence, there is a need to identify new prognostic markers of progression and the new potential drug targets. A possible negative marker of metastasis is the CD63 protein, belonging to the Tetraspanins superfamily. It is highly expressed in several normal tissues as well as in the early stage of melanoma. We have previously observed a progressive decrease of CD63 protein levels in tissue samples of human melanocytic nevi, dysplastic nevi, thin melanomas, thick melanomas and metastasizing melanomas in advanced stages of melanoma progression. This suggests that the gene coding CD63 could be a potential *metastasis suppressor.*

Working hypothesis:

We hypothesized that CD63 could play a functional role in controlling cell proliferation, motility and invasion, probably by means of functional interactions with other proteins playing key role in these processes. Based on data reported in literature, the pro-metastatic proteins uPAR, MMP-2, MMP-9 and the tissue inhibitor of metalloproteinases-1 (TIMP-1) seemed the best candidates. We also supposed that CD63 could play a role in the Epithelial-Mesenchymal transition (EMT), acting as negative marker of this process.

Results:

We demonstrated that the exogenous modulation of CD63 in human melanoma cell line A375 was correlated with cell proliferation, invasiveness and motility. In particular, A375 cells overexpressing CD63 shown a significant reduction in cell proliferation, motility and invasiveness compared to untreated control and compared to cell lines where CD63 has been reduced with siRNA. We also demonstrated that CD63 was inversely correlated with the pro-metastatic proteins uPAR, MMP-2 and MMP-9, whose levels were reduced in cells overexpressing CD63.

We also observed that B16 murine melanoma cells stimulated by inflammatory cytokines shown a reduction of CD63 tetraspanin expression and this was associated with the promotion of a metastatic phenotype, characterized by a high capacity to colonize host lungs and expressing a high level of uPAR and MMP-9 (*Bianchini F et al., Onc Rep 2006*). The reduction of CD63 expression was dose-dependent and proportional to the increase of IFNγ stimulation. However the molecular mechanism awaits to be unrevealed.

Recently, many studies reported the role of epithelial-mesenchymal transition in matrix degradation and cell invasiveness. We observed that CD63 expression in A375M6 cells, established from a colony isolated from the liver of immunodeficient mouse and derived from A375 iv inoculation, that shown a non-EMT profile, was higher compared to A375 and significantly decreased after treatment with the EMT inducer TGFβ. To explain and confirm this observation, we performed an invasion assay. We observed an increased invasiveness of clones overexpressing CD63 stimulated with medium derived from silenced cells as compared to invasion capacity of non-stimulated cells, probably due to a major content of MMPs.

Conclusions:

In summary, these results strongly point the high expression of CD63 as a major contributor to a non-invasive phenotype in melanoma. As the invasive phenotype of cells involves induction of molecular changes associated with EMT, we have reason to

believe that CD63 may play a key role in these processes, acting as a negative marker of EMT.

Finally, we conclude that CD63 has a potential to be a suppressor gene playing an important role in inhibition of melanoma progression, and as such could be a target for pharmacological and drug developing strategies.

Acknowledgments

We are grateful to Ente Cassa di Risparmio di Firenze and Regione Toscana for their supports to this research.

I

Introduction

1.1 Malignant Melanoma

1.1.1 Etiology and Pathogenesis

Malignant melanoma is a type of cancer arising from the melanocyte cells of the skin **(Figure 1)**. Melanocytes, that derive from a structure in the human embryo called neural crest, produce a pigment called melanin and are responsible for racial variations in skin colour as well as the colour of moles. They are distributed in the epidermis throughout the skin. Malignant melanoma develops when the melanocytes no longer respond to normal control mechanisms of cellular growth. They may then invade nearby structures or spread to other organs in the body, compromising their function.

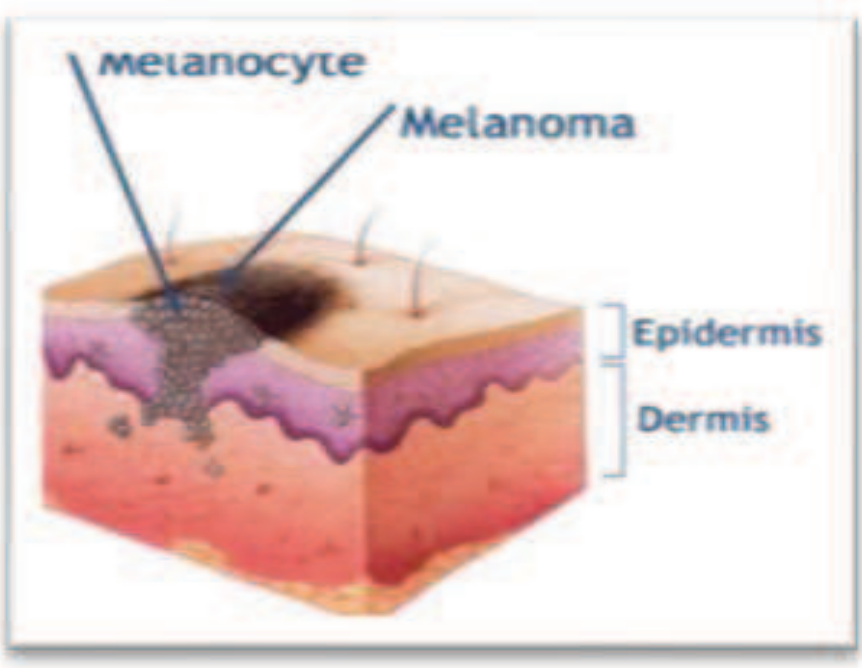


Figure 1. Malignant melanoma develops when the melanocytes no longer respond to normal control mechanisms of cellular growth. They may then invade nearby structures or spread to other organs in the body, compromising their function

There are four types of melanoma:

- *superficial spreading melanoma*:
- *nodular melanoma*;
- *lentigo maligna;*
- *acral lentiginous melanoma*,

accounting for 50 to 75%, 15% to 35%, 5% to 15%, and 5% to 10% of cases, respectively[1].

The *superficial spreading melanoma (SSM)* type often arises from a pigmented dysplastic nevus and typically develop after a long-standing stable nevus changes; typical changes include ulceration, enlargement or colour changes. A superficial spreading melanoma may be found on any body surface, especially the head, neck and trunk of males and the lower extremities of females.

Nodular melanomas (NMs) are found commonly on all body surfaces, especially the trunk of males. These lesions are the most symmetrical and uniform of the melanomas and are dark brown or black. The radial growth phase may not be evident in nodular melanomas; however, if this phase is evident, it is short-lived, because the tumor advances rapidly to the vertical growth phase, thus making this kind of tumor a high-risk lesion. Approximately 5% of all nodular melanomas are amelanotic melanomas.

Lentigo maligna melanomas (LMMs) are typically found on sun-exposed areas (hand, neck); they may have areas of hypopigmentation and often are quite large. It is basically in situ melanoma and is characterized by epidermal atrophy, extensive solar, lentiginous, and back to back proliferation of melanoma cells with nest formation extending into cutaneous adnexa. Only 5% of patients with lentigo maligna progress to lentigo malignant melanoma, and it usually takes several years.

Acral lentiginous melanomas (ALMs) are the only melanomas that have an equal frequency among blacks and whites. They occur on the palms, soles, and subungual areas. Subungual melanomas often are mistaken for subungual hematomas (splinter hemorrhages). Acral lentigo melanoma is extremely aggressive, with rapid progression from the radial to vertical growth phase.

Mucosal lentiginous melanomas (MLMs) develop from the mucosal epithelium that lines the respiratory, gastrointestinal, and genitourinary tracts. These lesions account for approximately 3% of the melanomas diagnosed annually and may occur on any mucosal surface, including the conjunctiva, oral cavity, esophagus, vagina, female urethra, penis, and anus.

Noncutaneous melanomas commonly are diagnosed in patients of advanced age. MLMs appear to have a more aggressive course than cutaneous melanomas, although this may be because they commonly are diagnosed at a later stage of disease than the more readily apparent cutaneous melanomas[2].

Melanomas have two growth phases, radial and vertical: during the radial growth phase, malignant cells grow in a radial direction, according to concentric circles, remaining confined to the epidermis (**melanoma in situ**), or showing an initial focal infiltration of the papillary dermis in the form of isolated cells and arranged in small nests (**microinvasive melanoma**). With time, most melanomas progress to the vertical growth phase, indicated by the appearance of one or more cell clones which acquire capacity for autonomous proliferation and invasive capacity with formation of aggregates or nodules which extend to the dermis or subcutaneous tissues (**Figure 2**).

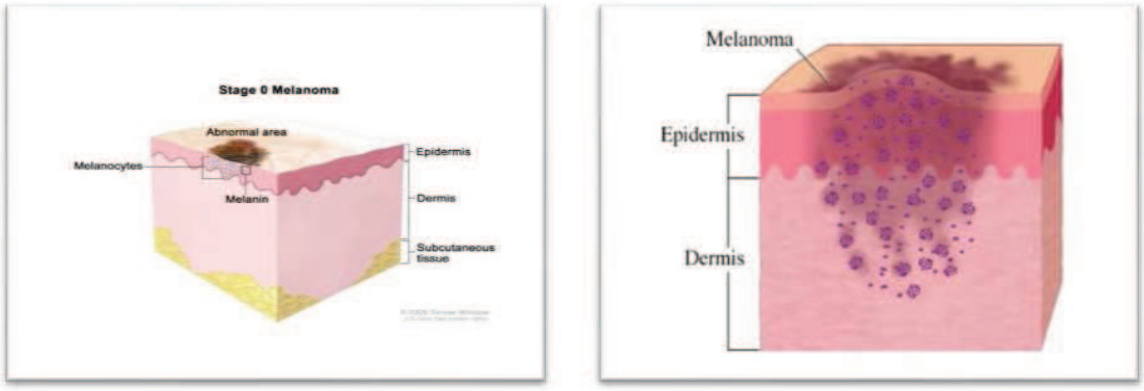


Figure 2. Melanomas have two growth phases, radial and vertical: during the radial growth phase, malignant cells grow in a radial direction, according to concentric circles, remaining confined to the epidermis (melanoma in situ), or showing an initial focal infiltration of the papillary dermis in the form of isolated cells and arranged in small nests (microinvasive melanoma). With time, most melanomas progress to the vertical growth phase, indicated by the appearance of one or more cell clones which acquire capacity for autonomous proliferation and invasive capacity with formation of aggregates or nodules which extend to the dermis or subcutaneous tissues

The locally invasive characteristic of this tumor involves vertical penetration through the skin and into the dermis and subcutaneous tissues of the malignant melanocytes. With the exception of the nodular variety of melanoma, there is often a phase of radial or lateral growth associated with these tumors. Since it is the vertical growth that characterizes the malignancy, the nodular variant of melanoma carries the worst prognosis. Fortunately, the superficial spreading type is most common. The primary tumor begins in the skin, often from the melanocytes of a pre-existing mole. Once it becomes invasive, it may progress beyond the site of origin to the regional lymph nodes or travel to other organ systems in the body and become systemic in nature.

Untreated malignant melanoma follows a classic progression: it begins and growths locally, penetrating vertically. It may be carried via the lymph to the regional nodes, known as regional metastasis. It may go from the lymph to the bloodstream or penetrate blood vessels, directly allowing it a route to go elsewhere in the body. When

systemic disease or distant metastasis occur, melanoma commonly involves the lung, brain, liver, or occasionally bone. The malignancy causes death when its uncontrolled growth compromises vital organ function.

1.1.2 Incidence and trend of Melanoma

The annual increase of cutaneous melanoma (CM) incidence varies between populations but has been estimated at between 3% and 7%[3, 4], with an estimated doubling of rates every 10 or 20 years. *Cutaneous malignant melanoma (CMM)* is the most rapidly increasing cancer in white populations: in the United States was reported to be 6 cases per 100,000 inhabitants at the beginning of the 1970s and 18 cases per 100,000 inhabitants at the beginning of 2000, demonstrating a threefold increase in incidence rates[3, 4]. A similar date was reported in central Europe where the incidence rate was increased, in the same time period, from 3 to 4 cases to 10 to 15 cases per 100,000 inhabitants per year[3].The Federal Health Authority in Germany used several regional population-based cancer registries to estimate the trend of incidence of CM, and a threefold increase resulted during a 3-decade period[3] **(Figure 3)**.

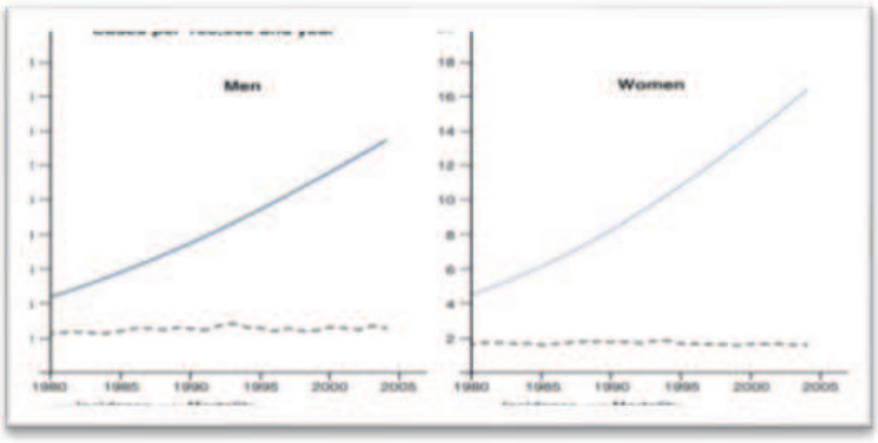


Figure 3. Age-standardized (European standard population) incidence and mortality rates in the Federal Republic of Germany during 25 years. Estimates of incidence rates are based on data from several cancer registries in different Federal States**[3]**.

The frequency of cutaneous melanoma is closely associated with the constitutive colour of the skin and depends on the geographic zone. From the 1990s onwards, studies from Europe, Canada, the United States and Australia reported that the increase of incidence rates was slowing or stabilizing[3,6-10]. In 2003 in the United States, 54,200 new diagnoses of *cutaneous melanoma* and 7600 deaths from melanoma were reported[4, 11].Incidence rate in central Europe are in the middle between the high-incidence countries and low-incidence countries (**Figure 4**). The highest rates of incidence in Europe were in Scandinavia countries [4,12,13], but significant increases of melanoma incidences were also found in central and southern Europe. The Mediterranean countries had lowest incidence rates[14-16]. The reason for this north – south gradient is a darker skin type (type III – IV according to Fitzpatrick) in the Mediterranean populations and different attitudes to recreational activities. The highest incidence rates was reported in Australia and New Zealand, with 30 to 60 cases per 100,000 inhabitants and year[10,17-19]. In these countries, cutaneous melanoma is one of the most frequent cancer types. The highest incidence rates were found in the northern equatorial areas of Australia such as in Queensland, where incidence rates up to 60 per 100,000 inhabitants and year were registered[4].

Figure 4. Age-standardized (world standard population) incidence rates from 17 countries worldwide for the year 2002. USA, United States of America[3].

1.1.3 Sun exposure and Melanoma

The incidence of primary cutaneous malignant melanoma has been steadily increasing, possibly related to increase of sun exposure. It affects all age groups but is most commonly seen in patients between 30 and 60 years of age. Sun exposure definitely increases the risk of developing melanoma, particularly in older males[5]. The melanocytes are part of the integument's photoprotective mechanism; in response to sunlight, they produce melanin that has a protective role from the sun's ultraviolet rays. For Caucasians, the amount of melanin present in the skin is directly related to sun exposure. However, it is not so much the total sun exposure that seems important, rather it is the history of sunburn, (especially if severe or at an early age), that correlates with the increased risk[22-25]. On this basis populations of fair-skinned people living in areas of high sun exposure such as the southwest United States or Australia are subject to increased risk. Malignant melanoma also affects non-Caucasians—though sun exposure probably does not play a role—at a rate of 10% that of Caucasians. The most common form of melanoma in Afro-Americans is *acral lentiginous melanoma*. An exposure substantial enough to result in a mild sunburn will be followed by melanin producing a tan that may last a few weeks. Both ultraviolet radiation and damaging oxygen radicals caused by sun exposure may damage cells, particularly their DNA. It is suspected that this damage induces mutations that result in the development of malignant melanoma. Though these mutations are alterations of the genome causing the melanoma, they are environmentally induced and account for sporadic or spontaneous cases of this disease[5].

1.1.4 The Biology and Genetics of melanoma

Malignant melanoma may arise in the skin anywhere on the body. It is estimated that 50%-70% develop spontaneously while the remainder start in a pre-existing mole.

The predisposing causes to the development of malignant melanoma are environmental and genetic. The environmental factor is excessive sun exposure. In 2003 some researchers proposed that there may be two pathways to malignant melanoma, one involving exposure to sunlight and the other with melanocyte proliferation triggered by other factors. This hypothesis is based on the difference in distribution of moles on the body between patients who develop melanomas on the face and neck, and those who develop melanomas on the trunk. A positive family history of one or two first-degree relatives having had melanoma substantially increases the risk[6]. A family tendency is observed in 8% to 12% of patients. There is a syndrome known as the *dysplastic (atypical) nevus syndrome* that is characterized by atypical moles with bothersome clinical features in children under age ten. Such individuals have to be observed closely for the development of malignant melanoma[7]. Two genes have been discovered in melanoma families: CDKN2A, which encodes for the tumor-suppressing proteins p16 and p19, on chromosome 9p21 and CDK4 on chromosome 12[1,26,28,29].

The CDKN2A gene acts as a tumor suppressor gene and plays a crucial role in cell cycle regulation and senescence[30]. Mutations of this gene confer susceptibility to familial melanoma. There are mutations in up to 50% of familial melanoma patients of the tumor-suppressing gene CDKN2A. Partial or complete loss of p16 expression has also been identified in sporadic melanomas.

In 2003 a group of Swedish researchers reported that 63 out of 71 melanoma patients, or 89% of the group, had mutations in either the NRAS or the BRAF gene. The researchers found that these mutations occur at an early point in the development of melanoma and remain as the tumor progresses.

Other genes, such as MC1R (Melanocortin 1 Receptor) and DNA repair genes, are likely to be more important in determining susceptibility for melanoma in the general population[8].

Although nevi and melanomas share initiating genetic alterations such as oncogenic mutations in BRAF and NRAS, melanoma often shows recurrent patterns of chromosomal aberrations such as losses of chromosomes 6q, 8p, 9p, and 10q along with gains of chromosomes 1q, 6p, 7, 8q, 17q and 20q, while benign nevi tend to have no detectable chromosomal aberrations by comparative genomic hybridization (CGH) or kariotyping[31,32]. Alteration of cell cycle proteins (CD1, pRb, p16) has a role in transformation and progression in melanocytic tumors. It has been shown that progressive loss of p16 can be seen in transformation of benign nevi to melanoma and to metastatic melanoma. Progressive increase in expression of cyclin D1 and pRb is associated with progression to melanoma cells; however, cyclin D1 and pRb show relative decrease in thick melanoma and metastatic melanoma[33]. Higher expression of PAR-1 (protease-activated receptor-1) is seen in melanoma cell lines and tissue specimens. Upregulation of PAR-1 mediates high levels of Cx-43 (gap junctional intracellular communication molecule connexine) expression. This protein is involved in tumor cell diapedesis and attachment to endothelial cells[34]. Type 1 collagenase and PAR-1 activating functions of MMP-1 (matrix metalloproteinase-1) is suggested to be involved in progression of non-invasive melanoma to invasive vertical growth by degrading type I collagen of skin[35]. Protein Kinase C (PKC) mediates signals for cell growth and is a target of tumor-promoting phorbol esters in malignant transformation[9]. Down regulation of E-chaderin and upregulation of N-chaderin may be seen in melanoma cells. Such shift of chaderin profile may have a role in uncontrolled proliferation, invasion and migration[10]. S100A1, S100B, Bcl-2 and CD44 have been associated with the transformation of melanocytes to melanoma cells. S100A1 expression is increased in contrast to S100B, which shows higher expression in benign nevi. It has been demonstrated that CD44 antigen shows higher expression in melanomas with known metastases than in those without metastases[38]. Interaction of the transcription factor E2F-1 with RGFR can act as driving force in melanoma progression[11]. Another important study demonstrated that vascular endothelial growth

factor (VEGF) and its receptors (VEGFR1, VEGF-R2 and VEGF-R3) are higher in melanomas and advanced melanomas than in benign nevi[40]. Immunochemistry analysis has shown significantly higher cortactin (a multidomain actin-binding protein important for the function of cytoskeleton) expression in melanomas than in nevi and higher expression in metastatic melanomas than in invasive primary melanomas[41].

MHC molecule overexpression in earlier stages of melanoma and down regulation in metastatic malignant melanoma have been observed[42].

Loss of PEDF (pigment epithelium-derived factor) appears to be associated with invasive phenotype and malignant progression[43].

It has also been demonstrated that deregulation of microRNAs (miRNAs) using cell lines from primary metastatic melanoma contributes in formation and progression of melanoma[44].

Melanoma chondroitin sulphate proteoglycan (MCSP) facilitates the growth, motility and invasiveness of tumor cells; it's expression is associated with increased expression of c-Met and HGF. c-Met inhibition limits growth and motility of melanoma cell lines[45]. Up-regulated expression of CRaf is seen in a subset of melanomas[46].

ATP-binding cassette (ABC) transporters regulate the transport of physiologic substrates; ABC-transporter mRNA expression profile may have some roles in melanoma tumorigenesis[12]. It has been suggested that there is transient upregulation of cDNA clone pCMa1 in neoplastic progression of melanocytes[48].

1.1.5 Signs and symptoms

The actual number of moles increases risk, but the size of the moles needs to be considered. Those with 10 larger moles of over 1 cm are at more risk than those with a higher number (50-99) of smaller moles. Finally, child that is born with a large congenital mole have an increased risk.

A good way to identify changes in a mole is the **ABCDE rule**:

- *asymmetry*,
- *border irregularity*,
- *colour variegation*,
- *diameter* greater then 0,6 mm,
- *elevation* about surrounding tissue.

Three of the criteria refer to viability of the lesion. Changes in a mole or the rapid development of a new one are very important symptoms.

Another way to analyse changes in a mole is the **Glasgow 7-points scale**:

- *change in size*,
- *change in shape*,
- *change in colour*,
- *inflammation*,
- *crusting and bleeding*,
- *sensory change* and
- *diameter* greater than 7 mm.

Symptoms related to the presence of regional disease are mostly those of nodules or lumps in the areas containing the lymph nodes draining the area. Thus nodularity can be found in the armpit, the groin or the neck if regional nodes are involved. There is also a special type of metastasis that can occur regionally with malignant melanoma; it is known as an *in-transit metastasis*. If the melanoma is spreading through the lymph system, some of the tumor may grow there, resulting in a nodule part way between the primary site and the original lymph node. These in-transit metastasis are seen both at the time of original presentation or later after primary treatment has been rendered, the latter being a type of recurrence. Finally, in those who either present with or progress to widespread or systemic disease, symptoms and

signs are related to the affected organ. Thus neurologic problems, lung problems, or liver problems develop depending on the organ involved.

The key to successful treatment is early diagnosis. Patients identified with localized, thin, small lesions (typified by superficial spreading subtype) nearly always survive. For those with advanced lesions, the outcome is poor in spite of progress in systemic therapy.

1.1.6 Clinical staging

Malignant melanoma is locally staged based on the depth of penetration through the skin and its appendages. There are two ways of looking at the depth of penetration.The ***Clarke system*** originally developed by W. H. Clark, back in 1966, utilizes the layers of the dermis and the skin appendages present at that layer to identify the depth of penetration. Clark levels are defined as follows:

- Level I: confined to the epidermis, called "in situ" melanoma;
- Level II: invasion of the papillary dermis
- Level III: filling of the papillary dermis, but no extension in to the reticular dermis
- Level IV: invasion of the reticular dermis
- Level V: invasion of the deep, subcutaneous tissue

The ***Breslow system***, first reported by Alexander Breslow, in 1970, uses the absolute measurement of depth. The Breslow thickness is defined as the total vertical height of the melanoma, from the “granular layer" to the area of deepest penetration into the skin **(Table 1)**.

The Clarke system is used less frequently because of the fact that skin is of different thickness in different regions of the body. The depth of penetration is much greater when the tumor reaches the subcutaneous fat when the skin involved is the

back as opposed to the face. The Breslow measurement is more reproducible and thus more useful. In Breslow classification, stage I and stage II have no involvement of the regional lymph nodes and are thus localized to the site of origin. These stages are subdivided on the basis of penetration. Stage Ia is 0.75 mm or less, and Stage Ib is 0.75-1.5 mm penetration. Stage IIa is 1.5-4.0 mm and Stage IIb is over 4.0 mm or into the subcutaneous fat. In stage III and IV, there is disease beyond the primary site. Stage III is defined by the presence of in-transit or regional nodal metastasis or both. Stage IV is defined by the presence of distant metastasis.

Once the diagnosis of malignant melanoma has been established by biopsy and the stage has been identified using the results of the examination and studies, a treatment plan is developed. Melanoma is not cured unless it is diagnosed at a stage when it can be isolated and removed surgically. Considerations revolve around the extent of the local and regional nodal surgery for stages I through III. For stage IV patients, or those that are treated and then develop recurrence at distant sites, chemotherapy or immunotherapy is planned. Adjuvant therapy (auxiliary drug treatment used to make possibility of relapse less for those at high risk) is also considered.

Surgical therapy for the primary site is the excision of the skin including subcutaneous tissue surrounding the lesion. In the past, wide excisions were large and encompassed 5 mm of tissue in all directions wherever feasible. It has been shown that such wide local excisions are not necessary; studies from the World Health Organization Melanoma Group and by the Melanoma Intergroup Committee in the United States have provided general guidelines based on the depth of penetration of the melanoma. In some cases regional lymph nodes are also removed. A possible problem associated with this resection is the lifelong edema or swelling in the extremity. In patients with no signs of regional disease, depth of penetration of the primary tumor helps guide the decision. If the tumor penetrates less than 1mm, dissection is not usually done. If it is 1-2 mm, node dissection may be done at the time

of primary treatment or the patient may be observed and only undergo lymph node dissection if the area later shows signs of disease. If the patient has enlarged lymph nodes resection of the nodes will be considered. In the latter case, more extensive imaging of the lung, liver, or brain may be appropriate to be sure that the patient does not already have stage IV disease.

Questions related to which patients should have resection of regional lymph nodes have led to an intermediary procedure known as sentinel node mapping and biopsy. Intermediate thickness melanomas between 1 and 4 mm deep may have nodal involvement even if the examination and any other studies done are normal. If a radioisotope tracer or blue dye is injected into the area of the primary tumor, it will travel to the lymph nodes draining that area. These sentinel nodes are thus identifiable and are the most likely to harbour any regional metastatic disease. If these nodes alone are biopsied and are normal, the rest of the lymph node group can be spared. If they show microscopic deposits of tumor, then the full resection of the lymph node group may be completed. This procedure allows selection of those patients with intermediate thickness melanoma who will benefit from the regional lymph node dissection.

Some patients, such as those with IIb or stage III melanoma, are at high risk for the development of recurrence after treatment. Although these patients are clinically free of disease after undergoing primary treatment, they are more likely to have some microscopic disease in the body that studies have not yet been able to identify. In an effort to decrease the rate of relapse, adjuvant therapy may be considered. Interferon alpha 2a is an agent that stimulates the immune system. This adjuvant therapy may slightly increase the duration of a patient's disease-free state and lengthen overall survival. However, interferon alpha 2a has high toxicity and patients may not tolerate the side effects.

Unfortunately, treatment for those patients who present with or go on to develop systemic disease usually fails; melanoma that has metastasized to the brain is particularly difficult to treat.

Some researchers are investigating the reasons why melanomas are so resistant to chemotherapy. One suggestion as of late 2003 is that the genes ordinarily responsible for apoptosis (cell self-destruction) do not function normally in melanomas. The development of new drugs to treat melanoma depends on a better understanding of the complex processes involved in apoptosis.

Almost all patients survive stage Ia malignant melanoma, and the survival for stage I overall is more than 90%. Survival drops in stage IIa to about 65% at five years and is worse yet for stage IIb at slightly over 50%. Stage III has a survival rate at 5 years of 10%-47%, depending on the size and number of regional nodes involved. Stage IV malignant melanoma is almost always a fatal disease.

The staging system for cutaneous melanoma was revised by the American Joint Committee on Cancer (AJCC) in early 2002; it is based on TNM classification **(Table 2)**.

Table 1. Clark and Breslow classification

Clark Classification	Breslow Classification (thickness)
Level I: all tumor cells above basement membrane (*in situ*) **Level II:** tumor extends into papillary dermis **Level III:** tumor extends to the interface between papillary and reticulum dermis **Level IV:** tumor extends between bundles of collagen of reticulum dermis **Level V:** tumor invasion of subcutaneous tissue	**Stage I:** less than or equal to 0,75 mm **Stage II:** 0,76 – 1,5 mm **Stage III:** 1,51 – 4 mm **Stage IV:** greater than or equal to 4 mm

Table 2. TNM classification

Primary tumor (T)		Regional Lymph nodes (N)			
Tx	Primary tumor cannot be assessed (eg, shave biopsy or regressed melanoma)	**Nx**	Regional lymph nodes cannot be assessed	**N2**	Metastasis in two to three regional nodes or intra-lymphatic regional metastasis without nodal metastis
T0	No evidence of primary tumor	**N0**	No regional lymph nodes metastasis	**N2a**	Clinically occult (microscopic) metastsis
Tis	Melanoma in situ	**N1**	Metastasis in one lymph node	**N2b**	Clinically apparent (macroscopic) metasitasis
T1	Melanoma ≤ 1 mm in thickness, with or without ulceration	**N1a**	Clinically occult (microscopic) metastasis	**N2c**	Satellite or in-transit metastasis without nodal metastasis
T1a	Melanoma ≤ 1 mm in thickness and Clark's II or III levels, no ulceration	**N1b**	Clinically apparent (macroscopic) metastasis	**N3**	Metastasis in four or more regional nodes, or matted metastatic nodes, or in-transit metastasis or satellites with metastatic nodes
T1b	Melanoma ≤ 1 mm in thickness and Clark's IV or V levels or with ulceration	**Distant metastasis (M)**			
T2	Melanoma 1.01 – 2.0 mm in thickness with or without ulceration	**Mx**	Distant metastasis cannot be assessed		
T2a	Melanoma 1.01 – 2.0 mm in thickness, no ulceration	**M0**	No distant metastasis		
T2b	Melanoma 1.01 – 2.0 mm in thickness with ulceration	**M1**	Distant metastasis		
T3	Melanoma 2.01 – 4 mm in thickness, with or without ulceration	**M1a**	Metastasis to skin, subcutaneous tissues or distant lymph nodes		
T3a	Melanoma 2.01 – 4 mm in thickness, no ulceration	**M1b**	Metastasis to lungs		
T3b	Melanoma 2.01 – 4 mm in thickness, with ulceration	**M1c**	Metastasis to all other visceral sites or distant metastasis at any site associated with an elevated serum lactic dehydrogenase (LDH) level		
T4	Melanoma ≥ 4 mm, with or without ulceration				
T4a	Melanoma ≥ 4 mm, no ulceration				
T4b	Melanoma ≥ 4 mm, with ulceration				

1.2 The Tetraspanins

1.2.1 The Tetraspanins Family

Tetraspanins, also called tetraspans or the transmembrane 4 superfamily (TM4SF), are cell-surface proteins that span the membrane four times; they are ubiquitously expressed and highly conserved and are found on many different cell types in many organisms, from sponges to mammals[49,50]. The tetraspanin superfamily consists of 4 subfamilies; the CD-, CD63-, uroplakin- and RDS families. The CD family of tetraspanins is the largest and contains all CD tetraspanins except for CD63. CD63 constitutes its own subfamily as it has a more ancient origin than the other CD tetraspanins[51].

Tetraspanins are involved in a multitude of biological processes, such as fertilization, parasite and viral infection, synaptic contacts at neuromuscular junctions, platelet aggregation, maintenance of skin integrity, immune response induction, metastasis suppression and tumor progression[52-55].

Tetraspanins were discovered in 1990[53,56,57], when comparison of the sequences of the newly cloned CD37, CD81 and sm23 genes with the tumor-associated gene CD63 revealed sequence homology and a conserved predicted structure[56,58,59] (**Figure 5**).

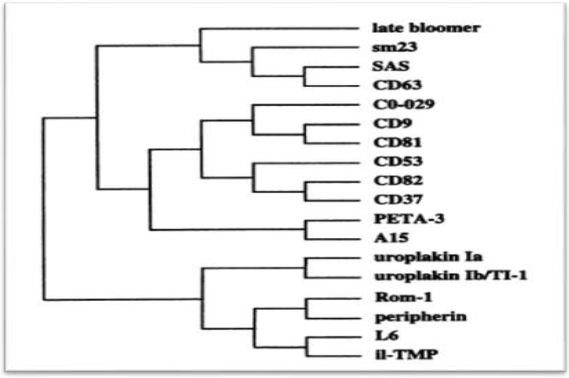


Figure 5. Evolutionary tree of the tetraspanin superfamily drawn as a dendogram with constant branch lengths.

Tetraspanins cross the membrane four times: they have four transmembrane domains (TM1-TM4) with short intracytosolic amino- and carboxy-terminal tails and two extracellular (EC) loops. The EC1 loop is short, between TM1 and TM2; the EC2 loop is large, between TM3 and TM4, with more than 100 aminoacids and some distinctive features, like a conserved CCG motif of unknown function and several conserved cysteines present in all members of the family (**Figure 6**).

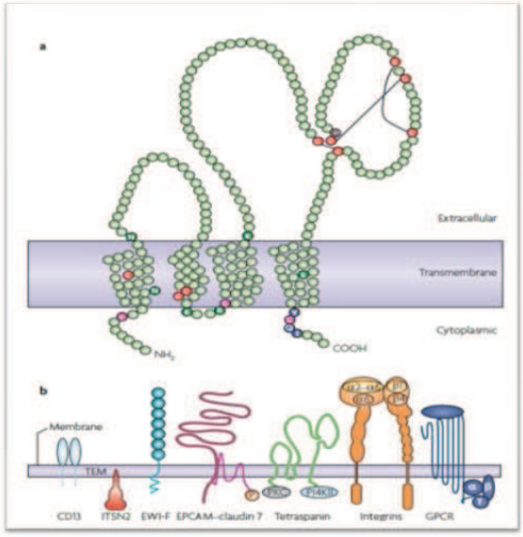


Figure 6. Tetraspanins cross the membrane four times: they have four transmembrane domains (TM1-TM4) with short intracytosolic amino- and carboxy-terminal tails and two extracellular (EC) loops. The EC1 loop is short, between TM1 and TM2; the EC2 loop is large, between TM3 and TM4, with more than 100 aminoacids and some distinctive features, like a conserved CCG motif of unknown function and several conserved cysteines present in all members of the family.

EC2 can be subdivided into a constant region and a variable region: the constant region may account for dimerization, the variable region for the interactions with non-tetraspanin partner molecules. The EC2 structure identifies at least three tetraspanin subgroups based on their different folding characteristics [60, 61]. Polar residues in the TM regions stabilize the tertiary structure[62, 63] and the short C-terminal region is likely to provide a link to intracellular signaling molecules[13].

Palmitoylation is an important mechanism for the initiation of the tetraspanin-tetraspanin web formation and also protects tetraspanins from lysosomal degradation, provides a link to cholesterol and gangliosides, and promotes increased cell-cell contact. Palmitoylation of specific integrins also contributes to tetraspanin complex formation[64-67].

Palmitoylation is the covalent attachment of fatty acids, such as palmitic acid, to cysteine and less frequently to serine and threonine residues of proteins, which are typically membrane proteins. Palmitoylation enhances the hydrophobicity of proteins and contributes to their membrane association. Palmitoylation also appears to play a significant role in subcellular trafficking of proteins between membrane compartments, as well as in modulating protein-protein interactions[68]. Palmitoylation is usually reversible, because the bond between palmitic acid and protein is often a thioester bond. The reverse reaction is catalysed by palmitoyl protein thioesterases. Because palmitoylation is a dynamic, post-translational process, it is believed to be employed by the cell to alter the subcellular localization, protein-protein interactions, or binding capacities of a protein.

Tetraspanin palmitoylation occurs in the Golgi complex[69]. After palmitoylation, tetraspanins often form homodimers, which are subsequently transported to the cell surface to function as building blocks for TEMs[14]. In addition to palmitoylation, tetraspanins are post-translationally modified with several N-glycans[71].

It has been demonstrated that palmitoylation also controls the association between tetraspanins and intracellular signalling molecules. In these complexes, their ligation results in phosphorylation of signalling molecules. Palmitoylation is regulated by the cellular redox state, and under oxidative stress it is inhibited favouring signalling by 14-3-3 adaptor proteins[72]. Palmitoylated proteins (CD9, CD81 and CD82) coexist and colocalize with palmitoylated integrin β4, which promotes the incorporation of CD151 into these tetraspanin complexes[73]. It has been demonstrated that loss of

integrin palmitoylation results in reduction of association among these tetraspanins and in an increase in CD9 complexes, as well as signalling by p130 (CAS) when cells are grown on laminin[73] (**Figura 7**). There are two different types of complexes: those with palmitoylated tetraspanins that favour their association with integrins and their integration into cholesterol-rich fractions, and non-palmitoylated tetraspanins that are accessible to binding with different signalling molecules, such as 14-3-3, p130 (CAS) or EWI proteins.

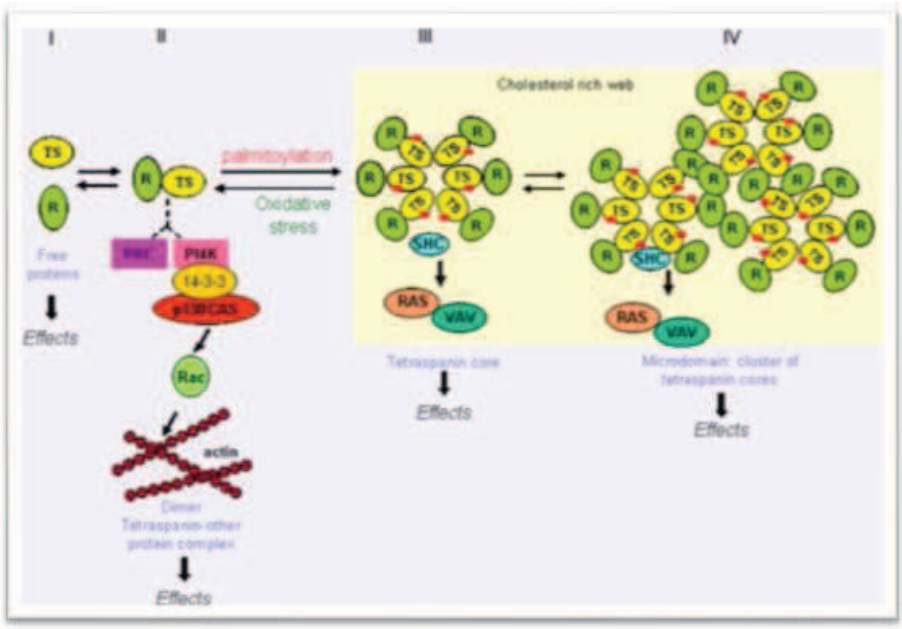


Figure 7. Dynamics of the formation of a tetraspanin microdomain. The tetraspanin protein binds to a receptor, which may be specific for each tetraspanin, on the plasma membrane. This heterodimer can be incorporated into a larger complex, where the tetraspanin proteins form the core. The participating tetraspanins are determined by their pattern and level of expression in a given cell type, as well as by the presence of their partners, which may also vary from cell to cell. These variations confer a great number of combinations that can be reflected in functional differences. The biological effects will depend on the situation of a specific tetraspanin at the time of stimulation.

The web assembly of tetraspanins and their specific interactions are dynamic and complex processes (**Figure 8**). The complexity depends on participation of the tetraspanins in the core and on the type and number of associated proteins. Thus variability in the combination of proteins can confer great flexibility to allow for specificity and functional differences depending on cell type. Therefore, tetraspanin

complexes in specific cell types can be very different despite sharing several of their components. In that way the association of tetraspanin with membrane receptors may exist as an isolated complex on the cell membrane, or form part of a larger tetraspanin-core complex. Obviously the association–dissociation kinetics represent an additional level of regulation, where palmitoylation and redox state play an important role. Individual associations of tetraspanin with other proteins may take place either in intracellular vesicles before moving to the membrane[15] or in the membrane before incorporation into the complex, and might even associate with partner proteins during early biosynthesis[16].However, initiation of signaling is very likely to be different if activated by either free molecules, in heterodimers, or a larger complex, such as the tetraspanin web.

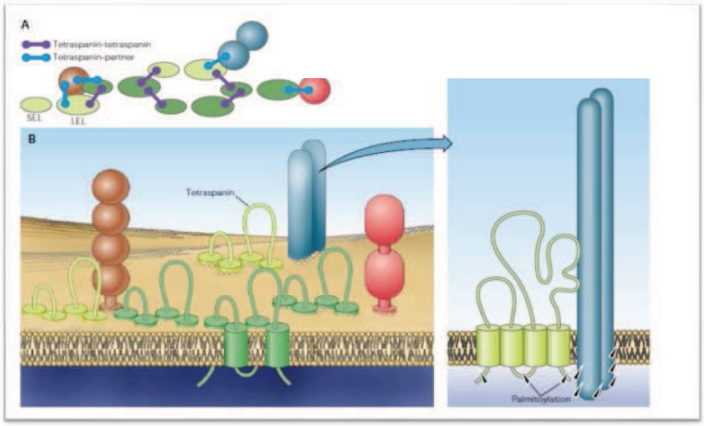


Figure 8. ***A*****:** aerial view of tetraspanin-partner and tetraspanin-tetraspanin interactions. ***B*****:** side view of lateral associations of tetraspanins with partner molecules. Clustering is facilitated by palmitoylation of conserved cysteines in the intracellular domains of the interacting proteins (*right*). Tetraspanins are shown in shades of green; partners of the immunoglobulin superfamily are shown in red and brown, and those of the integrin are in blue.

Tetraspanins are found in the endosomal system. Endocytosis starts with the building of endocytic vescicles from the plasma membrane that fuse with an early endosome. From early endosomes, proteins either recycle to the cell surface or become incorporated into intraluminal vesicles (ILVs) that bud into the endosomal

lumen. During their maturation, endosomes acquire increasing numbers of ILVs, which is why late endosomes are also called multivesicular bodies (MVB). Late endosomes/MVBs can mature into- or fuse with lysosomes[75, 76]. Within the endosomal system tetraspanins are predominantly found within MVBs and lysosomes. The significant levels of tetraspanins in these organelles suggest that tetraspanins are relatively protected from lysosomal proteolysis.

Tetraspanins are also present in so-called lysosome-related organelles or secretory lysosomes. These include the dense

granules and α-granules in platelets, melanosomes in melanocytes, cytotoxic granules in T-cells, Weibel–Palade bodies in endothelial cells and Major Histocompatibility Complex II (MHCII) compartments in dendritic cells[77-80]. Upon stimulation, these lysosome-related compartments can fuse with the cell surface, releasing their content into the extracellular environment. In many cell types, late endosomes/MVBs can be triggered to fuse with the cell surface and release their ILVs. The released ILVs are then indicated as 'exosomes'[81,82].

Exosomes are 30-100 nm vescicles released by many cells[17]. They derive from multivescicular bodies, which either fuse with lysosomes or fuse with the plasma membrane and release their intraluminal vescicles as exosomes[18]. The molecular composition of exosomes reflects their origin from intraluminal vescicles and includes several tetraspanins[17]. The exosomal proteins maintain their functional activity, as shown by their capacity to present peptides in MHC complex class I and II molecules[17]. Exosomes can contain mRNA and microRNA, which are transferred to the target cell where they can be translated and mediate RNA silencing[84, 85]. This process is specific to the target cell, such the RNA is transcribed in one but not another type of cell[86]. Thus, exosomes are thought to constitute a potent mode of intracellular communication that is important in the immune response[19], cell-to-cell spread of infective agents[17] and tumor progression[20]. The relative abundance of proteins, mRNA and microRNA differs between exosomes and donor cells. This implies active

sorting into multivescicular bodies, which for proteins can be achieved by monoubiqutylation, localization in cholesterol-rich membrane microdomains or higher-order oligomerization[88,89]. In fact, tetraspanins and their associated proteins are enriched in exosomes.

Since exosomes are secreted by many types of cancer cells, they have been a main subject in the cancer field. Tumor cell derived exosomes might have great potential in eliciting an anti-cancer immune response, since they can deliver specific tumor antigens to APC[21]. In mice, tumor-peptide-pulsed dendritic cells secrete exosomes that elicit anti-tumor immune responses and reduce the size of established tumors in vivo[21]. Recent evidence, however, suggests that tumor derived exosomes could also have immune-suppressing features and bear proteins that are involved in angiogenesis promotion and chemo resistance, hence promoting tumor progression[22]. The function of tetraspanins in exosomes is currently not known, although they have been implicated in the adhesion of exosomes to target cells[77]. The intrinsic distribution pattern of tetraspanins indicates that their transport to the various cellular locations must be tightly regulated.

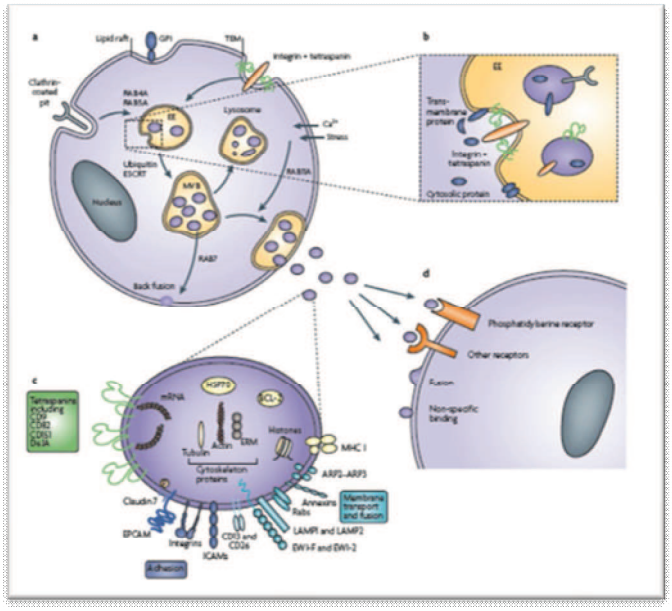


Figure 9. The generation and composition of exosomes. **a**,**b.** Clathrin-coated pits, clustering of proteins in tetraspanin-enriched membrane microdomains (TEMs) act as sorting signals, where endocytosed membrane proteins follow several routes from early endosomes (EEs) to recycling endosomes or multivesicular bodies (MVBs). MVBs

derive from membrane invagination of EEs, the inward budding vesicles being defined as intraluminal vesicles. Ubiquitylation, Rab proteins and the endosomal sorting complexes required for transport (ESCRT) machinery, among others, are involved in the intracellular transport of the vesicles and help to sort cargo into the MVBs. The intraluminal vesicles either fuse with lysosomes for degradation, are released in the cytoplasm in a process called back fusion or are delivered as exosomes. In exosomes certain stimuli, such as Ca2+ flux and stress, support the transport of the MVB to the plasma membrane. **c.** The composition of exosomes varies with the cell type, but contains some common components. Rab proteins, annexins and ARP2–ARP3 are involved in membrane transport and fusion. The figure shows only those membrane-located proteins that are directly or indirectly linked to tetraspanins, such as integrins, epithelial cell adhesion molecule (EPCAM) –claudin 7, CD13 or CD26, EWI-F and EWI-2. The cytoskeletal proteins associated with tetraspanins are ezrin–radixin–moesin (ERM) proteins and actin. The exosomal signal transduction molecules that are involved in tetraspanin-initiated pathways are small heterotrimeric GTP binding proteins, Src proteins, ERK1, ERK2, Rho family protein members, SH2 phosphatase and catenins, including β-catenin. **d.** The uptake of exosomes follows different routes: exosomes can bind to specific receptors, particularly phosphatidylserine receptors, bind unspecifically or fuse with the target cell membrane.

1.2.2 Tetraspanin genomic organization

The genomic organization of many tetraspanin members is known: there is a tight conservation of intron/exon structure[92]; a large intron lies between the first and second exones, whereas the remaining exones 2-8 are clustered more tightly at the 3' and of the locus. Many of the tetraspanin genes have been mapped, and they lie on a number of different chromosomes: CD81 and CD82 genes are found on human chromosome 11 (11p15.5 and 11p11.2, respectively), and CD9, CD63 and SAS are found on chromosome 12 (12p13, 12q12-q13 and 12q13-q14, respectively)[93, 94]; CD53 was found on chromosome 1p13[23] and CD37 on chromosome 19 in humans. In the mouse, CD63 has two loci: one on chromosome 10 in a region with linkage homology to human chromosome 12; the other maps to chromosome 18[92]. There is some evidence for divergence from a single ancestral locus that can be gleaned from gene mapping. In the mouse, CD53 on chromosome 3 and CD37 on chromosome 7 are surrounded by other genes that are structurally or functionally related[92]. Two of the tetraspanin loci, CD63 and CD81, have been conserved as part of a syntenic group (genes whose

chromosomal order is preserved between species, in this case between mouse and human)[24].

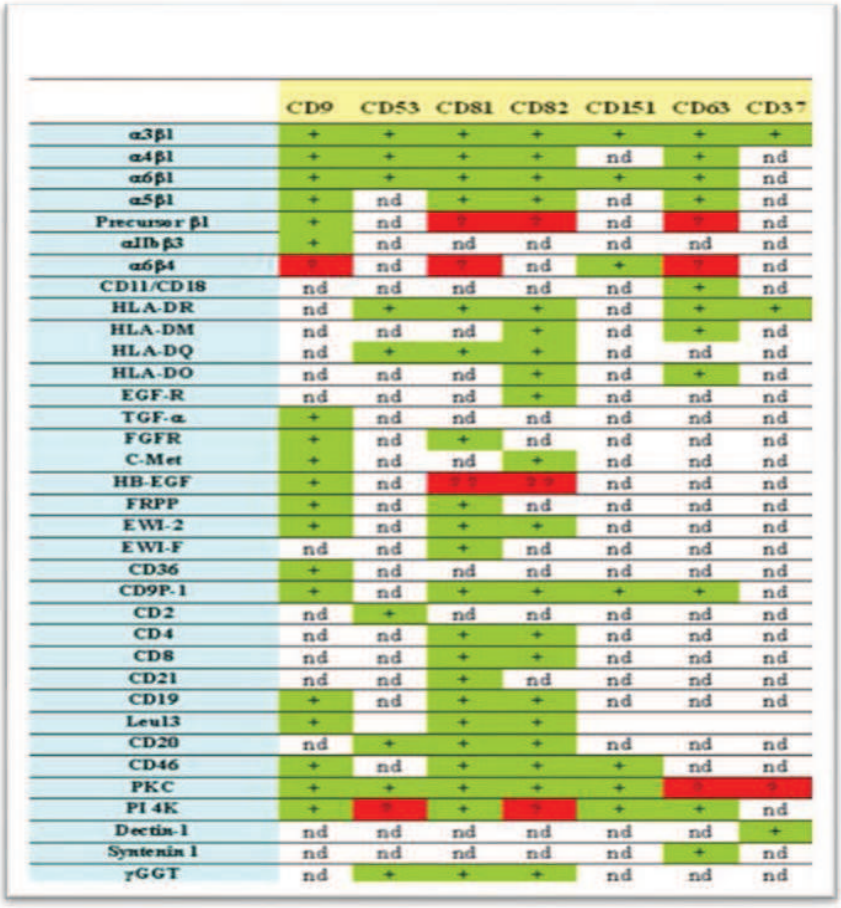
No many studies have been done on the regulation of tetraspanin gene transcription: it has been demonstrated the absence of a TATA box and the presence of Sp1 binding site[25]. In CD63, the promoter region is G-C rich and contains three transcription initiation sites, as well as potential binding sites for the transcription factors AP-1, Sp1 and ETF[97]; it has also been identified a cryptic promoter within the first intron that may be a responsive element[98]. The 5'- flanking region of CD81 is also G-C reach and contains putative Sp1 binding site[26]. Unlike CD9 and CD63, it does contain a TATA box at position -25.

Most of the tetraspanins are modified by N-glycosylation[100]: CD9 contain a glycosylation site in EC1, whereas other tetraspanins contain glycosylation sites in EC2. There is no conservation of glycosylated sites between different tetraspanin molecules; however, within individual members most glycosylation sites are conserved between species: for example, CD9 of mouse, rat, primates and cow have identical single glycosylation sites, whereas the feline molecule lost this site altogheter. In CD63, the glycosylation sites are conserved in mouse, rat, human and rabbit.

1.2.3 Tetraspanins interactions

The hydrophobic nature of the tetraspanins indicates that they associate with themselves or with other transmembrane and cytosolic proteins[50] that are required for their function. Tetraspanins can form microdomains rich in cholesterol and gangliosides on the plasma membrane, known as tetraspanin-enriched microdomains (TEMs), that are different from lipid rafts and can organize different types of membrane proteins[27]. The combination of tetraspanin expression differs depending on cell type and differentiation. (**Table 3**).

Table 3. Tetraspanins interactions



	CD9	CD53	CD81	CD82	CD151	CD63	CD37
α3β1	+	+	+	+	+	+	+
α4β1	+	+	+	+	nd	+	nd
α6β1	+	+	+	+	+	+	nd
α5β1	+	nd	+	+	nd	+	nd
Precursor β1	+	nd	?	?	nd	?	nd
αIIbβ3	+	nd	nd	nd	nd	nd	nd
α6β4	?	nd	?	nd	+	?	nd
CD11/CD18	nd	nd	nd	nd	nd	+	nd
HLA-DR	nd	+	+	+	nd	+	+
HLA-DM	nd	nd	nd	+	nd	+	nd
HLA-DQ	nd	+	+	+	nd	nd	nd
HLA-DO	nd	nd	nd	+	nd	+	nd
EGF-R	nd	nd	nd	+	nd	nd	nd
TGF-α	+	nd	nd	nd	nd	nd	nd
FGFR	+	nd	+	nd	nd	nd	nd
C-Met	+	nd	nd	+	nd	nd	nd
HB-EGF	+	nd	?	?	nd	nd	nd
FRPP	+	nd	+	nd	nd	nd	nd
EWI-2	+	nd	+	+	nd	nd	nd
EWI-F	nd	nd	+	nd	nd	nd	nd
CD36	+	nd	nd	nd	nd	nd	nd
CD9P-1	+	nd	+	+	+	+	nd
CD2	nd	+	nd	nd	nd	nd	nd
CD4	nd	nd	+	+	nd	nd	nd
CD8	nd	nd	+	+	nd	nd	nd
CD21	nd	nd	+	nd	nd	nd	nd
CD19	+	nd	+	+	nd	nd	nd
Leu13	+		+	+			
CD20	nd	+	+	+	nd	nd	nd
CD46	+	nd	+	+	+	nd	nd
PKC	+	+	+	+	+	+	+
PI 4K	+	?	+	?	+	+	nd
Dectin-1	nd	nd	nd	nd	nd	nd	+
Syntenin 1	nd	nd	nd	nd	nd	+	nd
γGGT	nd	+	+	+	nd	nd	nd

The most important non-tetraspanin partners are integrins that play and important role in adhesion to the extracellular matrix, cell motility, invasion and angiogenesis[60,101-103]. Tetraspanins associate mainly with integrins containing the β1 chain, a major component for attachment to the extracellular matrix, with variation in α chain, such as α3β1, α4β1 and α6β1, which are detected in most cell types. Some interactions seem to be specific for a tetraspanin, as in the case of α1β1 with CD9 that is promoted by the GM3 ganglioside. Tetraspanins appear to affect postligand effects such as modulating actin dynamics, which functionally reflect in migration and cell adhesion properties. The tetraspanin complex with integrin is in a low-affinity conformation, and changes in affinity do not alter integrin interaction with tetraspanis. The tetraspanin-integrin

complexes are functional in adhesion assay in a chaderin- independent manner, and might provide spatial cues for cellular polarization.

Tetraspanins also associate with growth factor receptors, G-protein-coupled receptors (GPCRs) and their associated intracellular heterotrimeric G-proteins, several peptidases, transmembrane proteins associated with tumor progression such as CD44 and epithelial cell adhesion molecule (EPCAM; also known as tumor associated calcium signal transducer 1 (TACSTD1)) and immunoglobulin (Ig) superfamily members including EWI-F (also known as prostaglandin F2 receptor negative regulator) and EWI-2 (also known as IGSF8)[50]. Cytosolic signal transduction molecules that co-immunoprecipitate with tetraspanins include protein Kinase C (PKC), a type II phosphatidylinositol 4-Kinase (PI4KII) and phospholipase Cγ (PLCγ)[55,104,105]. These primary interactions can be classified as types I, II and III according to the strength of detergent that is required to disrupt the association. Direct protein–protein interactions — type I interactions — are rare: these include tetraspanin homodimers, homotrimers and homotetramers, and some heterointeractions between CD151 and integrins and between CD9, CD81 and tetraspanin 8 and EWI proteins. These direct interactions may proceed through EC2 and/or TM2, TM3 or TM4[14]. The majority of tetraspanin–integrin and tetraspanin–tetraspanin interactions are type II interactions, which are maintained under milder lysis conditions. Palmitoylation of tetraspanins, and possibly of the associating proteins, is essential for this type of interaction, which may be initiated in the Golgi and provide a targeting sequence for co-associations[65, 73]. Weak type III interactions, such as those that occur with several kinases, are also stabilized by palmitoylation. Importantly, the functional activity of the tetraspanins does not depend only on these primary interactions.

1.2.4 Functions of Tetraspanins

The role and activity of the tetraspanins are closely related to the type of interaction that they create on the plasma membrane as well as the activation state of the cell and the surrounding tissue. *Maeker et* al described them as "molecular facilitators": they proposed that tetraspanins serve just such an organizing function; tetraspanins interact with many other molecules and participate in activation, adhesion and cell differentiation by stabilizing them an allowing them to function more efficiently[53,57]. This idea was supported by the fact that tetraspanins can act either directly, through their laterally associated partner molecules or, rarely, through ligand binding. In addition, as a major component of exosomes, tetraspanins are likely to be involved in cross-talk between distant cells.

Several studies demonstrated the role of tetraspanins in cell signaling and integrin-dependent adhesion strengthening, a process in which cells become increasingly resistant to detachment from immobilized integrin ligands: this might explain the effects of tetraspanins on integrin-dependent cell spreading, morphology and motility (**Figure 10**). For examples, it has been demonstrated that a mutation in the tetraspanin CD151 reduced integrin α6β1–dependent adhesion strengthening. A possible explanation for this process is the fact that many tetraspanins can regulate the organization of cellular actin[28]. PCK could have a key role, as tetraspanins such as CD9 and CD82 can associate with PKC isoforms and recruit them into complexes with integrins. Conventional PKC isoforms are known to regulate the actin cytoskeleton[29], and so would be well positioned to affect integrin-dependent adhesion strengthening. Also important are the three C-terminal residues in the cytoplasmic tails of many tetraspanins that can be recognized by PDZ domains (a protein-interaction domain that often occurs in scaffolding proteins, and is named after the founding members of this protein family)[30]. Mutation of the short C-terminal tail of CD151 almost completely eliminated the enhancement of adhesion strengthening[31].

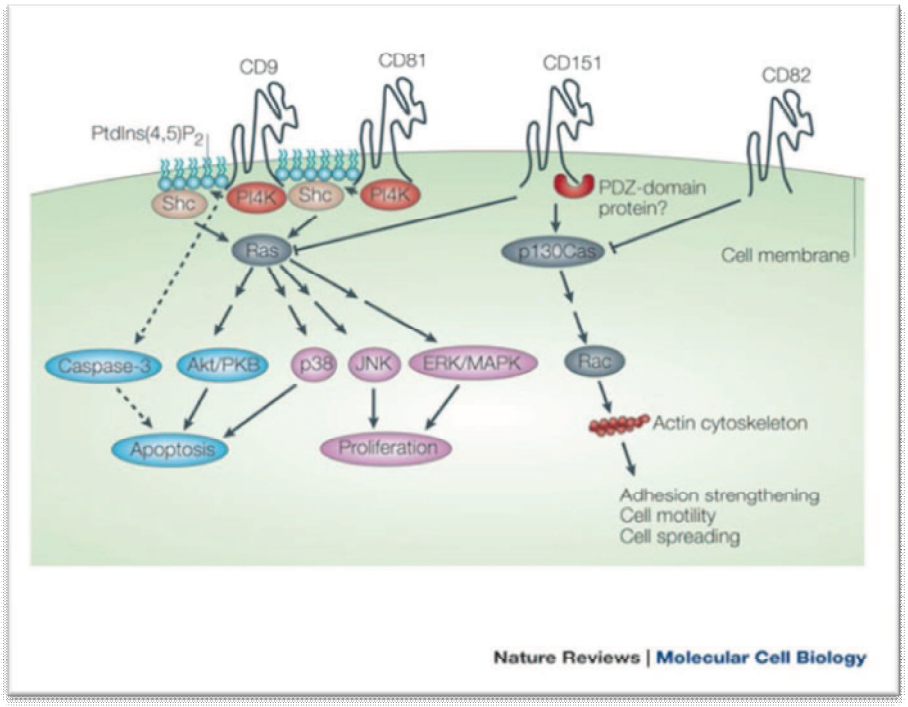


Figure 10. Tetraspanin signaling

Furthermore, tetraspanins such as CD81 and CD9 (which can associate with CD151) associate directly with EWI2 (a transmembrane protein with a conserved Glu–Trp–Ile motif), and bring it into proximity with integrins. Because EWI2 can affect integrin-dependent cell spreading, ruffling and motility, it is also likely to affect adhesion strengthening. Another possibility is that tetraspanins could affect adhesion strengthening by modulating the cell-surface expression levels of integrins. However, in the cases mentioned above, down regulation of the relevant integrins was not observed[31]. Tetraspanins might modulate adhesion strengthening not only by affecting integrins, but also by influencing integrin ligands. For example, CD9 could have a crucial role in optimizing the location of ICAM-1 and VCAM-1 (ligands for integrins áLâ2 and á4â1) on endothelial cells to support leukocyte adhesion strengthening under shear low[109].

It has been demonstrated that tetraspanins also affect processes such as cell proliferation, apoptosis and tumor metastasis. Anti-proliferative effects of anti-CD81

and anti-CD9 antibodies on lymphoid cells were first documented in 1990[13]. In both cases the regulation of proliferation is positively associated with activation of the extracellular signal-regulated kinase 1/2 (ERK1/2)/MAPK pathway. CD81 overexpression can activate ERK1/2 while promoting proliferation, whereas anti-proliferative CD9 antibodies caused the suppression of ERK1/2 activity; overexpressed CD81 binds to type II PI4K. The subsequent synthesis of phosphoinositides leads to phosphoinositide-dependent recruitment of the p46 and p52 isoform of Shc, which then leads to ERK1/2 activation[110]. Similarly, the local production of phosphoinositides through type II PI4K could contribute to Shc activation by CD9. CD9 also associates with type IIPI4K[16]. However, for CD9 (in contrast to CD81), the activation of the p46 Shc isoform was associated with decreased proliferation[111]. PKC can support cell proliferation by activating, through Raf1, the ERK/MAPK pathway[112,113]. CD81 and CD9 has also been linked to apoptosis, ere Shc signaling, effects on MAPK pathways and caspase-3 activation are implicated[111]. While suppressing ERK1/2 signaling, anti-CD9 antibodies activated the Jun N-terminal kinase (JNK)/stressed-activated protein kinase (SAPK) and p38 MAPK pathways with a promotion of apoptosis. CD151 affects integrin-dependent adhesion strengthening and cell motility: cell morphology and motility are controlled by changes in cytoplasmic actin dynamics, which are regulated by the Rho family GTPases Rac, Rho and Cdc42. Laminin-10/11 promotes cell migration while preferentially activating Rac over Rho, through the p130Cas-CRKII-DOCK180 pathway[114]. As CD151 is closely associated with laminin receptors, it might be expected to contribute to laminin-binding integrin-dependent regulation of cell proliferation and apoptosis. The CD82/KAI1 molecule was initially found to be a suppressor of tumor cell metastasis in 1995[115]. Recent studies explained the possible mechanisms by which CD82 inhibits cell motility and/or invasiveness. Activation of the FAK– Src–p130Cas–CRKII pathway (for example, during cell migration) leads to DOCK180-dependent activation of Rac1 membrane ruffling and directional cell migration, with the p130Cas–CRKII complex functioning as a key ‘molecular

switch'[116,117]. Ectopic CD82 expression in a metastatic prostate cancer line down regulated levels of p130Cas protein, thereby suppressing p130Cas–CRKII coupling and decreasing cell migration[118]. The association of CD82 with EWI2, another molecule that suppresses cell ruffling and migration[32], could also potentially contribute to the suppressor functions of CD82. However, other researchers have not observed CD82–EWI2 complex formation[120]. In other studies, CD82 attenuated signaling by the epidermal growth factor receptor (EGFR), and the related receptor ERBB2, by diminishing ligand- induced dimerization and endocytosis[121,122]. This provides another avenue for CD82-mediated inhibition of cell migration and invasion. CD82 seems to exert broad effects on cell migration, which are not restricted to any specific integrin or integrin substrate[33]. In addition, CD82 exerts broad reorganizing effects on ganglioside- and cholesterol-containing membrane microdomains, leading to altered properties of caveolin-1, filamentous actin and other proteins[33]. These global functional effects of CD82, coupled with its effects on membrane microdomain organization, emphasize the importance of considering tetraspanin signaling in the context of TEMs.

Additional studies have implicated tetraspanins in the coordination of intracellular signaling pathways with the cytoskeleton. Rho-GTPase was shown to link the cytoskeleton with CD82; G protein-coupled receptors were demonstrated to be dynamically regulated by CD9 and CD81 in human cells[34] and by sun in *Drosophila melanogaster*[124]; phosphatidylinositol 4-kinase and protein kinase C, the signaling components that interact with integrins, were also shown to associate with several tetraspanins[104]. The authors have suggested that TEM are microenvironments analogous to transportation ports. However, unlike seaports and airports, which function as hubs of activity but occupy fixed positions, tetraspanin-signaling ports are dynamic and quick to respond and to coordinate intracellular and intercellular activities. Tetraspanins might also regulate cellular invasiveness through their association with peptidases, ADAMs, matrix metalloproteinases (MMPs) and urokinase plasminogen

activator surface receptor (uPAR)[50]. Moreover, tetraspanins can modulate MMP transcription and secretion[35].

Tetraspanins play an important role in the viral life cycle; anti-tetraspanin antibodies inhibit syncytium formation and/or virus production. This was demonstrated for the tetraspanins CD81 and CD82 with human T-lymphotropic virus 1 (HTLV-1), and for the tetraspanin CD9 with the feline immunodeficiency virus and the canine distemper virus[36]. CD81 might also play a role in the aetiopathogenesis of hepatic C virus (HCV), that is responsible for hepatitis C, which can evolve to a hepatocellular cirrhosis and carcinoma, and also for immune diseases related to lymphoid B cells. It has been shown that HCV particles fix CD81, probably via binding of the viral envelope protein E2 to the tetraspanin EC loop, and in this way could allow the virus entry into the cell; on the basis of sequence comparison between human and monkey CD81, combined with mutagenesis studies, it was demonstrated that certain aminoacids are essential for CD81 recognition of E2[36].

1.2.5 Tetraspanins and tumor: metastasis suppression and cell motility

Tetraspanin proteins are involved in tumorigenesis process by supporting or inhibiting tumor growth, invasion, metastasis and angiogenesis.

Tumor progression includes different steps such as the invasion before intravasation, intravasation, extravasation and invasion after extravasasion. Success in these steps requires the coordination between tumor cell-cell adhesion, cell-matrix adhesion, matrix degradation and cell migration; interestingly, all these cellular events are regulated by tetraspanin superfamily[37]. Tetraspanins regulate not only the motility-related behaviors of tumor cells, but also the interactions between tumor cells and their microenvironment. Of the 33 human tetraspanins, approximately half have been experimentally studied, and several have been shown to correlate with tumor patient prognosis and regulate tumor progression and metastasis.

Some tetraspanins have been viewed as useful markers for the characterization of tumoral cells. CD9, for example, was initially described on the surface of cells of B-lineage acute lymphoblastic leukaemia[38]. It is expressed on 90% of B-lineage acute leukaemias and on 50% of acute myeloid leukaemias and B-lineage chronic lymphoid leukaemia. In particular, CD9 is a constant marker of acute promyelocytic laeukaemia, in association with $CD13^+/CD33^+/HLA\text{-}DR^-$ phenotype. The tetraspanin TALLA-1 is expressed in acute neuroblastomas and T-lymphoid leukaemia[39]; the antigen CO-029 was discovered in colorectal carcinomas[40], while the antigen L6 is overexpressed in breast, lung, colon and ovary tumors[129].

Tetraspanins have also been associated with tumor progression and metastasis. CD82, for example is associated with prostate, lung, pancreas and colorectal cancers[130,131]; CD9 is expressed in breast and oesophagus carcinomas[41].

Malignant transformation is associated with changes in cell adhesion and motility. Many cell-surface molecules involved in cell-ECM adhesion or cell-cell interactions have been described, including cadherins, selectines. immunoglobulin-like receptors, integrin and proteoglycans. These molecules act in a complex and coordinated way to hold the cells in place or to sustain cell movement. Adhesion molecules function in bidirectional signaling pathways required for many cellular functions, such as transcription, cytoskeletal organization and proliferation. Among the adhesion molecules, integrins are major ECM receptors and are also involved in cell-cell interactions and they are probably linked to tetraspanins.

A link exists between tetraspanin expression and metastasis potential, involving effects on cell motility and molecular associations with integrins. A direct or indirect effect of tetraspanins on integrin function might lead to alterations of adhesion or migration properties of the malignant cells that could modify their metastatic potential. The possible influence of tetraspanins on integrin function has been suggested by the observation that ligation of anti-tetraspanin antibodies can affect tyrosine phosphorylation of focal adhesion kinase (FAK), either positively or negatively

depending on the experimental conditions[133]. Another interesting aspect is that, during cell motility, tetraspanins tend to be localised at the leading edge of spreading cells, like integrins, and also in intracellular vesicles. These data suggest that tetraspanins might interfere with integrin movements in cell motility. This hypothesis is supported by the association of tetraspanin–integrin complexes with phosphatidylinositol 4-kinase (PI 4-kinase)[134], which is involved in the production of phosphatidylinositol-4,5-bisphosphate (4,5-PIP2), a regulator of cytoskeletal architecture. Finally, anti-tetraspanin antibodies, such as anti-β3 integrin antibodies, induce PI3-kinase-dependent production of matrix metalloproteinase 2 (MMP-2) in MDA-MB231 breast carcinoma cells[135]; this indicates that tetraspanins are involved in the control of a matrix proteinase that allows malignant cells to invade adjacent tissues by destruction of the ECM. The observation that a reduced level of expression of tetraspanins is associated with an increased metastatic potential of tumors raises the question of how they are down regulated. In cancer of the oesophagus, in which prognosis is linked to the expression of CD82, no mutations have been observed in the gene encoding CD82[136]; thus, down regulation is not a result of mutation. For CD82/Kai1, it has been suggested that the gene encoding this protein is controlled by the p53 tumor suppressor such that, in the presence of mutant p53, CD82/Kai1 gene expression is down regulated[137].

1.3 CD63 Tetraspanin

The CD63 gene is located on human chromosome 12q13 and its product became the first characterized tetraspanin. It was discovered as a protein present on the cell surface of activated blood platelets, known as platelet glycoprotein 40 (Pltgp40)[51] and in early stage human melanoma cells, where it was known as melanoma antigen 491 (ME491)[93,138]. CD63 interacts with many different proteins either directly or indirectly. Interaction partners include integrins (α4β1, α3β1, α6β1, LFA-1 and β2 [139-141], other

tetraspanins (CD81, CD82, CD9, CD151[142,143], cell surface receptors (MHCII, CD3, FcεRI, CXCR4)[144-146], kinases (phosphatidylinositol 4-kinase and the Src family tyrosine kinases Lyn and Hck)[141], adaptor proteins (AP-2, AP-3, AP-4)[42] and other proteins, including L6 antigen[148], syntenin-1[149], TIMP-1[150], H, K-ATPase[151] and MT1-MMP[152].

CD63 is ubiquitously expressed, localized within the endosomal system and at the cell surface. Lysosomal membrane proteins that exit the TGN (trans-Golgi network) can travel to lysosomes via either a direct TGN-to-endosome pathway or via an indirect route, involving passage over the plasma membrane and subsequent endocytosis. In general, the sorting of lysosomal membrane proteins depends on tyrosine and dileucine-based consensus motifs within their cytosolic tails. CD63, like the type I integral lysosomal membrane proteins LAMP-1 and LAMP-2, bears a consensus motif in its carboxy terminal cytoplasmic domain that is required for endocytosis at the plasma membrane, but has also been implicated in direct TGN to lysosome targeting. For lysosomal targeting, however, a specific conformation seem to be required: a glycine residue might precede the critical tyrosine and the entire motif might be positioned 6–9 residues from the transmembrane domain[43].

Tyrosine-based sorting motifs are found in at least 12 of the 33 human tetraspanins and mediate binding to adaptor proteins (APs) and recruitment of a clathrin coat. The lysosomal targeting motif of CD63 is important for its association with AP-2, involved in clathrin-mediated endocytosis from the plasma membrane, and with AP-3, for targeting from recycling endosomes to lysosomes. However, silencing of the endocytic AP-2 complex leads only to a partial block in the direct transport of CD63 to lysosomes, suggesting the existence of a direct TGN-to-endosome pathway that is likely independent of AP-1[154,155].

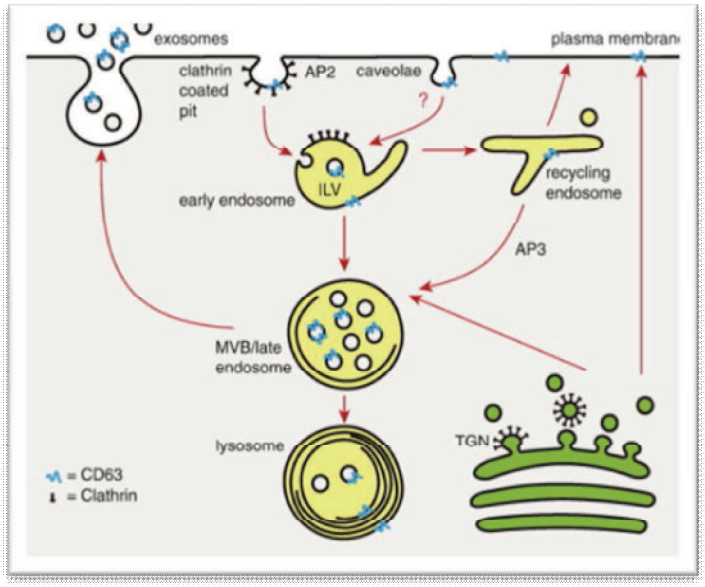


Figure 11. CD63 is transported from the TGN to either the cell surface via the exocytic pathway or to endosomes via a direct intracellular pathway. From the cell surface CD63 is endocytosed via AP-2 and clathrin coated pits. Another pathway that may contribute to CD63 internalization is caveolae-mediated endocytosis. After endocytosis, CD63 can either recycle to the cell surface or follow the endosomal route towards late endosomes and lysosomes. Lysosomal targeting may occur via incorporation into intraluminal vesicles (ILV) that bud inwards from the endosomal limiting membrane or via recycling endosomes, which depends on CD63 association with AP3. In late endosomes/MVBs CD63 is enriched in the ILVs that in certain conditions can be released into the extracellular space by fusion of late endosomes with the cell surface. The ILVs are then called exosomes. The red arrows indicate possible pathways of CD63 trafficking.

CD63 is enriched in exosome fractions as compared to whole cells[77]. In addition to the large endosomal/lysosomal fraction of CD63, a small pool is present on the cell surface. This pool may reflect CD63 passing over the cell surface on its way to late endosomes and lysosomes, but might also have a specific function. At least a portion of cell surface associated CD63 is constitutively endocytosed via interaction with the μ2 subunit of AP-2 and subsequent incorporation into clathrin coated vesicles[154,155]. After fusion of the endocytic vesicles with early endosomes, CD63 either recycles back to the cell surface or is transported to lysosomes[155].

An alternative pathway for clathrin-mediated endocytosis is provided by caveolae, 50–100 nm diameter plasma membrane invaginations that mediate endocytosis in a

dynamin-dependent, but clathrin-independent manner[44]. The best characterized entry via caveolae is for Simian virus 40 (SV40), also Echovirus (EV1) uses α2β1-integrin to gain entry into the cell via caveolae. Other proteins, such as cholera toxin and albumin can also be endocytosed via caveolae[157,158].

Klumperman et al found CD63 in caveolae of HeLa and endothelial cells. In addition, when human CD63 was exogenously expressed in mouse Mocha fibroblasts (derived from AP-3 deficient Mocha mice and displaying high cell surface levels of CD63), it was also found in caveolae. At least part of the CD63 antibodies are internalized in caveolin-1 positive compartments that are not connected to the cell surface, indicating that CD63, in addition to clathrin-mediated endocytosis, may also be endocytosed via caveolae.

Several studies indicate that in certain conditions the cell surface expression of CD63 is tightly regulated, probably by two proteins: syntenin-1 and L6-antigen. Syntenin-1 is a widely expressed PDZ domain-containing cytoplasmic protein that in physiological conditions regulates cell-migration and adhesion-dependent signalling. Syntenin-1 can interact with transport machinery proteins, such as the small GTPases Rab5, Rab7 and the SNARE syntaxin 1A, as well as with the serine/threonine kinases Ulk1 and 2[149]. In many types of human cancers the levels of syntenin-1 expression are up-regulated, which correlates with induced tumor metastasis. Interestingly, cell surface-associated CD63 can interact with the PDZ domains of syntenin-1 and overexpression of syntenin-1 decreases the rate of internalization of CD63. Since syntenin-1 binds CD63 at the C-terminal region where also AP-2 binds, binding of AP-2 and syntenin-1 may be mutually exclusive, indicating that syntenin-1 binding impairs clathrin mediated endocytosis. Moreover, it was postulated that binding of syntenin-1 might divert endocytosis of CD63 from a fast, AP-2 dependent route to a slow, AP-2 independent route. This latter pathway may involve the N-terminal 100 amino acids of syntenin-1, since deletion of this part has no effect on the formation of the syntenin-1–CD63 complex, but blocks CD63 internalization. Thus, interaction between CD63 and

syntenin-1 results in increased cell surface levels of CD63 and might divert CD63 to an alternative pathway of endocytosis[149].

L6-antigen (L6-Ag), also known as TM4SF1 is highly expressed on the cell surface of human epithelial tumor cells, such as lung, breast, colon and ovarian carcinomas. In addition, L6-Ag is present in MVBs/late endosomes, where it co-localizes with CD63. Augmented expression of L6-Ag increases cell motility and metastatic capacity[129]. On the cell surface, L6-Ag is recruited to CD63-positive TEMs, which is required for its pro-migratory effect. Very recently it was shown that down regulation of L6-Ag by RNA-interference (RNAi) results in a specific increase in the cell surface levels of CD63, as well as that of another tetraspanin, CD82[148]. Overexpression of L6-Ag leads to a decreased cell surface expression of CD63. Interestingly, while L6-Ag is overexpressed in various cancers, CD63 expression is often reduced in metastasizing cancers. Since L6-Ag can negatively regulate the cell surface expression of CD63 these two events may be related. The mechanism by which L6-Ag might regulate cell surface levels of CD63 is unknown.

1.3.1 CD63 and immune system

Professional APCs, like macrophages and dendritic cells (DCs) initiate an immune response by presenting antigens in the context of MHCII to CD4-positive T-cells. APCs exploit their late endosomes and lysosomes, together called MHCII compartments (MIICs), for binding exogenous antigenic peptides to the MHCII molecules. In DCs, CD63 interacts with MHCII molecules in MIICs as well as on the cell surface[49, 144]. Since CD63 cycles between the plasma membrane and MIICs, it was hypothesized that CD63 – as part of a tetraspanin web also containing CD82 – might chaperone MHCII molecules through the endosomal system[45]. However, MHCII may also traffic independently of CD63. In the case of ingestion of larger particles,

degradation takes place in phagolysosomes. CD63 after successful acidification of this compartment, which depends on the cargo that is internalized, recruits to the phagocytic vacuole. CD63 and MHCII/LAMP-I can traffic to the phagosomes via different pathways and MHCII can also traffic independently from CD63[160].

In gastric parietal cells, the heterodimeric ion pump H, K-ATPase is responsible for the secretion of gastric acid. In unstimulated cells, this ion pump is present in so-called intracellular tubulo-vesicular storage compartments, which are positive for CD63. Upon stimulation, the tubulo-vesicular membranes fuse with the plasma membrane. They thereby deliver the ion pump to the cell surface, initiating gastric acid secretion into the lumen of the stomach. When the β-subunit of H, K-ATPase is expressed in COS cells, it travels to the plasma membrane. However, co-expression of CD63 causes a redistribution of the β-subunit from the cell surface to intracellular compartments. This occurs by CD63-mediated linkage of the H, K-ATPase β-subunit to AP-2, thereby facilitating its clathrin-mediated endocytosis. These data suggest that CD63 might be involved in the recycling of the H, K-ATPase pump between the plasma membrane and intracellular storage compartments[151].

A recent study links CD63 to the trafficking of the cell surface associated chemokine receptor CXCR4, which facilitates HIV-1 entry into T-lymphocytes. In a screen for HIV-1 inhibitors in T-cells and CD4-expressing HeLa cells, the expression of an N-terminal deletion mutant of CD63, still containing its C-terminal GYEVM motif, resulted in the miss-targeting of CXCR4 to late endosomes and lysosomes and consequently in an inhibition of HIV-1 infection. Notably, this effect was specific for CD63, since other tetraspanins present in TEMs did not influence the cell surface expression of CXCR4. In seeming contrast with the N-terminal deletion mutant, depletion of endogenous CD63 by RNAi increased the cell surface levels of CXCR4[146]. These data indicate that CD63 may normally function in suppressing CXCR4 cell surface levels and that this capacity is enhanced by deletion of the N-terminal part of the protein.

CD63 has also been identified in association with neutrophils, hematopoietic cells that are important mediators of the innate immune system. They store the serine protease 'neutrophil elastase' (NE) in secretory lysosomes (primary granules) that are positive for CD63. Targeting of NE to primary granules requires AP-3, but is independent of mannose-6-phosphate receptors that mediate transport of most lysosomal hydrolases to the lysosomes[46]. Since lysosomal targeting of CD63 also requires AP-3, a recent study investigated a putative role for CD63 in NE transport by co-expression studies in COS cells[161]. CD63 overexpression led to an increased intracellular retention of NE, which required the large extracellular loop of CD63. Pull down experiments demonstrated that CD63 and NE are present in a complex and when in neutrophil progenitor cells CD63 levels were reduced by RNAi, less NE was retained intracellularly whereas these cells contained morphologically altered granules devoid of NE. Together these data indicate a role for CD63 in the targeting of NE to primary granules.

1.3.2 CD63 and cancer

CD63 was first discovered as an abundantly expressed surface antigen in early stage melanoma cells[47]. A correlation between decreased CD63 expression and increased malignancy is observed in many tumors. In ovarian cancer tissues, increasingly lower expression levels are found to correlate with increasingly higher chances of tumor metastasis[48]. In lung adenocarcinoma, low CD63 expression correlates with more tumor growth and a poor prognosis and also in breast- and colon cancers a negative relation between CD63 expression and cancer invasiveness and metastasis is observed[163-165].

Taken together, these studies show that CD63 expression often decreases in late stages of cancer development, which correlates with increased tumor cell motility and metastasis. CD63 involvement in cell motility could be related to its association with

integrins mediating binding to the ECM. CD63 may interact with the integrins α4β1, α3β1, α6β1, LFA-1 and β2[49,139,141], but the effect of these interactions on the integrins is largely unknown although in colon cancer expression of CD63 appears positively correlated with the expression of integrin α3β1[165]. One possibility is that CD63 influences the activity and stability of integrins via its interaction with other tetraspanins in TEMs. Alternatively, CD63 might be involved in the endocytosis of integrins, like for the ion pump H, K-ATPase in gastric parietal cells.

CD63 has also been implicated in regulation of transport of other proteins involved in tumor development. The 'membrane-associated type-1 matrix metalloprotease' (MT1-MMP) functions in extracellular matrix turnover and thereby may increase tumor invasiveness and metastasis[152]. Upon overexpression of CD63 in human embryonic kidney cells, the cell surface levels of MT1-MMP are decreased. MT1-MMP binds to the N-terminal region of CD63, which then leads to its endocytosis and subsequent degradation of MT1-MMP in lysosomes. The targeting of MT1-MMP to lysosomes depends on the C-terminal GYEVM lysosomal targeting motive of CD63[152]. Hence, CD63 seems to promote lysosomal degradation of MT1-MMP.

'Tissue metalloprotease inhibitor protein-1' (TIMP-1) is a cytoplasmic protein that inhibits MMP activity, thereby decreasing extracellular matrix turnover and remodelling. Furthermore, TIMP-1 interacts with β1 integrins at the cell surface and keeps them in an activated conformation[150]. In breast cancer epithelial cells, CD63 interacts with TIMP-1 at the cell surface, thereby facilitating its interaction with β1 integrins, resulting in cell survival signalling and inhibition of apoptosis. Concomitantly, CD63 knockdown reduces binding of TIMP-1 to the cell surface and its interaction with β1 integrins[150].

1.4 Metastasis and Epithelial-Mesenchymal transition: a possible role of tetraspanins

Primary tumors consist of heterogeneous populations of cells with genetic alterations that allow them to surmount physical boundaries, disseminate, and colonize a distant organ. Metastasis is a succession of these individual processes[166,167]. Each tissue has a physical structure and an established functional anatomy complete with compartmental boundaries, a vascular supply and a characteristic extracellular milieu of nutrients and stroma. Cancer cells that circumvent this organization become exposed to environmental stresses, including a lack of oxygen or nutrients, a low pH, reactive oxygen species, and mediators of the inflammatory response. Such pressures can select tumor cells with the capability of growth despite these challenges and in the process can cause them to acquire an aggressive phenotype. Genetic and epigenetic alterations in cancer cells in combination with a plastic and responsive microenvironment support metastatic process of evolution of tumor.

Metastasis progression can be viewed as a stepwise sequences of events, which is mediated by different classes of metastasis genes. For each type of cancer, the clinical course of these events occurs with distinct temporal kinetics and in unique organ sites. The long latency period of certain tumor types suggests the evolution of malignant cells in the microenvironments of a particular organ. The acquisition of pro-metastatic functions earlier during primary tumor formation might enable other cancer subtypes to relapse more quickly. The organ specificity of metastatic cells is determined by unique infiltrative and colonization functions required after their dissemination from a primary tumor.

In many primary tumors with invasive properties, intercellular adhesion is reduced, often because of a loss of E-cadherin, a direct mediator of cell-cell adhesive interactions. The cytoplasmic tail of E-chaderin is tethered, via α-catenin and β-catenin, to the actin cytoskeleton; one of actin's properties is to maintain cell junctions. Loss of

E-chaderin function is a process whereby epithelial cells switch to a mesenchymal progenitor-cell phenotype, enabling detachment and reorganization of epithelial-cell sheets during embryonic development, as well as tumor invasion and metastasis[166].

The epithelial-mesenchymal transition (EMT) is a biologic process that allows a polarized epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and increased production of ECM components. An EMT is complete when the underlying basement membrane is degraded and mesenchymal cells are formed; these cells are able to migrate away from the epithelial layer in which they originated.

Activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins, production of ECM-degrading enzymes, and changes in the expression of specific microRNAs are essentially to initiate and complete an EMT. In many cases, the involved factors are also used as biomarkers to demonstrate the passage of a cell through an EMT (**Figure 12**).

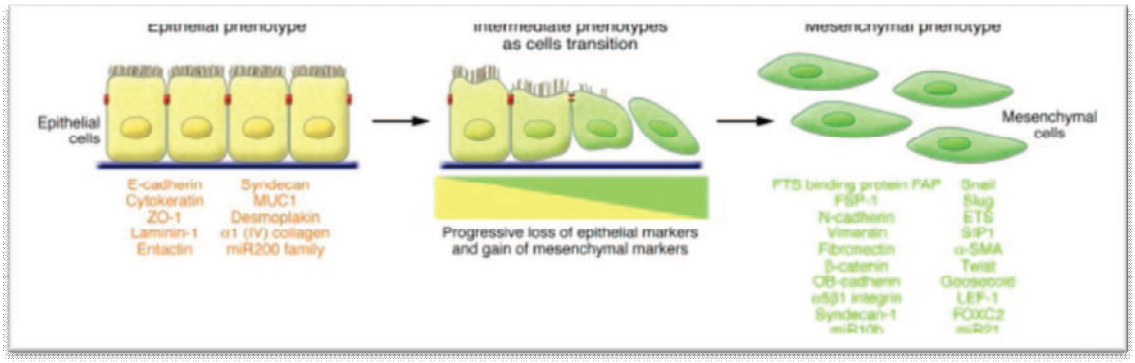


Figure 12. EMT involves a functional transition of polarized epithelium cells into mobile and ECM-component-secreting mesenchymal cells. The epithelial and mesenchymal cell markers commonly used by EMT researchers are listed. Colocalization of these sets of distinct markers defines an intermediate phenotype of EMT, indicating cells that have passed only partly through an EMT.

EMTs have a key role in different biological settings classified into three different biological subtypes based on the biological context in which they occur[168] (**Figure 13**):

- *Type I EMTs*, associated with implantation, embryo formation and organ development; it neither causes fibrosis nor induces an invasive phenotype resulting in systemic spread via the circulation.
- *Type II EMTs*, associated with wound healing, tissue regeneration, and organ fibrosis. The program begins as part of a repair-associated event that normally generates fibroblasts and other related cells in order to reconstruct tissues following trauma and inflammatory injury. However, in contrast to type 1 EMTs, these type 2 EMTs are associated with inflammation and cease once inflammation is attenuated. In the setting of organ fibrosis, type 2 EMTs can continue to respond to ongoing inflammation, leading eventually to organ destruction.
- *Type 3 EMTs*, that occur in neoplastic cells that have previously undergone genetic and epigenetic changes, specifically in genes that favor clonal outgrowth and the development of localized tumors.

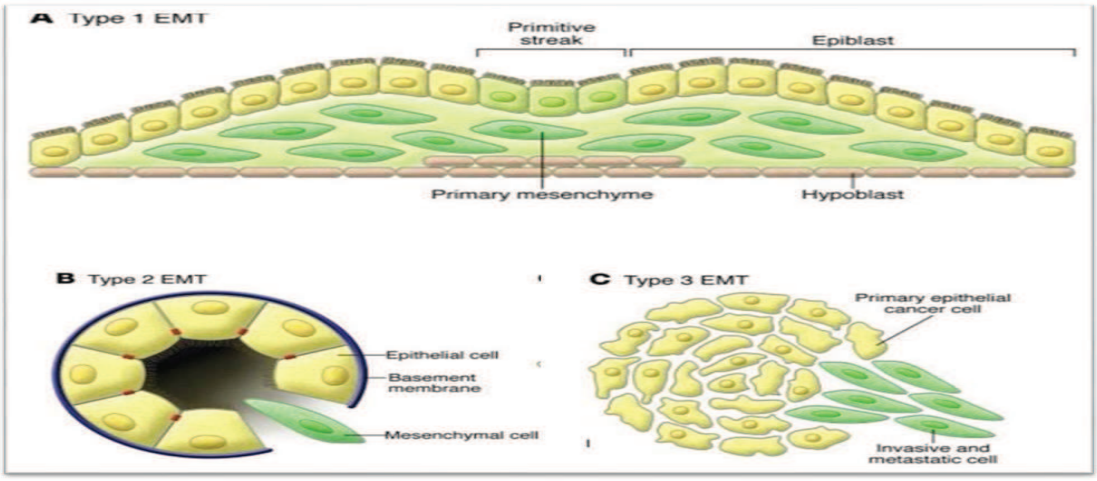


Figure 13. Different types of EMT. (**A**) Type 1, associated with implantation, embryo formation and organ development; it neither causes fibrosis nor induces an invasive phenotype resulting in systemic spread via the circulation; (**B**) Type II, associated with wound healing, tissue regeneration, and organ fibrosis; (**C**) Type III, that occur in neoplastic cells that have previously undergone genetic and epigenetic changes, specifically in genes that favor clonal outgrowth and the development of localized tumors.

Many mouse studies and cell culture experiments have demonstrated that carcinoma cells can acquire a mesenchymal phenotype and express mesenchymal markers such as α-SMA, FSP1, vimentin, and desmin. These cells typically are seen at the invasive front of primary tumors and are considered to be the cells that eventually enter into subsequent steps of the invasion-metastasis cascade (intravasation, transport through the circulation, extravasation, formation of micrometastases, and ultimately colonization).

An apparent paradox comes from the observation that the EMT-derived migratory cancer cells typically establish secondary colonies at distant sites that resemble, at the histopathological level, the primary tumor from which they arose; they no longer exhibit the mesenchymal phenotypes ascribed to metastasizing carcinoma cells. The idea is that metastasizing cancer cells must shed their mesenchymal phenotype via a MET during the course of secondary tumor formation[168]. The tendency of disseminated cancer cells to undergo MET likely reflects the local microenvironments that they encounter after extravasation into the parenchyma of a distant organ, quite possibly the absence of the heterotypic signals they experienced in the primary tumor that were responsible for inducing the EMT in the first place[169,170].

Several studies indicate that induction of an EMT is likely to be a centrally important mechanism for the progression of many tumors to a metastatic stage and implicates MET during the subsequent colonization process (Figure 14).

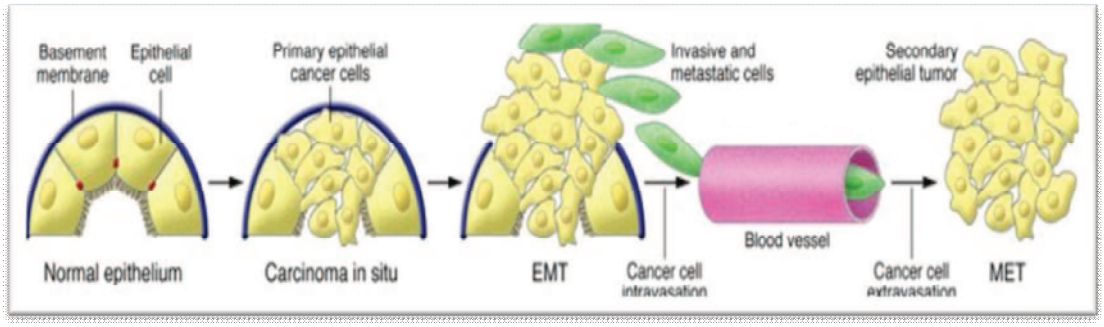


Figure 14. Contribution of EMT to cancer progression. Progression from normal epithelium to invasive carcinoma goes through several stages. The invasive carcinoma stage involves epithelial cells losing their polarity and detaching from the basement

membrane. The composition of the basement membrane also changes, altering cell-ECM interactions and signaling networks. The next step involves EMT and angiogenic switch, facilitating the malignant phase of tumor growth. Progression from this stage to metastatic cancer also involves EMTs, enabling cancer cells to enter the circulation and exit the blood stream at a remote site, where they may form micro- and macro-metastasis, which may involve METs and thus a reversion to an epithelial phenotype.

The induction or functional activation in cancer cells of EMT-inducing transcription factors, such as Snail, Slug, zinc finger E-box binding homeobox 1 (ZEB1), Twist, Goosecoid, and FOXC2[169,170] appear to be related to EMT-inducing signals emanating from the tumor-associated stroma, such as HGF, EGF, PDGF, and TGF-β. The activation of EMT programs is mediated by intracellular signaling networks, such as signal transducing proteins, ERK, MAPK, PI3K, Akt, Smads, RhoB, β-catenin, lymphoid enhancer binding factor (LEF), Ras, and c-Fos as well as cell surface proteins such as β4 integrins, α5β1 integrin, and αVβ6 integrin[171]. An important mechaninsm for EMT activation is the disruption of cell-cell adherens junctions and the cell-ECM adhesions mediated by integrins[168,172].

TGF-β is an important suppressor of epithelial cell proliferation and primary tumorigenesis; however, it is now clear that in certain contexts it can also serve as a positive regulator of tumor progression and metastasis[173,174]. Thus, in vitro studies have demonstrated that TGF-β can induce an EMT in certain types of cancer cells. Two possible signaling pathways have been identified as mediators of TGF-β–induced EMT. The first of these involves Smad proteins, which mediate TGF-β action to induce EMTs via the ALK-5 receptor[175,176] and this pathway facilitates motility. Inhibitory Smads modulate differential effects of relevant transcription factors and cytoplasmic kinases and induce the autocrine production of TGF-β, which can further reinforce and amplify the EMT program. Signaling pathways that mediate the action of β-catenin and LEF also cooperate with Smads in inducing an EMT. The second of these involves p38 MAPK and RhoA; it has been demonstrated that they mediate an autocrine TGF-β–induced EMT in NMuMG mouse mammary epithelial cells. This process also requires

integrin β1–mediated signaling and the activation of latent TGF-β by αVβ6 integrin[175,177]. Fibulin-5, an ECM molecule, augments TGF-β–induced EMT in a MAPK dependent mechanism[178].

It has been demonstrated that TGF-β can induce an EMT in Ras transformed hepatocytes, mammary epithelial cells (via MAPK), and MDCK cells; at the same time, Ras- activated PI3K inhibits TGF-β–induced apoptosis to facilitate this transition. Raf also mediates TGF-β–induced EMT and promotes invasiveness of cancer cells. In mouse models of skin carcinoma and human colon cancer, the absence of TGF-β receptor expression actually confers better prognosis[179,180].

The connection between loss of E-cadherin expression by cancer cells and passage through an EMT has been established by many studies[181,182]. For example, induction of the c-Fos oncogene in normal mouse mammary epithelial cell lines induces an EMT and is associated with a decrease in E-cadherin expression. Moreover, epithelial cell adhesion complexes reorganize and cell proliferation is suppressed when the full-length or the cytoplasmic portion of E-cadherin (containing the β-catenin binding site) is ectopically expressed in cells that have passed through an EMT, causing such cells to lose their mesenchymal phenotype[183]. Sequestration of β-catenin in the cytoplasm is important for the preservation of epithelial features of cancer cells, and acquisition of the mesenchymal phenotype correlates with the movement of β-catenin to the nucleus, where it becomes part of Tcf/LEF complexes. Such β-catenin accumulation in the nucleus, which is often associated with loss of E-cadherin expression, correlates with susceptibility to enter into an EMT and acquisition of an invasive phenotype[184]. Thus, cells that lose cell surface E-cadherin become more responsive to induction of an EMT by various growth factors[169]. Some studies have demonstrated that the epigenetic control of E-cadherin and β-catenin/LEF activity is important in establishing the metastatic potential of cancer cells. Cell lines that lack E-cadherin show increased tumorigenicity and metastasis when transferred into immunodeficient mice[184,185]. E-cadherin expression levels vary dramatically in different

human tumors, and an inverse relationship between levels of E-cadherin and patient survival has been documented. In this regard, mutations in the E-cadherin gene have been identified in cancer cells, making them more susceptible to EMT and metastasis[186]. The central role played by E-cadherin loss in the EMT program is further illustrated by the actions of several EMT-inducing transcription factors that facilitate acquisition of a mesenchymal phenotype, such as Snail and Slug, as well as those encoding two key zinc finger–containing basic helix-loop-helix transcription factors, survival of motor neuron protein interacting protein 1 (SIP1) and E12 (also known as E47-E2A). These transcription factors are induced by TGF-β exposure and, once expressed, repress E-cadherin expression. Loss of E-cadherin promotes Wnt signaling and is associated with high levels of Snail in the nucleus. SIP1 represses E-cadherin expression and binds, along with Snail, to the E-cadherin promoter in an overlapping fashion. The expression of Snail and E-cadherin correlates inversely with the prognosis of patients with breast cancer or oral squamous cell carcinoma[169].
It has been demonstrated that Twist and Goosecoid genes facilitate EMT and induce metastasis[187].

Several studies have reported that matrix-degrading enzymes such as MMP-3 facilitate EMT by inducing genomic instability via Rac1b and[188].

Noncoding microRNAs are also components of the cellular signaling circuitry that regulates the EMT program. For example, microRNA 200 (miR200) and miR205 inhibit the repressors of E-cadherin expression, ZEB1 and ZEB2, and thereby help in maintaining the epithelial cell phenotype[189,190].

EMT may play a role in the generation of high-grade invasive cells with stem cell–like features, and the latter phenotype, which includes self-renewal potential, may facilitate the formation of secondary tumors by disseminating cancer cells, a notion that still requires direct demonstration.

Recently, it has been demonstrated that the tetraspanin CD151, whose expression has been associated with increased invasiveness and poor prognosis in

hepatocellular carcinoma (HCC), when associated with α6 integrin, induces an epithelial-mesenchymal transition[211], *Ke et al* shown that α6 integrin formed a complex with endogenous CD151 in HCC cells. In cells that expressed high levels of α6 integrin and CD151, lamini-5 promoted cell spreading by inducing the epithelial-mesenchymal transition (EMT); this effect was not observed in cells that expressed high levels of only α6 integrin or CD151. Cells that expressed high levels of α6 integrin and CD151 underwent the EMT in response to laminin-5, through hyperactivation of phosphatidylinositol-3-kinase (PI3K), primarly induced via the PI3K-protein kinase B (Akt)-Snail-phosphatase and tensin homolog feedbask pathway. The EMT was reversed by PI3K inhibitors and antibodies against CD151 or α6 integrin *in vitro*, and was delayed by specific interference with CD151 and α6 integrin *in vivo*. This study suggested that tetraspanins could play an important role in regulate EMT and MET profile.

In our study, we propose that CD63 could play a role in regulation of cell motility and invasion, as well as proliferation and apoptosis. We also hypothesize that CD63 could have a role in EMT; in particular, it could function as a negative marker of EMT.

II

Aims of the thesis

Melanoma is one of the most aggressive tumor with higher metastatic potential and a high rate of mortality.

Over the last decades, the increase in incidence have mainly been reported for thin melanomas (≤1mm) associated with a good prognosis. Whereas the rate for thick melanomas (>1mm), with unfavourable prognosis and associated with a higher rate of distant metastases and worse survival, seems to be fairly stable. Hence, there is a need to identify new prognostic markers of progression and the new potential drug targets. A possible negative marker of metastasis is the CD63 protein, belonging to the Tetraspanins superfamily. It is highly expressed in several normal tissues as well as in the early stage of melanoma. We have previously observed a progressive decrease of CD63 protein levels in tissue samples of human melanocytic nevi, dysplastic nevi, thin melanomas, thick melanomas and metastasizing melanomas in advanced stages of melanoma progression.

This suggests that the CD63 gene could be a potential *metastasis suppressor gene*.

We hypothesized that CD63 could play a functional role in controlling cell motility and invasion, probably by means of functional interactions with other proteins playing key role in these processes. Based on data reported in literature, the pro-metastatic proteins uPAR, MMP-2, MMP-9 and the tissue inhibitor of metalloproteinase-1 (TIMP-1) seemed the best candidates. We also supposed that CD63 could play a role in controlling cell proliferation.

To confirm our hypothesis we acted modulating the expression of CD63 in A375 human melanoma cell line: cells in which CD63 has been overexpressed and silenced

have been monitored in order to assess significant differences in the ability of motility, invasion, proliferation and apoptosis.

A further confirmation of our hypothesis has come from the observation of CD63 expression levels in B16 murine melanoma cell line; in particular, we have observed that CD63 levels are significantly reduced in cells following treatment with IFN-gamma, known to be a metastatic cancer inducer.

We also hypothesised that CD63 could play a role in the Epithelial-Mesenchymal Transition (EMT), acting as negative marker.

III

Materials and Methods

3.1 Immunohistochemistry

Immunohistochemical analysis was performed on 50 archival sections of formalin-fixed, paraffin-embedded human samples including 10 melanocitic nevi, 10 displastic nevi, 10 thin melanomas, 10 thick melanomas and 10 metastasis (kindly provided by Dr. Urso - Pathological Anatomy Unit, Santa Maria Annunziata Hospital, Florence).

The sections were deparaffinized with toluene for 15 minutes and rehydrated with decreasing concentration of alcohol. The sections were then washed with ddH_2O and antigen retrieval was achieved by warming the sections for 15 minutes in commercial citrate buffer pH6 (as suggested by the protocol for the antibody in use) using microwave. There was no need to perform the inhibition of peroxidase because we used the FastRed chromogen instead of DAB. After two washes in PBS, tissues were incubated for 10 minutes at room temperature in Ultra V Block to block the nonspecific background staining, and subsequently incubated with primary antibody anti-CD63 (diluted 1:100) at 37 ° C in oven for 2h. sections were washed three times with PBS and incubated in the dark with secondary antibody for 30 minutes. After incubation the chromogen was added and kept for 10 minutes at room temperature. Samples were then washed in distilled water and mantained for 5 minutes in hematoxylin for staining nuclei. Finally, the slides were mounted using Canada balsam and examined using light microscopy.

3.2 Cell lines, treatments and antibodies

Human and murine cells were used in our study. Hela cells, human A375P and A375M6 melanoma cells and murine B16 melanoma cells were cultured in DMEM medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100µg/ml streptomycin and 1% glutamine. All cells were maintained in 5% CO_2 at 37°C in humified incubator. Stable clones overexpressing CD63 were maintained in DMEM supplemented with 10% FBS, 1%glutamin, 100 U/ml penicillin, 100µg/ml streptomycin and 1mg/ml geneticin (G418). Murine B16 cells were treated with Interferon γ 25U, 50U and 100U for 24 hours to induce a metastatic profile.

The mouse anti-CD63 monoclonal antibody used for immunohistochemistry analysis was obtained from Thermo Scientific (MA1-26352) and used at a concentration of 1:100. The rabbit anti-CD63 and anti-uPAR policlonal antibody were obtained by Santa Cruz (CD63 Antibody (H-193): sc-15363; uPAR Anticorpo(FL-290): sc-10815) and used at a diluition of 1:200. The mouse monoclonal anti-Flag M2 was obtained from Sigma and used at a diluition of 1:200. The fluorescent antibody used for confocal microscopy and flow citometry were obtained from Abnova (goat anti-mouse IgG secondary antibody – rhodamine (PAB10744) and goat anti-rabbit IgG secondary antibody – fluorescein (PAB10821)) and used at a concentration of 1:700. Mouse monoclonal anti-tubulin and anti-actin were used as housekeeping genes.

3.3 RNA extraction, PCR and plasmid contruction

Total RNA was isolated from cells with an NucleoSpin II Extraction Kit (Macherey-Nagel), analyzed using Q-bit spectrophotomer (Qiagen, Venlo, Netherlands) and its quality was assessed by running 500 ng of sample on a 1,5% polyacrylamide gel. 500

ng of RNA were retrotranscribed using ImProm-II reverse transcriptase (Promega, Madison, Wisconsin, USA) and random examers.

For PCR amplification, CD63FLAGpCDNA3fw 5'–CGCGGATCCGCCACCATGGACTACAAGGACGACGATGACAGGATGGCGGTGGAAGGAGGAATGAAATGTG -3' was used as the forward primer and CD63rv 5'-CCGGAATTCCATCACCTCGTAGCCACTTCTGATACTC – 3' as reverse primer. This primer pair amplifies a 720-bp fragment of CD63 cDNA. Reactions were carried out in 20 µl of a solution containing specific primers and 0.1 U/µl of GoTaq Polymerase, using a Biorad Thermocycler. Aliquots of 10 µl of each PCR mixture were applied to a 1,5% agarose gel, electrophoresed and visualized. cDNA products were evaluated on the basis of standard PCR markers.Our insert contained a Kozak translation initiation sequence, an ATG start codon for proper initiation of translation and a stop codon for proper termination of the gene. Subsequently, the cDNA was inserted into the pcDNA3.1+ vector, which was prepared for cloning by digestion with BamHI and EcoRI. The plasmid, containing the neomycin resistance gene for selection of stable cell lines, was then transformed into *E. coli* DH5α and transformants were selected on LB plates containing 50 to 100µg/ml ampicillin. Once we have identified the correct clone, we purified the colony and prepared a glycerol stock of our plasmid-containing *E.coli* strain for long-term storage.

3.4 Transfection of CD63 DNA and selection of stable clones

CD63 cDNA was subcloned into the BamHI/EcoRI sites of a pcDNA3.1+ vector (Invitrogen), downstream of a cytomegalovirus promoter. The CD63 cDNA expression construct was transfected into A375 human melanoma cells by using Lipofectamine LTX Plus Reagent (Invitrogen) according to the manufacturer's instructions. pcDNA3.1+ vector only was also transfected as a control. Cells ($5x10^5$) were seeded in

35 mm dishes and allowed to reach 80% confluence. Lipofectamine reagents and DNA were diluited in Opti-Mem and incubated at room temperature for 30 minutes. DNA - lipofectamine complex was then added to the cells in DMEM serum-free and incubated for 48 hours. Western Blot was performed to verify the efficiency of transfection

For stable expression of CD63 mutants, plasmid DNA was transfected into A375 human melanoma cells using Lipofectamine LTX Plus Reagent (as previously described). After 48 h, cells were then cultured in media containing Genetycin (G418, 1mg/ml; MMedical) for selection. After 2 weeks of selection, colonies were pooled, and CD63-positive cells were analysed by flow cytometry and western blot.

3.5 CD63 silencing

siRNA for CD63 (esiRNA) and control siRNA were purchased from Sigma-Aldrich (MISSION esiRNA are endo-ribonuclease prepared siRNA. MISSION esiRNA are a heterogeneous mixture of siRNAs that all target the same mRNA sequence. These multiple silencing triggers lead to highly specific and effective gene silencing). Transfection of RNA was performed using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells ($5x10^5$) were seeded in 35 mm dishes and allowed to reach 50-60% confluence. The final siRNA concentration used for transfection was 100nM. Lipofectamine reagent and siRNA were diluited in Opti-Mem and incubated at room temperature for 20 minutes. siRNA - lipofectamine complex was then added to the cells in DMEM serum-free and incubated for 48 hours. Western Blot was performed to verify the efficiency of transfection.

3.6 Western Blot analysis

Cells were collected by centrifugation and RIPA lysis buffer [50 Mm Tris (pH 7.4), 150 mM NaCl, 1% Tritonx-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptinin] and cocktail proteinase inhibitor were added to the pellet for 30 minutes on ice; cells were then centrifugated at 14,000 rpm for 20 minutes and the supernatant were collected and stored at -80°C. Protein quantification were performed by an Eppendorf spectrophotometer. Fifty micrograms of total protein were used for each sample that was denaturated at 90°C for five minutes and separated on Any KD MiniProtran TGX precast gels (Biorad) with 200 V for 30 minutes under nonreducing conditions. After separation, the proteins were electrophoretically transferred to nitrocellulose membranes (Millipore) using constant 0,33 A, 20 V for 30 minutes. Transfer were verified with ponceau solution and membranes were incubated in a blocking solution consisting of PBS/Odyssey Blockyng Buffer 1:1 (PBS/OBB) (Lycor Bioscience) for 1 h at room temperature. Membranes where then incubated overnight at 4°C in the appropriate antibody. Membranes were then washed four times with PBS- Tween 0,1% solution (five minutes) and probed with the secondary fluorescent antibody (Goat anti-rabbit Lycor) according to the manufacturer's instructions. Proteins were detected using the Odyssey scanner (Lycor).

3.7 Flow-cytometric analysis

Cells were incubated with 10 μg/ml rabbit anti-CD63 antibody H-193 (Santa Cruz) and mouse monoclonal anti-Flag M2 (Sigma) for 30 minutes, washed with cold PBS and then incubated with saturating concentration of goat anti-rabbit IgG secondary antibody – fluorescein (PAB10821) and goat anti-mouse IgG secondary antibody – rhodamine (PAB10744) respectively for 30 minutes at 4°C. After being washed with PBS, the cells were analysed by flow cytometry performed on FACS Canto.

3.8 Confocal analysis

Cells were grown on glass coverslip in complete media for 24 hours. Spread cells were fixed with 3,7% paraformaldehyde-PBS for 15 minutes. Specimens were then blocked with phosphate-buffered saline (PBS)/10% FBS for 1 hour at room temperature to saturated nonspecific sites. Cells were then incubated with 10 µg/ml rabbit anti-CD63 antibody H-193 (Santa Cruz) and mouse monoclonal anti-Flag M2 (Sigma) for 1 hour, washed with PBS, and stained goat anti-rabbit IgG secondary antibody – fluorescein (PAB10821) and goat anti-mouse IgG secondary antibody – rhodamine (PAB10744) respectively for 30 minutes at room temperature. Specimens were washed with PBS and mounted in the dark using mounting medium. Confocal images were acquired.

3.9 Wound Healing Assay

Cell migration was evaluated by an in vitro wound healing assay. Cells were cultured near (>90%) confluence in 35 mm dishes and and incubated overnight to allow monolayer cell formation. The cell layer was wounded with a sterile 200 µl pipet tip and then incubated in the presence of 1% FBS culture medium for 24 h. cell migration toward the wounded are was observed and photographed. Pictures were taken at 0, 6, 12 and 24 hours, using phase contrast and 10X and 20X. Time lipse was also used for this kind of experiment.

3.10 Matrigel Invasion Assay

A cell invasion assay was conducted by using BD Matrigel™ Basement Membrane Matrix and Millicell cell culture Insert (24-well PCF 8.0 µm, Millipore). The Matrigel (10 mg/mg) was diluted at 35 µg/ml and coated onto the membrane of the

Millicell upper chamber (100 µl in DMEM serum-free) in a 24-well plate overnight. Matrigel was rehydrated with 200 µl DMEM serum-free and cells (1 x 10^5) were seeded into the upper chamber in 400 µl DMEM serum-free. The lower chamber contained complete culture medium, which include 10% FBS to trap invading cells. Cells were incubated in 5% CO_2 at 37°C for 12 h (cells transfected with siRNA) and 24 h (cells overexpressing CD63). Then, the non-invading cells on the upper surface of Matrigel matrix were removed by cotton swabs. The cells that invaded across the matrix to the lower surface of the membrane were fixed and coloured with a Diff Quick Kit and counted. Pictures of the stained invaded cells (5 random fields/culture) were taken under an inverted microscope at 10X and 20X and the mean number of cells of the fields was recorded. The data were then expressed as the average number (±SD) of cells from 5 fields that invaded from each of 3 experiments performed.

3.11 Wst1 Proliferation Assay

Cell proliferation was evaluated in triplicate by a colorimetric assay. This technique can determine cellular viability by measuring the metabolic conversion of a water-soluble tetrazolium salt, Wst-1 (Roche), into farmazan by mitochondrial dehydrogenases: it forms a dark red product that is soluble in tissue culture medium. The amount of formazan produced is proportional to the number of live cells and is expressed as cellular viability. For this procedure, cells were seeded at a density of 3000 cells/well in 96-well plates and incubated in serum-free medium for 24, 48 and 72 hours and assays were performed by adding Wst-1 directly to the culture wells and incubating them for 15 minutes to 120 minutes at 37°C. Plates were then read by a scanning multiwall spectrophotometer by measuring the absorbance of the dye with a wavelength of 450 nm and a reference wavelength of 630 nm.

3.12 Zymography Assay

Gelatine zymography was performed to determine the activity of MMP-2 and MMP-9. Cells were starved with serum-free medium for 24 and 48 hours. Medium was collected and protein were concentrated using Amicon Ultra-0.5 ml centrifugal filter devices (Millipore) and quantified by a pectrophotometer (Eppendorf). Proteins in medium were then separated in 10% SDS-page gel containing 1 mg/ml gelatine. HT1080 was used as MMPs standard. After running, the gel was incubated in the 2.5% triton-X 100 in deionized water for renaturing with gentle agitation for 30 minutes at room temperature. Then the gel was incubated in developing buffer (50 mM Tris-HCl, 0,2 M NaCl, 5 mM $CaCl_2$, pH 7.4) overnight with gentle shaking at 37°C. The gel was stained with a fixing-staining solution (0,5% Comassie Blue in methanol/water/acetic acid in a ratio 5:5:1) for 2 hours at room temperature and then washed with a destaining solution (methanol/water/acetic acid without Comassie blue). The gel was visualized under a Licor Scanner Odyssey and pictures were captured. Analysis was performed with ImageJ software.

3.13 Tumor Cell Invasion and Spontaneous Pulmonary Metastases

An *in vivo* assay was performed to determine the ability of A375 cells with low levels of CD63 to invade. Scid/Beige immunodeficient mice 6 weeks old (Charles River) were given iv injections of $1x10^6$ viable A375 human melanoma cells. 30 days later, the animals were killed and the presence of any pulmonary or other metastases was recorded.

3.14 Statistical Analysis

Error bars in all figures represent SEM. The Student's t-test was performed to evaluate pair wise differences with a $p<0.05$ being considered significant.

IV

Results and Discussion

4.1 CD63 expression in human melanoma

CD63 belongs to the transmembrane 4 superfamily of membrane proteins and it is expressed in several normal tissues as well as in melanoma cells; Several studies suggested that CD63 may play an important role in inhibiting melanoma progression. CD63 expression is strong in the early stage of melanomas, weak or absent in the late stage[138,140]. To understand the role of CD63 in melanoma, we analyzed the CD63 expression in diverse samples of human melanoma tissues. Immunohistochemistry analysis was performed on 50 samples divided into five experimental groups: 10 melanocytic nevi, 10 dysplastic nevi, 10 thin melanomas, 10 thick melanomas and 10 metastases (kindly provided by Dr. Urso - Pathological Anatomy Unit, Santa Maria Annunziata Hospital, Florence). As shown in **Figure 1R**, CD63 is highly expressed in nevi and melanomas, whereas its expression is reduced in metastasis. In melanocytic and dysplastic nevi, CD63 is mainly confined to the epidermis, although some positive cells can be found in the underlying layer. In thin melanoma, in agreement with the definition of superficial melanoma, CD63 positive cells are confined in the surface layer of the dermis. Instead, in thick melanoma, cells expressing CD63 are spread evenly throughout the epidermis and the dermis. In metastases, the expression of CD63 is limited to a few cells distributed heterogeneously in the tissue. These data suggest that the tetraspanin CD63 could play a role of negative suppressor, and a potential marker of melanoma progression.

The observation that reduction of CD63 expression is correlated with increased malignancy is also supported by several other studies. 1) In ovarian cancer tissues,

increasingly lower expression levels of CD63 were found to correlate with increasingly higher chances of tumor metastasis[162]; 2) In lung adenocarcinoma, low CD63 expression correlated with increased tumor growth and a poor prognosis[163]; 3) A negative relation between CD63 expression and cancer invasiveness and metastasis was also found in breast and colon cancers[164,165].Taken together, these studies and our data show that CD63 expression often tends to decrease in the late stages of cancer development, in association with increased tumor cell motility and metastasis.

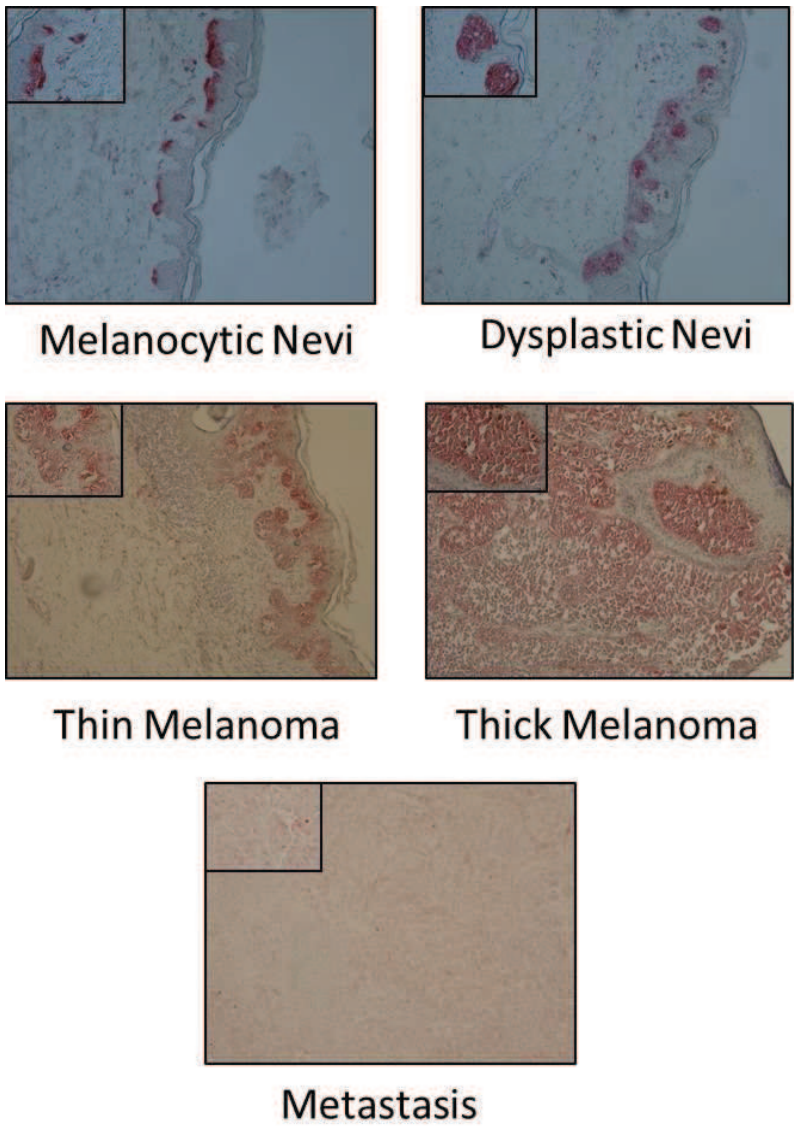
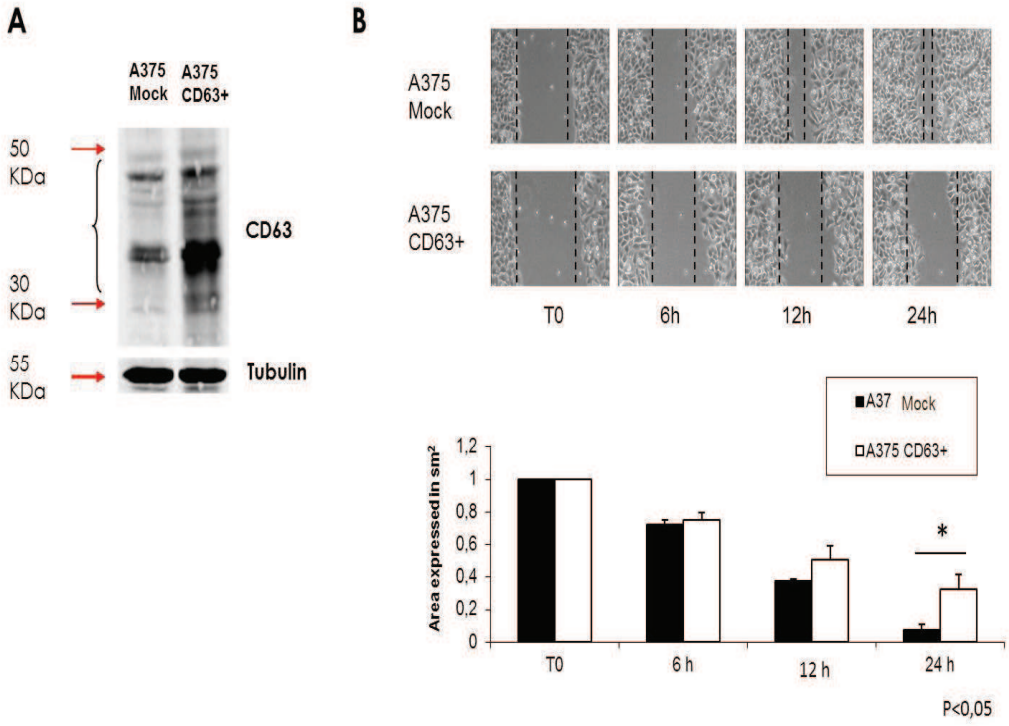


Figure 1R. Distribution of CD63 positive cells in: Melanocytic Nevi, Dysplastic Nevi, Thin Melanoma, Thick Melanoma and in a Melanoma metastasis. IHC α-hCD63, magnification 10x and 40x.

4.2 Role of CD63 in cell motility and metastasis

In order to understand the effect of CD63 on cellular motility, we performed a wound healing assay on human stable melanoma cell line A375. The cells were transiently transfected with a human CD63 cDNA or with siRNA in order to overexpress the CD63 protein or to silence it, respectively. Using a time lapse microscopy the cells were video-registered for 24 hours and evaluated for their ability to repair the wound. As shown in **Figure 2R** the exogenous modulation of CD63 in human melanoma cell line A375 was effective and correlated with a change in cell motility. Indeed, A375 cells overexpressing CD63 showed a significant reduction in cell motility (**2R-B)** compared to control ($P<0.05$); whereas A375 with silenced CD63 were able to close the wound more rapidly as compared to control ($P<0.05$) (**2R-D**).



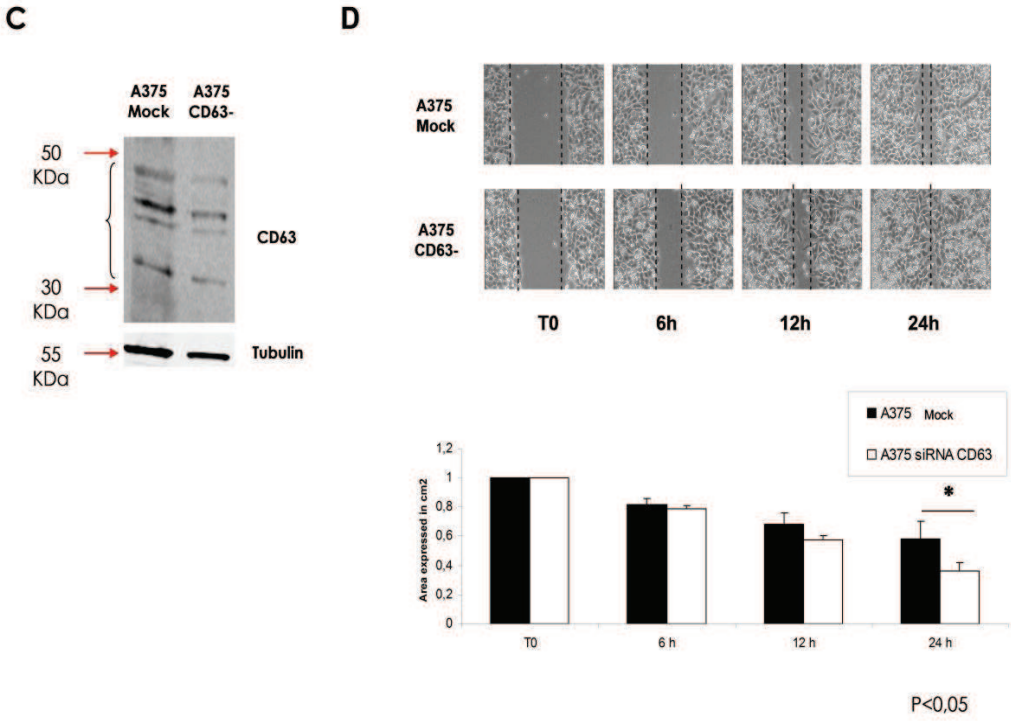


Figure 2R. Wound healing assay. A375 cells overexpressing CD63 (**2R-A,** evaluated by western blot) show a significant reduction in cell motility (**2R-B**) compared to control ($P<0.05$); whereas A375 with silenced CD63 (**2R-C,** evaluated by western blot) are able to close the wound (**2R-D**) more rapidly as compared to control ($P<0.05$)

The ability of tumor cells to invade is one of the hallmarks of the metastatic phenotype. During the development of invasive cancer, tumor cells have to penetrate the extracellular matrix including the basement membrane (BM), a usually continuous layer composed of a dense meshwork of collagens, glycoproteins and proteoglycans. It normally underlies epithelia and lacks any pores large enough to allow epithelial cells to pass through. In consequence, the invasion of tumor cells through the BM must be either an active process effected by the tumor cells themselves or is mediated by structural alterations of the BM occurring during carcinogenesis and cancer progression. It was supposed by many authors that invading and metastasizing tumor cells are able to degrade actively the continuous, uninterrupted BM by secreting collagenases and other proteolytic enzymes[191].

To elucidate the mechanisms by which tumor cells acquire an invasive phenotype, *in vitro* assays have been developed that mimic the *in vivo* process. The most commonly used *in vitro* invasion assay is a modified Boyden chamber assay using a basement membrane matrix preparation called Matrigel. The results obtained using this assay show a strong correlation between the ability of tumor cells to invade *in vitro* and their invasive behaviour *in vivo*, and are widely recognized as a measure of invasive potential[192].

It has been demonstrated that the tetraspanins CD82 andCD9 mostly suppress tumor progression. By their interactions with a variety of proteins including integrins, signaling proteins and immunoglobulin superfamily members they suppress motility and promote adherence to the surrounding matrix. Their expression is often reduced in late-stage human tumors. On the other hand, two tetraspanins, CD151 and Tspan8, are overexpressed in several human tumors and seem to promote and support tumor progression. CD151 regulates cell migration, mostly through its association with α3β1, α6β4 and matrix metalloproteinases. Additional transmembrane and cytosolic proteins in multimolecular contribute to the formation of complexes in TEM. The opposing effects of CD82 and CD9 versus CD151 and Tspan8 on metastasis suppression and promotion cannot be fully explained by differences in the composition of the TEM. Indeed, there are strong hints that, by their enrichment in exosomes, tetraspanins and associated molecules become engaged in intercellular communication, where their involvement in membrane fusion facilitates message, including mRNA and microRNA, delivery[50].

Few studies have been performed in order to demonstrate a role of the tetraspanin CD63 in invasiveness control. The following results demonstrate that exogenous modulation of CD63 in human melanoma cell line A375 correlated with cell invasiveness. The invasive capacity was quantified using the Matrigel *in vitro* invasion assay. The number of cells that invaded through the membrane after incubation was counted. As shown in **Figure 3R** the A375 cells overexpressing CD63 showed a

decreased invasiveness (more than four-fold) compared to control (**3R-A**), whereas A375 with CD63 silenced showed an increased invasiveness (two-fold) compared to control. (**3R-B**) after an incubation of 24 hours. Taken together, these studies showed that reduced CD63 expression mimicking the late stages of cancer development, correlated with increased tumor cell motility and metastasis.

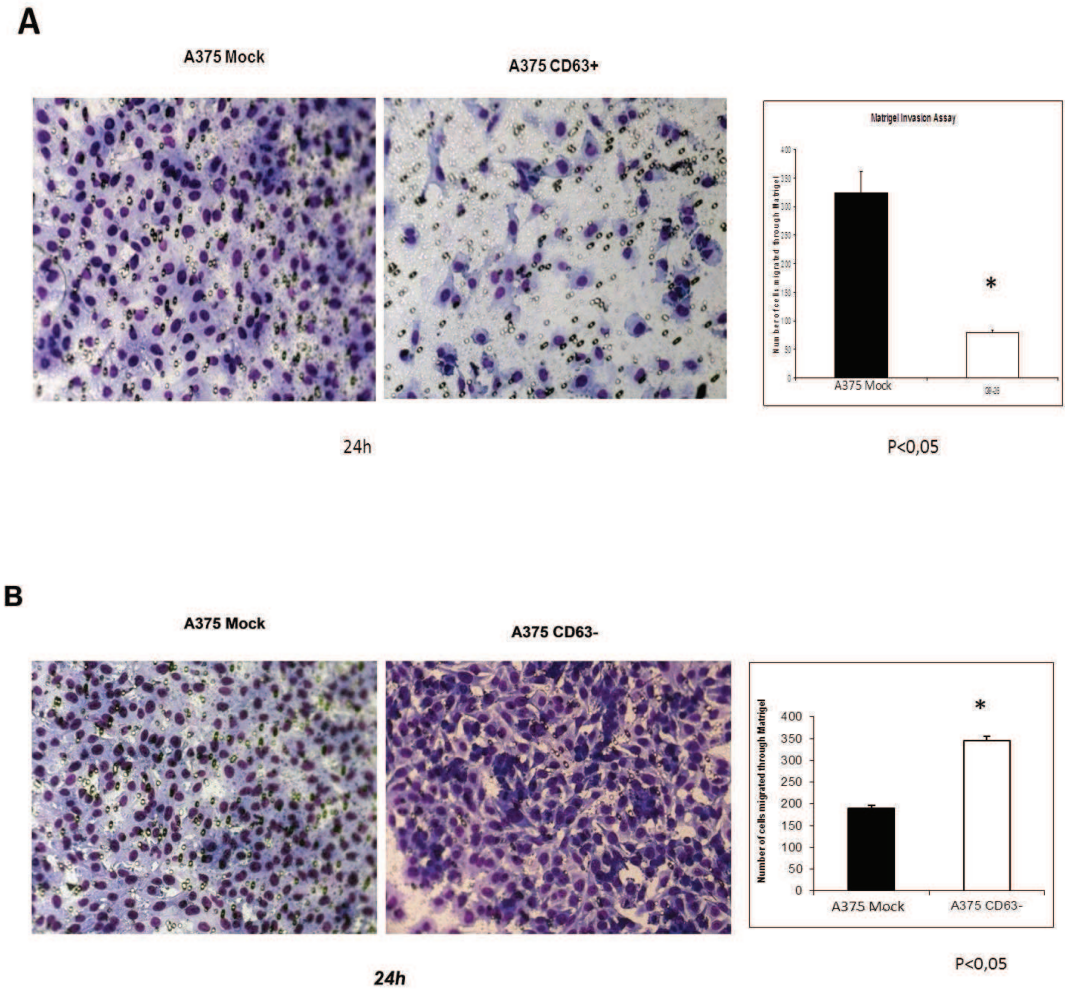


Figure 3R. Matrigel invasion assay. The A375 cells overexpressing CD63 show a decreased invasiveness (more than four-fold) compared to control (**3R-A**), whereas A375 with CD63 silenced showed an increased invasiveness (two-fold) compared to control (**3R-B**) after an incubation of 24 hours.

4.3 Overexpression of CD63 in relation to invasiveness of melanoma cells

In order to better understand the biological significance of increased CD63 expression in melanoma cells, we produced by stable transfection and selected G418-resistant stable A375 clones overexpressing different levels of CD63. As shown in **Figure 4R**, we have chosen four stable A375 clones, called G9-21, G9-24, G9-26 and G9-61, based on their different levels of expression of CD63 protein. Each clone phenotype was determined by both flow cytometry and western blot analyses. The localization of exogenous CD63 was evaluated by immunocytochemistry in order to examine and quantify the expression of cytoplasmic and membrane forms, but no differences were observed compared to control.

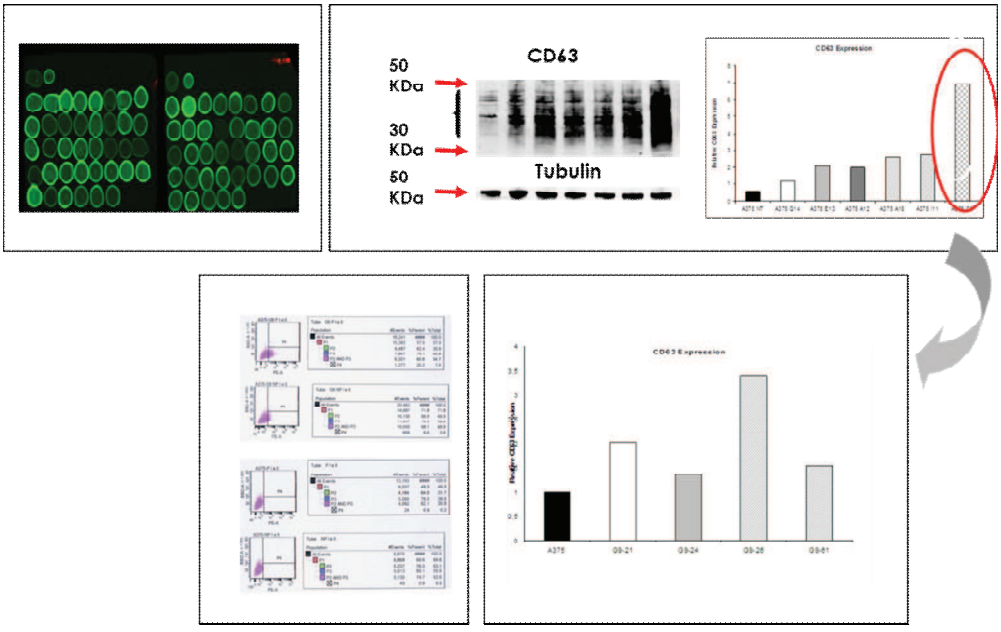


Figure 4R. Selection of stable clones overexpressing CD63. Each clone phenotype was determined by both flow cytometry and western blot analyses.

Next, we reproduced the motility and invasiveness assays on these clones, confirming what we have previously observed on A375 cells transiently transfected with CD63 cDNA. Analogically to previous experiments with transient transfectants, the stable

overexpression of CD63 in A375 cells significantly reduced the cellular invasiveness capability, and interestingly, negatively correlated with the levels of over expression of CD63 (**Figure 5R**).

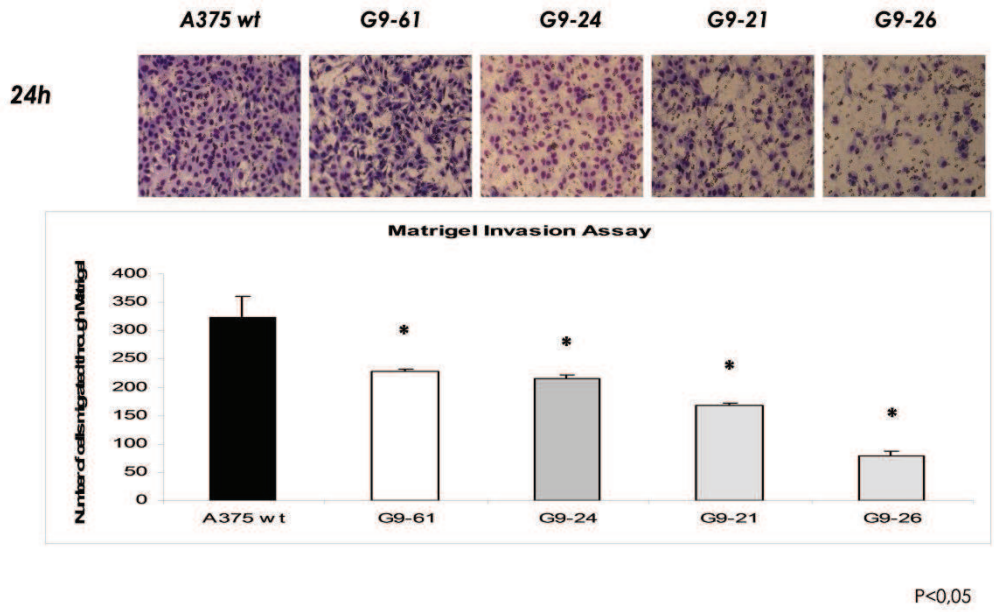


Figure 5R. Matrigel invasion assay. The stable overexpression of CD63 in A375 cells significantly reduced the cellular invasiveness capability, and interestingly, negatively correlated with the levels of over expression of CD63

4.4 Correlation between CD63, uPAR and MMPs expression

As previously demonstrated, endothelial basement membranes are barriers normally impermeable to migrating cells; however metastatic cells can breach them to enter the parenchyma of the target organ and proliferate, forming a secondary tumor or metastasis. Metastatic cells have been found to express significant amounts of matrix degrading enzymes, particularly metalloproteinases, serine proteases, cathepsins and others[193]. Alterations in the balance between proteolytic enzymes and their inhibitors appear to be a key feature of invasion. Although tumor cells themselves can show increased protease production, often these are derived

from the host tissue in response to tumor cell stimulation[194]. Inhibitors of metalloproteinases have been proven to be particularly effective in regulating invasion and metastasis.

In melanoma, the expression of MMP-2 has been found to correlate with unfavourable prognosis and tumor progression[195]. However, not only the presence of active MMP, but also the cellular localization of the active form plays a crucial role in cell invasion. It has been shown that collaboration between cell adhesion molecules, such as integrins and CD44, and proteolytic active MMPs at the cell surface, may result in highly localized and tightly regulated pericellular degradation of the ECM[196]. In established melanoma, the MMPs, in particular MT1-MMP and MMP-2, interact with specific cell adhesion molecules and growth factors, thereby promoting cell motility and invasion[197]. MMP-9 also plays a critical role in tumor cell invasion.

It has been shown that MT1-MMP associates with various tetraspanins, resulting in possible negative regulation of expression, and both negative and positive functional regulation. *Lafleur et al* also described how multiple tetraspanins associate with MT1-MMP in cancer cells, preventing their lysosomal degradation, supporting cell surface expression and uniformly enhancing MT1-MMP functions in multiple proteolysis assays[198].

The tetraspanin membrane protein CD151, in contrast to other tetraspanin members as CD9, CD63 and CD81, associated with a negative control of cell motility and invasiveness, has been suggested to regulate positively cancer invasion and metastasis by initiating signaling events. *Hong et al*[199] found that stable transfection of CD151 into MelJuSo human melanoma cells lacking CD151 expression significantly increased cell motility, matrix metalloproteinase-9 (MMP-9) expression, and invasiveness. The enhancement of cell motility and MMP-9 expression by CD151 over expression was abrogated by inhibitors and small interfering RNAs targeted to focal adhesion kinase (FAK), Src, p38 MAPK, and JNK, suggesting an essential role of these signaling components in CD151 signaling pathways. Also, CD151-induced MMP-

9 expression was shown to be mediated by c-Jun binding to AP-1 sites in the MMP-9 gene promoter, indicating AP-1 activation by CD151 signaling pathways. Meanwhile, CD151 was found to be associated with α3β1 and α6β1 integrins in MelJuSo cells, and activation of associated integrins was a prerequisite for CD151-stimulated MMP-9 expression and activation of FAK, Src, p38 MAPK, JNK, and c-Jun. Furthermore, CD151 on one cell was shown to bind to neighboring cells expressing CD151, suggesting that CD151 is a homophilic interacting protein. The homophilic interactions of CD151 increased motility and MMP-9 expression of CD151-transfected MelJuSo cells, along with FAK-, Src-, p38 MAPK-, and JNK-mediated activation of c-Jun in an adhesion dependent manner.

In order to examine the role of CD63, we evaluated the expression of metalloproteinases MMP-2 and MMP-9 in control A375 cells and in stable clones overexpressing CD63. As shown in **Figure 6R**, MMPs expression diminished in clones overexpressing higher levels of CD63. The reduction MMPs expression was proportional to the increase in the levels of over expression of CD63 and reduction of cell motility and invasiveness.

Based on the data reported in literature, we hypothesize that CD63 could play an important role in regulating tumor progression, probably by a negative control of cellular matrix degradation mediated by metalloproteinases. However, it is not yet clear how this mechanism occurs.

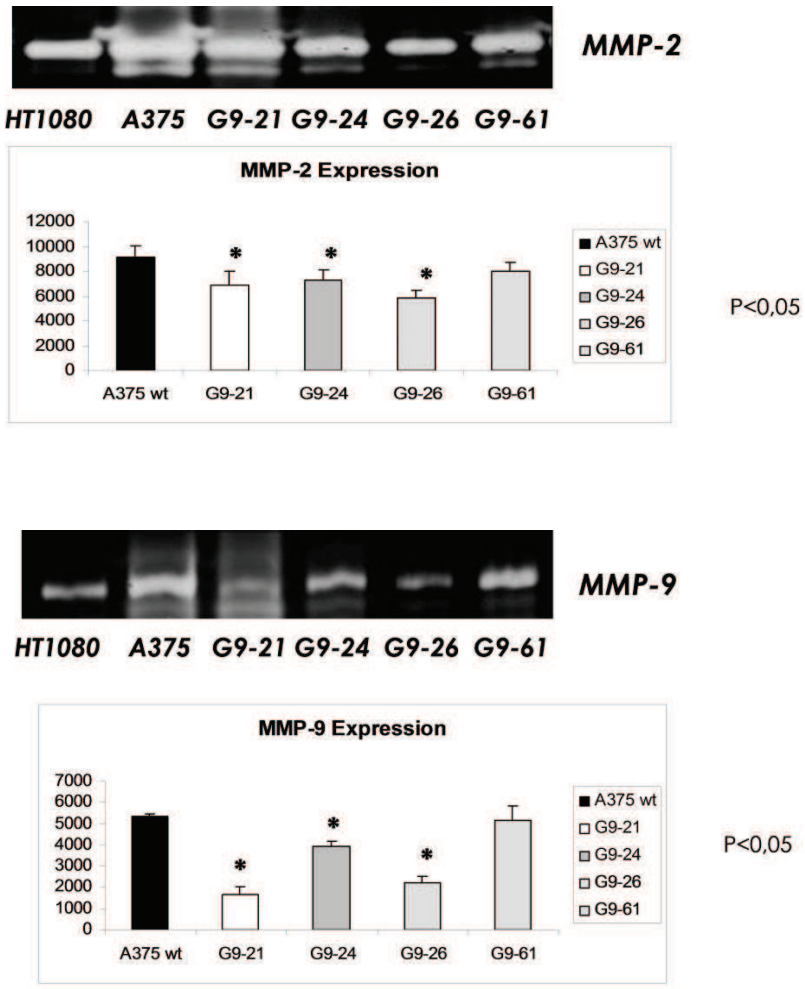


Figure 6R. MMPs expression. MMPs expression diminished in clones over expressing higher levels of CD63. The reduction MMPs expression was proportional to the increase in the levels of over expression of CD63 and reduction of cell motility and invasiveness.

The metalloproteinases also interact with another major proteinase system regulating invasion: the plasminogen activator system[200]. This consists of plasminogen activator (uPA, also produced as a latent proenzyme), its receptor (uPAR) and its inhibitor (PAI's). uPA cleaves plasminogen to plasmin, which has a broad substrate specificity and degrades many components of the extracellular matrix. uPA can also directly activate metalloproteinases. Inhibitors of plasmin have been found to inhibit invasion into matrigel, while inhibitors of uPA delay invasion and stimulators

of uPA production enhanced invasion. uPA must be bound to cell surface uPAR to affect invasion; interruption of this interaction leads to decreased invasion.

The role of uPAR in melanoma progression and metastasis promotion has been well established, and several studies have been performed. *Sthal et al*[201] demonstrated that uPAR is expressed in a highly metastatic melanoma cell line (M24met) and that uPAR is functionally involved in invasion and migration of these cells.

It has been shown that tetraspanins interact with uPAR system and can modify uPAR function. Bass et al[202] demonstrated that expression of the tetraspanin CD82 (a tumor metastasis suppressor) affects uPAR function. Pericellular plasminogen activation was reduced by 50-fold in the presence of CD82, although levels of components of the plasminogen activation system were unchanged. uPAR was present on the cell surface and molecularly intact, but radioligand binding analysis with uPA and anti-uPAR antibodies revealed that it was in a previously undetected cryptic form unable to bind uPA. This was not due to direct interactions between uPAR and CD82, as they neither co-localized on the cell surface nor could be co-immunoprecipitated. However, expression of CD82 led to a redistribution of uPAR to focal adhesions, where it was shown by double immunofluorescence labeling to co-localize with the integrin α5β1, also redistributed with CD82. Co-immunoprecipitation experiments showed that, in the presence of CD82, uPAR preferentially formed stable associations with α5β1, but not with a variety of other integrins. These data suggested that CD82 inhibited the proteolytic function of uPAR indirectly, moving together uPAR and α5β1 to focal adhesions and promoting their association with a resultant loss of uPA binding.

We examined the interaction of uPAR and CD63 by evaluation of the expression of uPAR in A375 controls and in CD63 overexpressing stable clones. It demonstrated that uPAR expression was significantly reduced in cells with high levels of CD63, as shown in **Figure 7R**. Interestingly, the reduction of uPAR appeared to be proportional

to the increase in CD63 over expressin. The mechanism underlying this reduction is now under investigation.

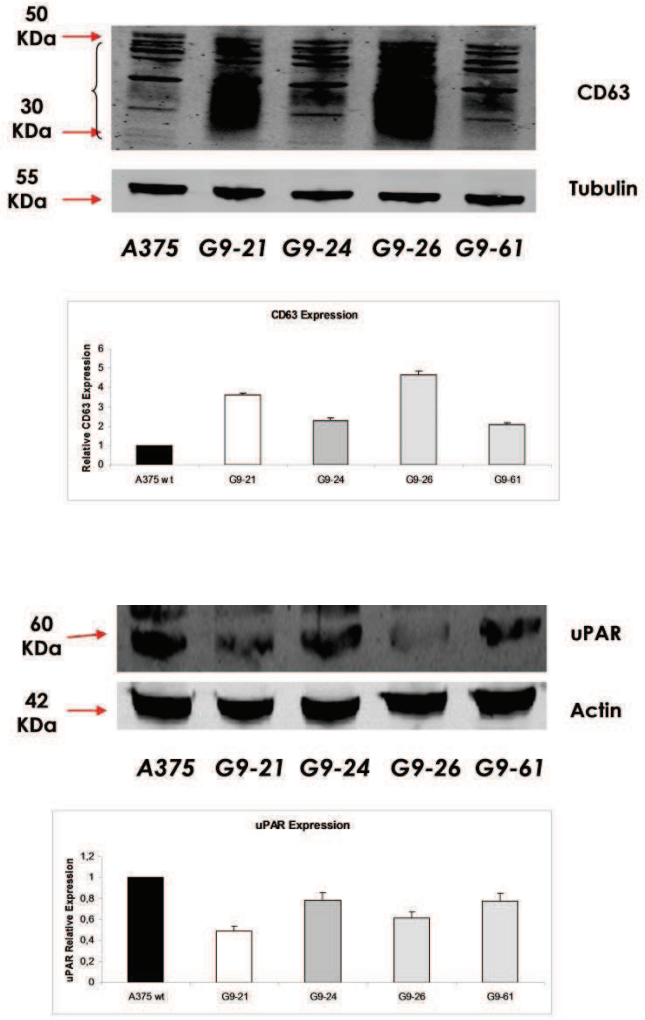


Figure 7R. uPAR expression was significantly reduced in cells with high levels of CD63. The reduction of uPAR appeared to be proportional to the increase in CD63 over expressin. The mechanism underlying this reduction is now under investigation.

Altogether, these results strongly encourage to consider the MT1-MMP/MMP-2 and uPA/uPAR systems for possible application as a prognostic marker in cutaneous melanoma, and CD63 as a key player in regulating matrix degradation by modulating MMPs and uPAR systems.

4.5 Proliferation assay

In stark contrast to normal cells, which only divide a finite number of times before they enter into a permanent state of growth arrest or simply die, cancer cells never cease to proliferate. So far, few studies have been carried out on the role of tetraspanins in the control of cellular proliferation and apoptosis. *Hemler et al*[52] proposed that CD81 and CD9 could associate with phosphatidylinositol 4-kinase (PI4K), which locally produces phosphoinosites and this causes the recruitment and activation of SHC (SRC-homology-2-domain-containing transforming protein). Subsequent Ras-mediated activation of extracellular signal-regulated kinase (ERK) or p38 or Jun N-terminal kinase (JNK) pathways leads to proliferation or apoptosis, respectively.

An interesting study was performed by *Ovalle et al*[203] to characterize the effects of expression of the tetraspanin CD9 on proliferation and signalling in colon carcinoma. They demonstrated that the expression of CD9 resulted in a marked decrease in cell proliferation. Inhibition of cell growth can result either from a slower progression through the cell cycle or from an increase in the rate of apoptosis and/or necrosis. This data were confirmed by *Powner et al*[204] that demonstrated how downregulation of CD9 increases proliferation of MDA-MB-231 cells in 3D MatrigelTM.

To determine the effects of over expression of CD63 on human melanoma cells, we performed cellular proliferation assays using the WST-1 reagent. According to data reported in literature, we observed significant reduction of cellular proliferation rate which was proportional to the increase of the over expression levels of CD63. As shown in **Figure 8R** the clone G9-26, with the highest level of CD63 over expression showed a four-fold decrease in proliferation rate as compared to control.

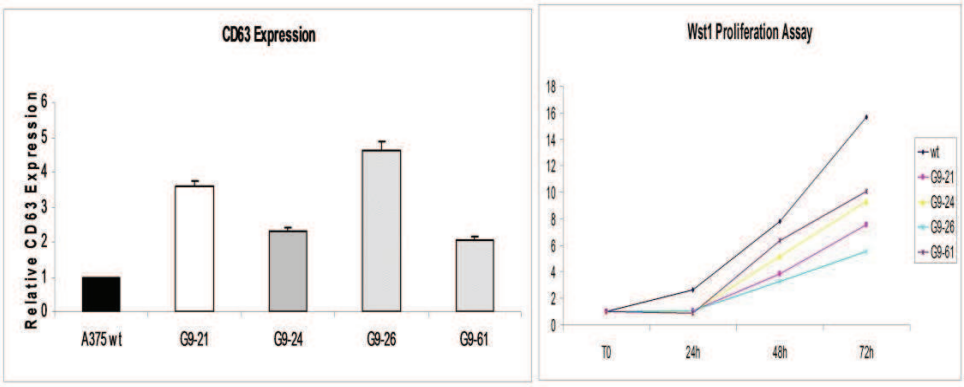
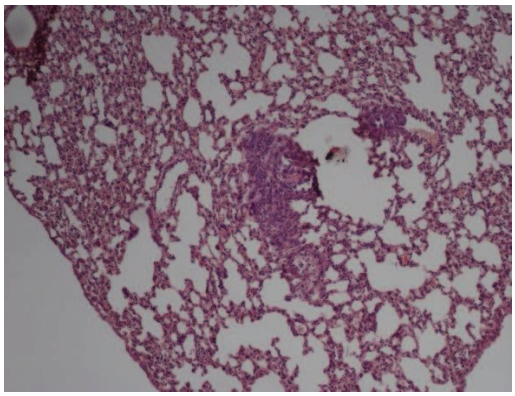


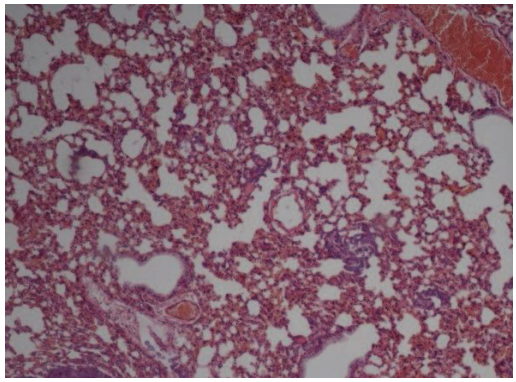
Figure 8R. Wst1 proliferation assay. Various levels of CD63 overexpression in A375 transfectants reduced cell proliferation as compared to wt control. The clone G9-26, with the highest level of CD63 over expression showed a four-fold decrease in proliferation rate as compared to control.

4.6 In vivo experiments

In order to understand the role of CD63 *in vivo* some Scid/Beige mice were injected into the vain tail with A375 or A375 CD63-silenced cells. Cells were obtained by transient transfection of A375 with a degenerated (control) or a CD63 specific siRNA. For the analysis $1x10^6$ cells A375 WT or A375 CD63-silenced were injected. Five mice for each group were used. After 5 weeks, all the animals were sacrified and their lungs used for histological analysis (**Figure 9R**). Several macrometastasis were found in the lungs of mice bothering at both groups. No statistical significance were found ($p>0.05$). For the analysis of micrometastas, mice's lungs were embedded in paraffin and cut. H&E staining shown the presence of cancer cells spread in the lung. No differences in the number of metastasis were found. To better understand the role of CD63 *in vivo* this experiment will be repeated. Much more animals and tissues will be analysed.



A375 CD63-



A375 wt

Figure 9R. Histological analyses of mice's lung. H&E staining shown the presence of cancer cells spread in the lung. No differences in the number of metastasis were found.

4.7 B16 murine melanoma cells

Proteases are crucial for the spread of cancer cells from primary tumor to the site of secondary growth. *Bianchini et al* [205] demonstrated that B16 murine melanoma cells enhanced their lung colonizing potential in vivo and invasiveness through Matrigel-coated filters upon co-stimulation with IFNγ and TNFα; but neither IFNγ nor TNFα alone, at the dose used in the experiments was able to elicit a change in the invasive/metastatic efficiency of melanoma cells. They also showed that the invasive phenotype of murine melanoma cells stimulated with IFNγ and TNFα was characterized by an enhanced uPA/uPAR and MMP-9 expression: TNFα promoted MMP-9 mRNA expression and pro-MMP9 protein secretion, and the co-stimulation with IFNγ and TNFα was required to potentiate the expression of mRNA and protein for uPAR, and to induce a redistribution of uPA from the soluble to the cell body associated form. Both monoclonal antibodies, anti-uPAR and anti-MMP9 caused a significant reduction of invasiveness in IFNγ/TNFα stimulated melanoma cells. These results indicated that invasiveness in B16 murine melanoma cells can be regulated in a

cytokine-specific fashion and is dependent on the synergism between the uPA/uPAR system and MMP9.

We have observed that B16 murine melanoma cells stimulated by inflammatory cytokines showed reduced expression of CD63 (**Figure 10R**). This was associated with the promotion of a metastatic phenotype, characterized by a high capacity to colonize host lungs and expression of a high levels of uPAR and MMP-9 (**Figure 10R**[205]).

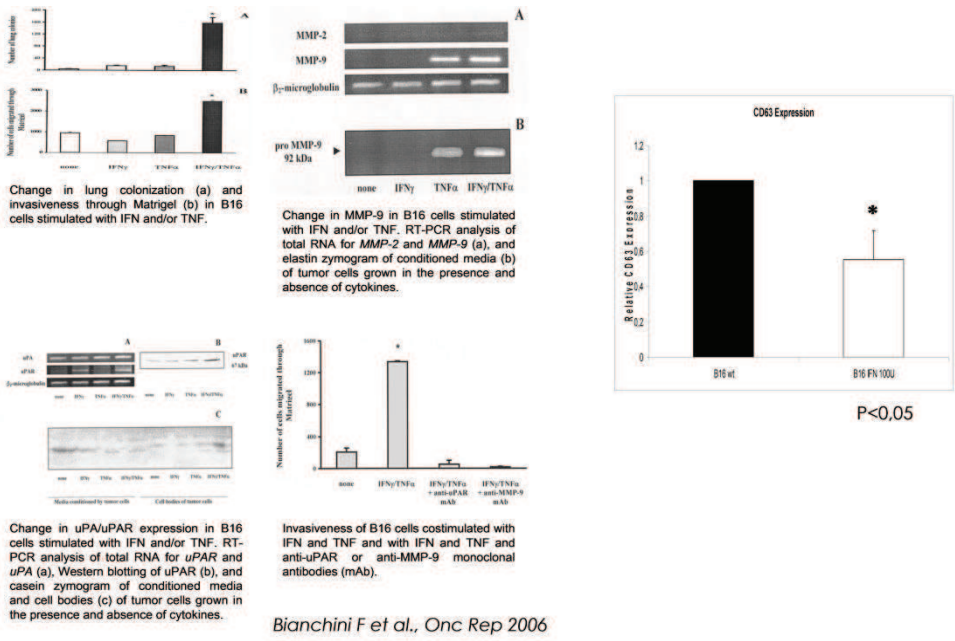


Figure 10R. B16 murine melanoma cells stimulated by inflammatory cytokines showed reduced expression of CD63. This was associated with the promotion of a metastatic phenotype, characterized by a high capacity to colonize host lungs and expression of a high levels of uPAR and MMP-9.

Next, we evaluated the expression of CD63 in B16 cells treated with different doses of IFNγ (25U, 50U and 100U) for 24 hours. The reduction of CD63 expression was dose dependent and proportional to the increase of IFNγ. A significant reduction was observed with 50 U and 100 U of IFNγ as shown in **Figure 11R**, so that we

hypothesized confirming our hypothesis of cytokine- dose-dependent effect on CD63 expression in melanoma .

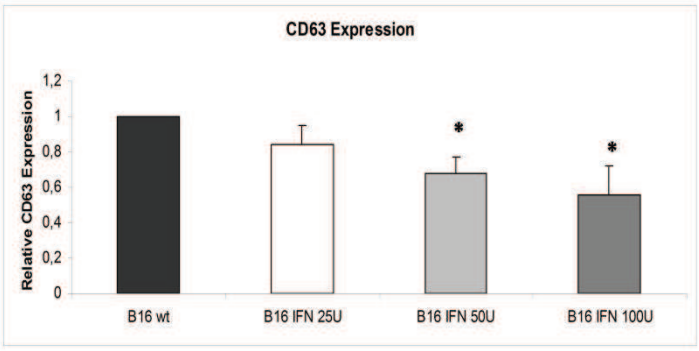


Figure 11R. Expression of CD63 in B16 cells treated with different doses of IFNγ (25U, 50U and 100U) for 24 hours. The reduction of CD63 expression was dose dependent and proportional to the increase of IFNγ. A significant reduction was observed with 50 U and 100 U of IFNγ

4.8 CD63 as negative marker of EMT

Several studies indicate that induction of an EMT is likely to be a highly important mechanism for the progression to a metastatic stage of many tumors.

EMT is the process by which an epithelial cell suffers transitory changes in cell structure and becomes a more motile mesenchymal cell with migratory and invasive properties[206,207]. As a result, cell-cell junctions are altered, cells lose polarity, express mesenchymal markers, and the actin cytoskeleton becomes reorganized. Consequently, tumoral cells lose contact with extracellular matrix, invading surrounding territories and acquiring capacity for metastasis. *Alonso et al*[206] suggested that migration and invasion in melanoma are the result of a specific interaction between tumoral and stromal cells, associated with the expression of a set of molecules involved in EMT. N-cadherin, lumican, glypican, osteonectin, osteopontin, metalloproteinases and integrins seems to be involved in the melanoma metastasis process. In particular, *Alonso*[206] described N-cadherin as a crucial molecule in the

EMT event, acting as an oncogene, by promoting tumor invasiveness and progression in many tumor types including carcinomas. Cell-matrix interactions are closely related to EMT category. The integrins are a family of cell surface adhesion molecules that coordinate cell-cell and cell-matrix interactions. Integrin αV was identified as marker of melanoma metastasis[208]. Closely related are the MMP family of proteins, which are involved in the breakdown of the extracellular matrix in normal physiologic processes and in cancer invasion and metastasis; MMP-2 and ADAM9 has been associated with melanoma progression[209].

Cano et al[210] identified the transcription factor Snail as a direct and strong repressor of E-cadherin expression. According to this hypothesis, stable expression of Snail in epithelial MDCK cells induces a dramatic phenotypic transition concomitantly with the loss of E-cadherin expression and an increase in the expression of mesenchymal markers. This phenotypic change is also accompanied by the acquisition of tumorigenic and invasive/migratory properties. Another Snail-family member, Slug, has a role in EMTs.

But EMT is not a permanent, irreversible process occurring during the course of tumor metastases; indeed, a reversible EMT model has been proposed to describe the transient activation of the EMT program that carcinoma cells undergo during tumor metastasis[169]. In this model, carcinoma cell activate the EMT program to achieve local invasion and dissemination to distant organs. Once they have reached those organs, this mesenchymal cells may revert via an MET to an epithelial identity and thereby regain proliferative ability and the ability to form epithelial growths in distant organ sites. Moreover, cancer cells may pass through a partial EMT program rather than a complete one; such cells may concomitantly express epithelial and mesenchymal markers.

A role of tetraspanin family in EMT has been recently suggested; *Ke et al*[211] demonstrated that the tetraspanin CD151, whose expression has been associated with increased invasiveness and poor prognosis in hepatocellular carcinoma (HCC), when

associated with α6 integrin, induces an epithelial-mesenchymal transition. They shown that α6 integrin formed a complex with endogenous CD151 in HCC cells. In cells that expressed high levels of α6 integrin and CD151, lamini-5 promoted cell spreading by inducing the epithelial-mesenchymal transition (EMT); this effect was not observed in cells that expressed high levels of only α6 integrin or CD151. Cells that expressed high levels of α6 integrin and CD151 underwent the EMT in response to laminin-5, through hyperactivation of phosphatidylinositol-3-kinase (PI3K), primarily induced via the PI3K-protein kinase B (Akt)- Snail-phosphatase and tensin homolog feedback pathway. The EMT was reversed by PI3K inhibitors and antibodies against CD151 or α6 integrin *in vitro*, and was delayed by specific interference with CD151 and α6 integrin *in vivo*.

Based on literature and data cited above, in collaboration with Professor Calorini, we hypothesized the role of tetraspanin CD63 to be a negative marker of EMT.

In order to examine our hypothesis, we firstly analyzed the cell line A375 M6, established from a colony isolated from the liver of immunodeficient mouse and derived from A375 iv inoculation. In this cell line the expression of factors involved in EMT such as transcriptional factor Snail, adhesion molecules E-cadherin, N-cadherin, and Vimentin. The parental A375 cells were used as control. As shown in **Figure 12R**, Snail mRNA level was reduced compared to control A375, whereas E-cadherin mRNA level was increased compared to A375 control; Vimentin mRNA level was slightly increased and no differences were observed in N-cadherin levels as compared to control. These data suggested an epithelial profile for A375M6 cell line as compared to its parental control A375, and were in agreement with Thiery et al observations.

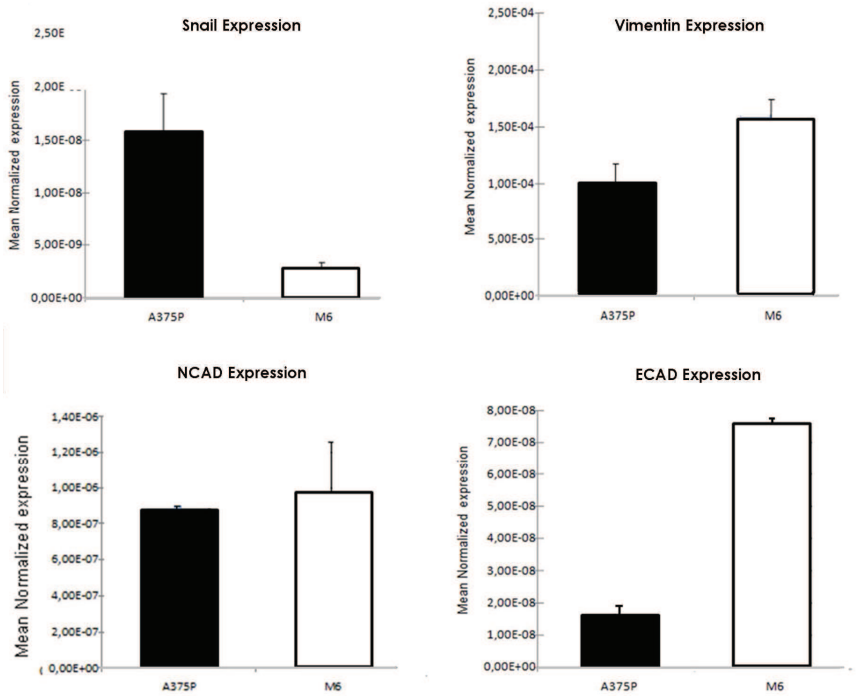


Figure 12R. Analysis of the expression of factors involved in EMT. Snail mRNA level was reduced compared to control A375, whereas E-cadherin mRNA level was increased compared to A375 control; Vimentin mRNA level was slightly increased and no differences were observed in N-cadherin levels as compared to control.

When treated with TGFβ, a potent inducer of EMT, A375 M6 undergone cell morphology modifications and acquired mesenchymal profile as compared to non-treated A375 M6 (**Figure 13R)**. Moreover, we also observed a significant increase of Slug and N-cadherin mRNA levels (**Figure 14R**) and a significant decrease in E-cadherin mRNA levels as compared to non TGFβ-treated A375 M6.

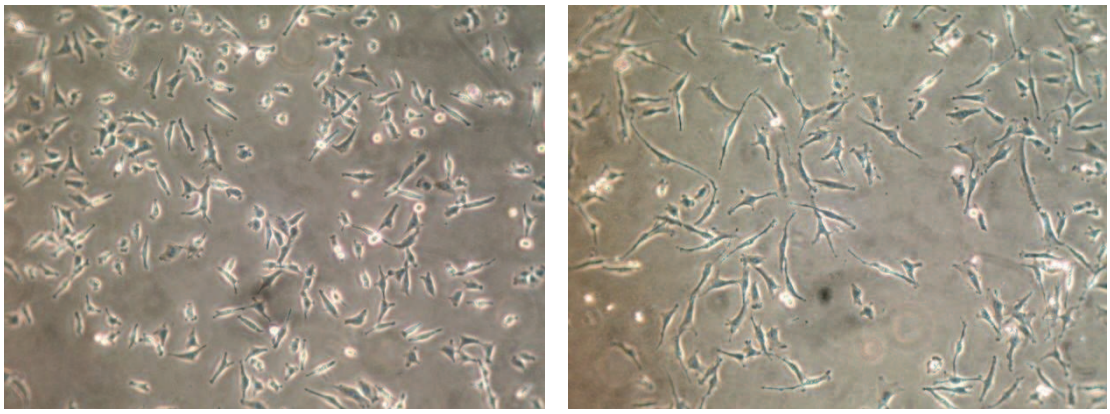


Figure 13R. EMT and morphology. When treated with TGFβ, a potent inducer of EMT, A375 M6 undergone cell morphology modifications and acquired mesenchymal profile as compared to non-treated A375 M6

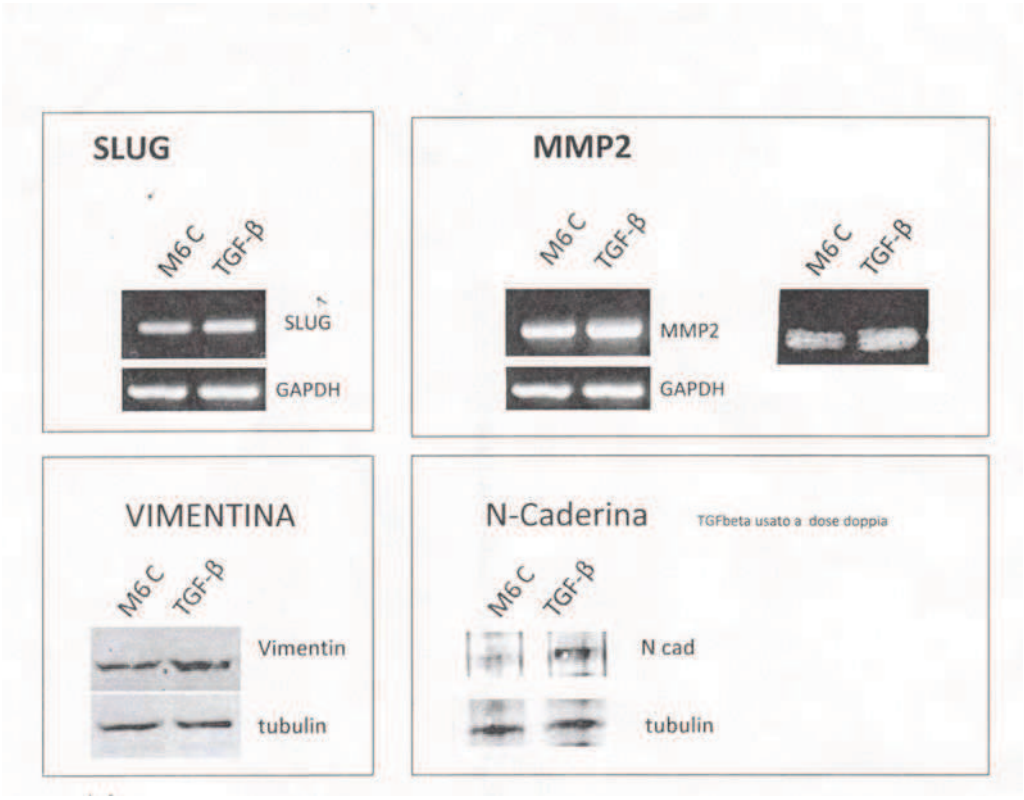


Figure 14R. When treated with the inducer oh EMT TGFβ, Slug and N-cadherin mRNA levels significantly increased compared to non treated cells.

The expression of MMPs (and particularly of MT1-MMP) is part of EMT processes. Accordingly, the regulation of several MMPs by transcription factors (Snail, ETS, β-catenin), known to regulate EMT pathways, have clearly been established. Consequently, MMPs are rather considered as target genes of EMT pathways and MMP expression as a late event of the EMT. This may be due, in part, to direct regulation of MMP gene transcription by the factors which drive EMT. Several MMPs have been shown to be able to cleave E-cadherin, thereby inducing E-cadherin complex fragility and EMT changes. Although stromal cells are a major source of MMP in tumors, expression of MMPs (particularly MT1-MMP) by parenchymal cells is a clear EMT step which can ensure a pericellular proteolysis of basement membrane components but also other substrates, and thereby facilitate migration and invasion.

In order to confirm these data, a Matrigel invasion assay was performed. The A375 M6 cells were treated with TGFβ to activate the EMT profile and then plated on Matrigel with or without Ilomastat, a selective inhibitor of metalloproteinases. As shown in **Figure 15R**, we observed a significant reduction of invasiveness in cells treated with Ilomastat as compared to non TGFβ-stimulated control.

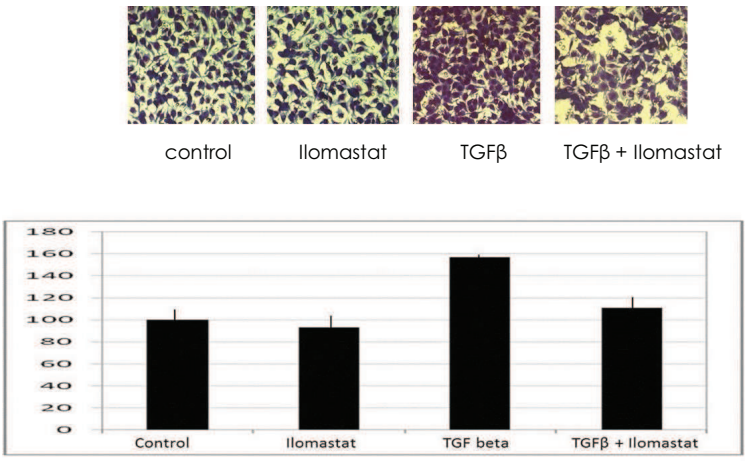


Figure 15R. Matrigel invasion assay. A375 M6 cells were treated with TGFβ to activate the EMT profile and then plated on matrigel with or without the inhibitor of metallorpoteinases Ilomastat. Cells treated with Ilomastast shown a significant reduction of invasiveness as compared to non-TGFβ-stimulated control.

Moreover, we observed that CD63 protein was highly expressed in A375M6 as compared to parental A375 control **(Figure 16R)**, but this expression rapidly decreased after a treatment with the EMT inducer TGFβ, supporting our hypothesis that CD63 could play a key role in EMT, acting as a negative marker of transition.

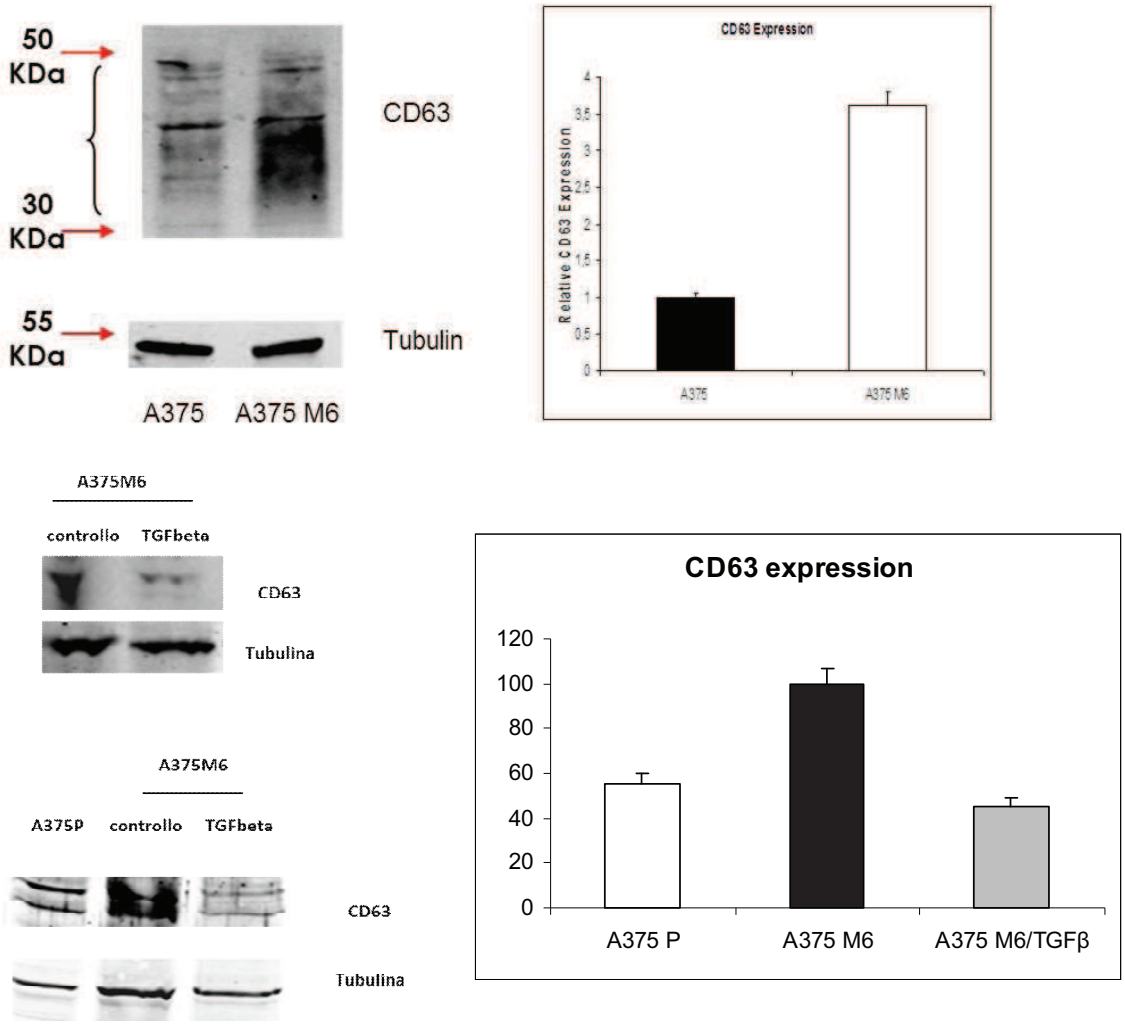


Figure 16R. CD63 expression in A375 M6 and parental A375 control. We observed that CD63 protein was highly expressed in A375M6 as compared to parental A375 control but this expression rapidly decreased after a treatment with the EMT inducer TGFβ

To confirm this, we performed a Matrigel invasion assay. The CD63 in A375 cells was transiently silenced with siRNA and the medium was used to plate stable on Matrigel. We observed an increased invasiveness of clones overexpressing CD63 stimulated with medium derived from silenced cells as compared to migration capacity of non stimulated cells **(Figure 17R)**. We believe that this effect was probably due to a major content of MMPs in medium used to stimulate clones overexpressing CD63. This experiment confirmed the importance of metalloproteinases in the invasion mechanism of A375, supporting the role of CD63 in the mesenchymal phenotype.

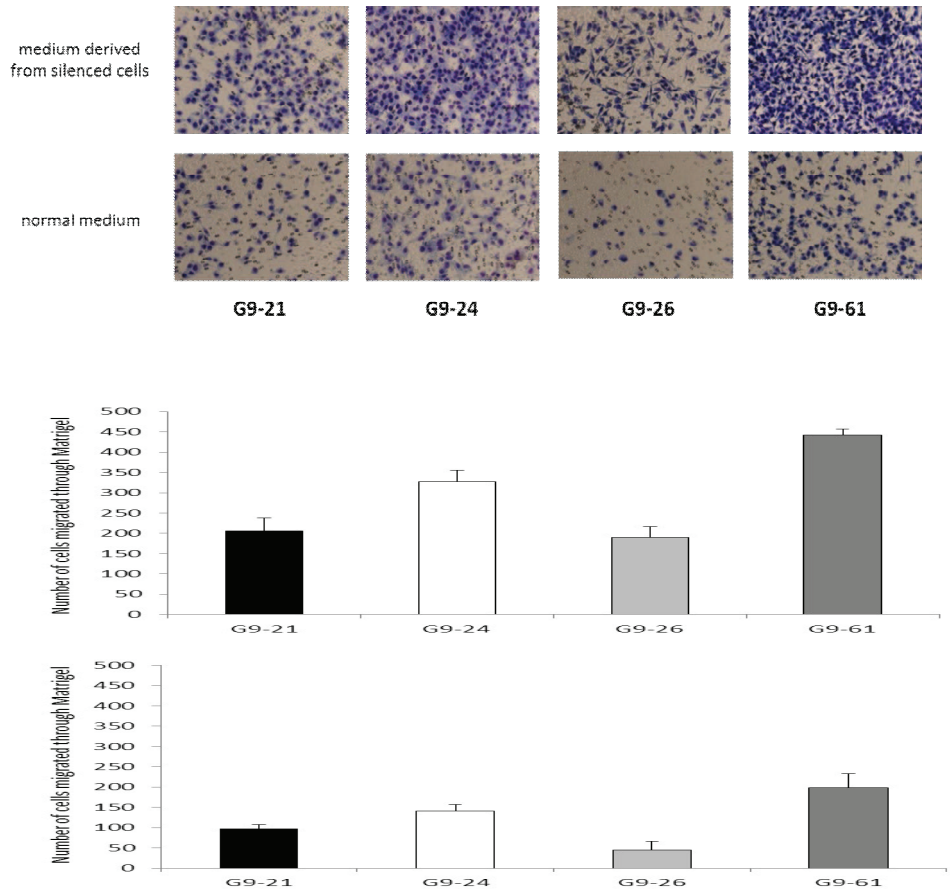


Figure 17R. Matrigel invasion assay. We observed an increased invasiveness of clones overexpressing CD63 stimulated with medium derived from silenced cells as compared to migration capacity of non-stimulated cells. This difference was probably due to a major content of MMPs in medium used to stimulate clones overexpressing CD63.

V

Conclusions

Melanoma is one of the most aggressive tumors with high metastatic potential and rate of mortality.

The key to successful treatment is early diagnosis. Patients identified with localized, thin, small lesions nearly always survive. For those with advanced lesions, the outcome is poor in spite of progress in systemic therapy. Hence, there is a need to identify new prognostic markers of progression and new potential drug targets.

Recently, many researchers have focused on a new class of membrane glycoproteins, the tetraspanins, involved in a multitude of biological processes, such as fertilization, parasite and viral infection, synaptic contacts at neuromuscular junctions, platelet aggregation, maintenance of skin integrity, immune response induction, metastasis suppression and tumor progression[50]. It has well established that tetraspanin proteins are involved in tumorigenesis process by supporting or inhibiting tumor growth, invasion, metastasis and angiogenesis.

A correlation between decreased CD63 expression and increased malignancy was observed in many tumors. In ovarian cancer tissues, increasingly lower expression levels were found to correlate with increasingly higher chances of tumor metastasis[162]. In lung adenocarcinoma, low CD63 expression correlated with more tumor growth and a poor prognosis[163] and also in breast and colon cancers a negative relation between CD63 expression and cancer invasiveness and metastasis was found[164,165].

We identified the tetraspanin CD63 as a possible negative marker of metastasis in melanoma.

Analysis of different human melanoma tissues led to observation that CD63 is expressed in nevi and in thin melanomas, whereas it was expressed at low levels in

metastasis. In melanocytic and dysplastic nevi, CD63 was mainly confined to the epidermis, although some positive cells could be found in the underlying layer. In thin melanoma, CD63 positive cells were confined in the surface layer of the dermis. Instead, in thick melanoma, cells expressing CD63 were spread evenly throughout the epidermis and the dermis. In metastases, the expression of CD63 was limited to a few cells distributed heterogeneously in the tissue.

In order to understand the biological effect of CD63 on tumor progression, we performed *in vitro* assays on the human melanoma cell line A375.

We demonstrated that exogenous modulation of CD63 expression correlates with cell proliferation, motility and invasiveness. High levels of CD63 were associated with reduced cell proliferation, motility and invasiveness and with increased apoptosis. Furthermore, the reduction of invasiveness in CD63 over expressing cells, compared to non modulated parental control cells, tightly correlated with a lower expression of pro-metastatic proteins uPAR, MMP-2 and MMP-9, the key players in the matrix degradation.

Altogether, these results support our hypothesis that CD63 could be a negative suppressor of metastasis in melanoma. We are currently perform the experiments in order to demonstrate the role of CD63 *in vivo* in a mouse model of melanoma.

We have also observed that B16 murine melanoma cells stimulated by inflammatory cytokines showed a reduction of CD63 tetraspanin expression and this was associated with the promotion of a metastatic phenotype, characterized by a high capacity to colonize host lungs and expressing a high levels of uPAR and MMP-9[205]. The reduction of CD63 expression was dose dependent and proportional to the increase of IFNγ stimulation. However, the molecular mechanism awaits to be unraveled.

Recently, many studies reported the role of epithelial-mesenchymal transition in matrix degradation and cell invasiveness. It has been shown that EMT and non-EMT cells cooperate to complete the spontaneous metastasis process. EMT cells are responsible for degrading the surrounding matrix to lead the way of invasion and intravasation.

Non-EMT cells, probably because of a growth advantage compared to MET cells, enter the blood stream and re-establish colonies in the secondary sites[212].

According to these observations, *Calorini et al* isolated a lung colony derived from A375 cells injected to an immunodeficient mouse, called A375M6. The cells show a non-EMT profile, characterized by low levels of the transcriptional factor Snail, high levels of E-cadherin and increased levels of Vimentin. When treated with TGFβ, a potent inducer of EMT, the cell morphology changed, cells acquired and epithelial profile as compared to non treated A375M6. At a molecular level a significant increase of Slug mRNA levels and a significant decrease in E-cadherin mRNA levels compared to non treated A375 M6 were observed.

Furthermore, because MMPs expression is a part of EMT process, a Matrigel invasion assay was performed on A375M6 cells induced with TGFβ and treated with Ilomastat, that selectively blocks MMPs. The cells with inhibited metalloproteinases showed a reduced capacity to invade the matrix as compared to non treated control.

We have also observed that CD63 expression in A375M6 was higher compared to parental A375 and significantly decreased after treatment with TGFβ. To explain and confirm this observation, we performed a Matrigel invasion assay. A375 cells were transiently silenced and the medium was used to plate stable clones overexpressing CD63 on Matrigel. We observed an increased invasiveness of clones overexpressing CD63 stimulated with medium derived from silenced cells as compared to invasion capacity of non stimulated cells, probably due to a major content of MMPs. This experiment confirmed the importance of metalloproteinases in the invasion mechanism of A375, supporting the role of CD63 in the mesenchymal phenotype.

In summary, our results strongly point to the high expression of CD63 as a major contributor to a non-invasive phenotype in melanoma.

As the invasive phenotype of cells involves induction of molecular changes associated with epithelial-mesenchymal transition, we have reason to believe that CD63 may play a key role in these processes, acting as a negative marker of EMT.

Finally, we conclude that CD63 has a potential to be a suppressor gene playing an important role in inhibition of melanoma progression, and as such could be a target for pharmacological and drug developing strategies.

VI

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