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**PLASMA MEMBRANE SIALIDASE NEU3
SILENCING EFFECTS ON THE MOLECULAR
PHENOTYPE OF MELANOMA CELLS**

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1. MELANOMA

Melanoma arises from the malignant transformation of melanocytes. Melanocytes are a heterogeneous group of cells that originate from the neural crest and are able to synthesize melanin (Cichorek M, 2013). In addition to epidermis, melanocytes are present also in hair and iris, determining their color, in the inner ear, nervous system, gastrointestinal tract, oral and genital membranes, and heart (Cichorek M, 2013). The life of melanocytes begins with the differentiation of some embryonic neural crest cells into melanoblasts. Then melanoblasts migrate, proliferate, and differentiate into mature melanocytes, which are characterized by a dendritic morphology and acquire special organelles called “melanosomes” where melanin synthesis occurs (Cichorek M, 2013). The proliferation of melanocytes in the skin can give rise to common, benign neoplasms called “melanocytic nevi”.

Both genetic and environmental factors, including ultraviolet (UV) radiation, can lead to the appearance of melanoma. Melanoma is a very aggressive tumor, accounting for most skin cancer deaths and, unfortunately, its incidence is increasing.

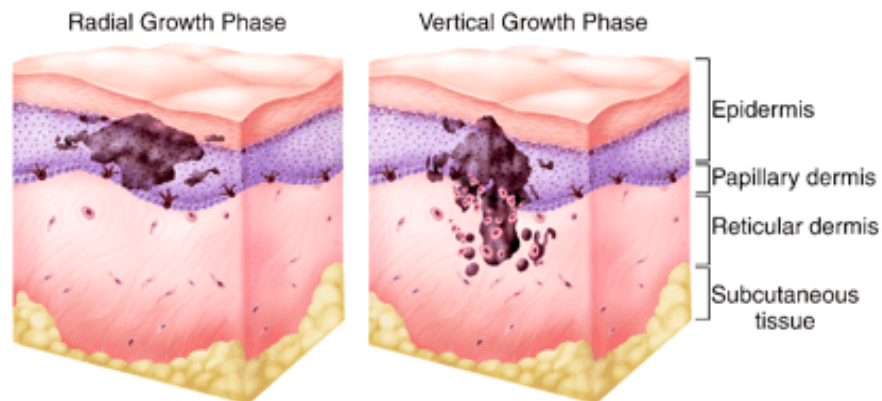


Figure 1: The progression of melanoma (Hodi FS, 2006).

The typical development of melanoma begins with its growth along the epidermis (radial growth phase). When diagnosed at this stage, melanoma can be fully eradicated surgically. But, rapidly, the radial growth phase can progress into the vertical-growth phase, in which invasion of malignant cells is deeper into the dermis (Fig. 1) and, then, the

metastatic stage and the spreading of melanoma cells into distal organs come after (Miller AJ, 2006). It must be observed that not all melanomas develop following this scheme but some tumors can progress directly to the metastatic stage (Gray-Schopfer V, 2007). Even if, recently, there were significant improvements in the treatment of metastatic melanoma due in particular to immunotherapy, the overall survival of patients after 5-years is about 15%.

1.1. CLINICAL SUB-TYPES OF MELANOMA

Four main subtypes of melanoma endowing with different pathogenic behavior were described (Gray-Schopfer V, 2007):

1. Nodular melanoma: cancer lesion typically consists of raised nodules that rapidly enlarge over time. Proliferation takes place through the skin in vertical growth phase manner (Gray-Schopfer V, 2007).

2. Acral lentiginous melanoma: this form is common in people with dark skin and tends to be found on the palms of the hands, the soles of the feet and in the nail bed. It is not related to UV radiation. 50% of melanoma patients in non-Caucasian populations report this type of melanoma (Gray-Schopfer V, 2007).

3. Lentigo maligna: this form develops due to the chronic exposure of skin, mostly in elderly people. Generally, this form is flat and irregular in appearance and enlarges slowly (Gray-Schopfer V, 2007).

4. Superficial spreading melanoma: this form is the most common form and it is found in the edges and scattered in the epidermal region. Severe sunburn can be one cause of this tumor. It is the third most common cancer in USA and UK (Gray-Schopfer V, 2007).

1.2. COMMON ALTERATIONS OF SIGNALLING PATHWAYS ASSOCIATED WITH MELANOMA

Alterations of the Ras/Raf/Mek/Erk (MAP kinases) pathway are characteristically involved in the development and progression of melanoma. MAP kinases (MAPK) play a

central role in key cell functions including proliferation, gene expression, differentiation, survival, and apoptosis. In melanocytes, MAPK are mostly activated by stem-cell factor, fibroblast growth factor and hepatocyte growth factor. Hyper-activation of MAPK is found in 90% of melanoma patients (Gray-Schopfer V, 2007).

Mutations in NRAS gene are responsible for the activation of the MAPK pathway in 15-30% of patients (Gray-Schopfer V, 2007). Instead, the most frequently mutated oncogene related to MAPK, in melanoma, is BRAF, one among the three human RAF genes. Mutated ^{V600E}BRAF is able to constitutively activate the MAPK pathway (Gray-Schopfer V, 2007). Moreover, mutated BRAF is responsible for the progression toward the metastatic stage by also promoting the synthesis of vascular endothelial growth factor (VEGF) (Gray-Schopfer V, 2007). NRAS and BRAF mutations are mutually exclusive in melanoma (Gray-Schopfer V, 2007).

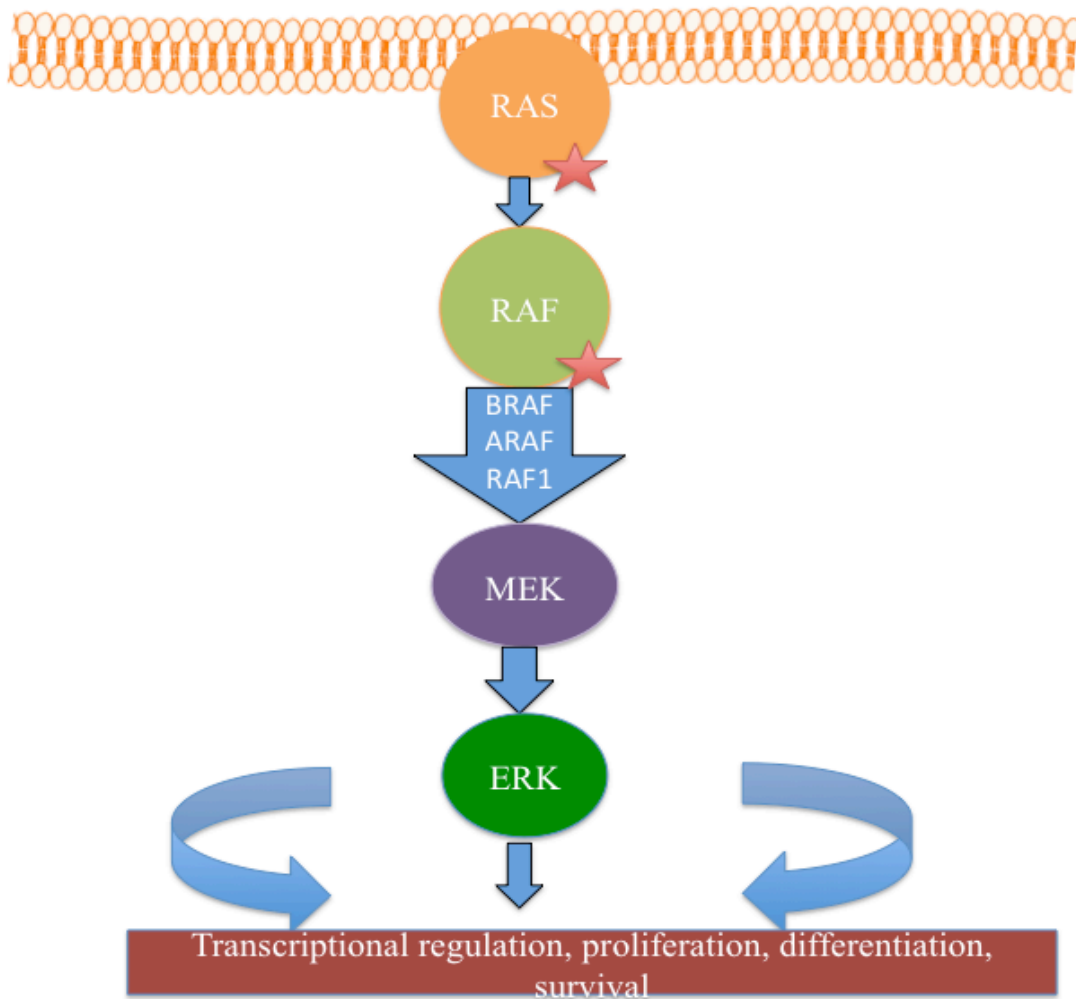


Figure 2: MAPK Pathway.

Another signaling pathway important for melanoma development and progression is the phosphoinositide-3-OH kinase (PI3K) pathway. PI3K is involved in the regulation of many cellular functions such as cell growth, survival, and migration (Gray-Schopfer V, 2007). PI3K phosphorylates and activates the serine–threonine protein kinase B (AKT) (Fresno Vara JA, 2004). Notably, AKT is up-regulated in 60% of melanomas (Gray-Schopfer V, 2007). PTEN is responsible for the negative regulation of PI3K/AKT pathway (Slipicevic A, 2005). Inactivation of PTEN enhances melanoma cell growth and survival. The loss of function of PTEN is observed in 5-20% of melanoma patients (Gray-Schopfer V, 2007). Both NRAS and BRAF mutations can lead to the up-regulation of the PI3K pathway (Gray-Schopfer V, 2007).

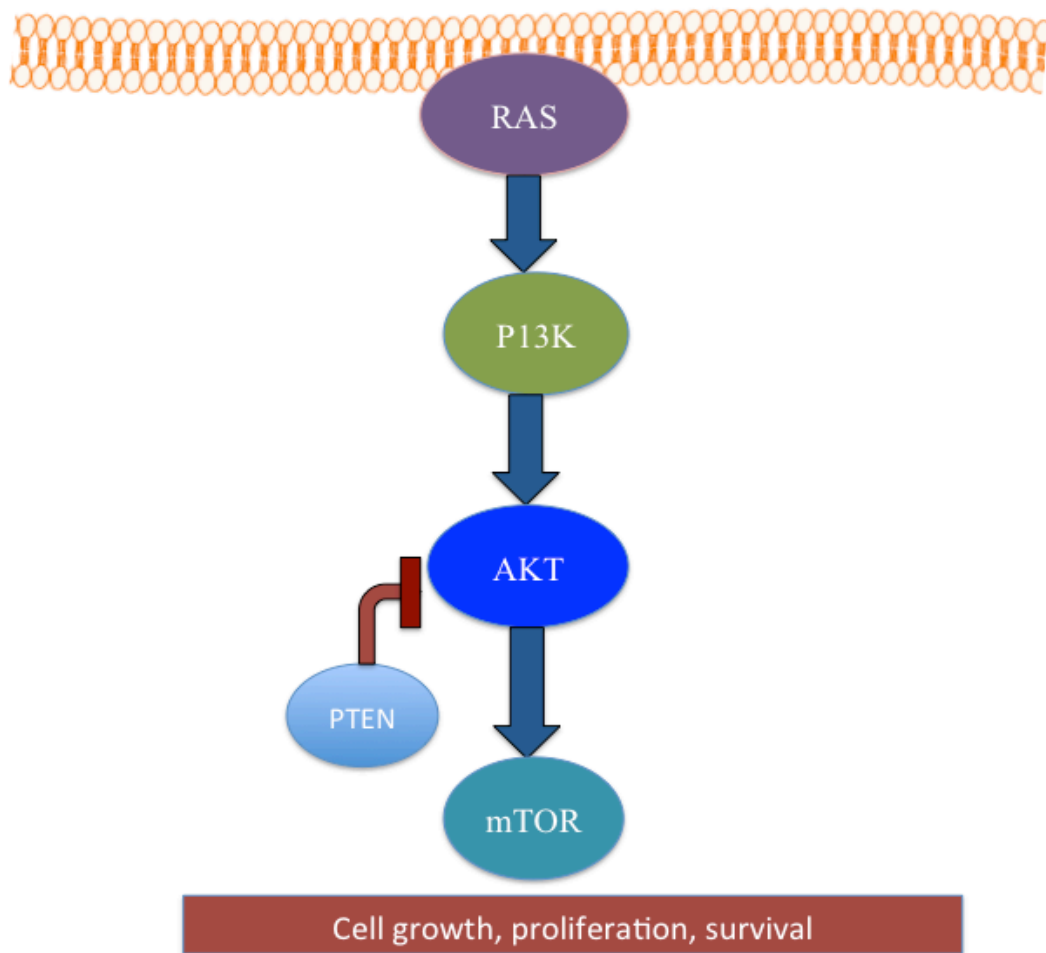


Figure 3: AKT Pathway.

The microphthalmia-associated transcription factor (MITF) is recognized as a key regulator in the commitment of melanocyte lineage and acts a central part in melanoma

(Gray-Schopfer V, 2007). MITF is frequently less expressed in melanomas than in melanocytes. The increase of MITF expression in melanoma reduces the proliferation rate and cell tumorigenicity (Gray-Schopfer V, 2007). The expression levels of MITF seems to be critical for cell survival: MITF suppression leads to cell senescence while high levels induce differentiation; low levels are related to an invasive phenotype (Hartman ML, 2015). Thus, it has been hypothesized that high levels of MITF induce cell cycle block and differentiation whereas intermediate levels favoure proliferation and survival (Gray-Schopfer V, 2007; Hartman ML, 2015). It has been demonstrated that MITF can synergize with ^{V600E}BRAF to induce the malignant transformation of melanocytes; therefore, it is needed for melanoma development. Nevertheless, because MITF is addressed toward the degradation by MAPK phosphorylation, its expression is down-regulated at intermediate levels needed to maintain cell proliferation and invasiveness (Gray-Schopfer V, 2007).

Other alterations of signaling pathways occurring in melanoma and involved in the control of cell growth and survival include the over-expression of Bcl-2, NF-kB, and AKT3, the up-regulation of β catenin, and the silencing of APAF-1 (Gray-Schopfer V, 2007). KIT, the cyclin dependent kinase inhibitor 2A (CDKN2A) and the telomerase reverse transcriptase (TERT) are also frequently mutated (Shain AH, 2016)

Mutations concerning p53 are less common (10%) (Gray-Schopfer V, 2007).

1.3. THE THERAPIES AGAINST MELANOMA

Wide excision represents the definitive treatment of early stage melanomas. Instead, advanced melanoma is very difficult to be treated because it is known to be highly resistant to many common chemotherapeutic drugs. Interferon- α and interleukin-2 have been used as adjuvant immunotherapy for advanced melanoma but their efficacy is low. Dacarbazine is the reference approved chemotherapeutic drug for advanced melanoma (Gray-Schopfer V, 2007). Currently, the most promizing tools to treat advanced melanoma seem to be targeted therapies and immunotherapy. Many inhibitors targeting BRAF were tested against melanomas carrying BRAF mutations. Sorafenib shows low efficacy against melanoma alone; when it is administred in addition to other chemiotherapeutic drugs, it becomes more efficacious (Gray-Schopfer V, 2007). Vemurafenib was approved by FDA in 2011 for

patients affected with melanoma having ^{V600E}BRAF mutation. Vemurafenib significantly improved the overall survival of these patients but the duration of its effects is limited because strong resistance against it occurs (Russo A, 2014).

Inhibitors targeting MEK seem to be able to reduce the in vitro proliferation of melanomas carrying RAF mutations. However, clinical trials demonstrated that only trametinib can improve the patients' survival (Russo A, 2014).

Also, PI3K and mTOR inhibitors were explored against melanomas; however, their employment alone was demonstrated to be ineffective. They are used in combination with MEK inhibitors (Russo A, 2014).

Immune checkpoint inhibitors are drugs targeting pathways responsible for the activation of the immune response toward cancer cells. In fact, melanoma cells can escape the attack from T cells inactivating these "immune checkpoint". Pembrolizumab and nivolumab belong to this class of novel drugs: they inhibit PD-1, a protein which prevent T cells to attack melanoma cells. Pembrolizumab was approved by FDA in 2014: the first clinical trials (Keynote-001 study) demonstrated that 55% of patients with advanced melanoma was still alive after 2 years (Robert C, 2016). Currently, this drug seems to be the most promising tool against metastatic melanoma.

1.4. MELANOMA AND GANGLIOSIDES

Gangliosides are glycosphingolipids carrying sialic acid residues. They are present in the plasma membranes of all cells, particularly in the nervous system. The lipid moiety of gangliosides is ceramide and it is constituted by sphingosine linked to a fatty acid by an amide linkage (Sonnino S, 2006). Different oligosaccharide chains define the uniqueness of gangliosides species, their biological properties, and their ability to interact with other molecules (Sonnino S, 2006). Gangliosides play an important role in many events occurring at the plasma membrane such as signal transduction (through their ability to interact and modulate receptors and integrins), adhesion, and interaction with other cells. Therefore, they are involved in the control of cell differentiation, growth, and immune response (Furukawa K, 2008). Tumorigenesis leads to many modifications in ganglioside synthesis and

metabolism, resulting in the appearance of atypical gangliosides on cancer cell surface. Cancer gangliosides reveal an immune suppressive action and the ability to enhance cell motility, proliferation, survival, and angiogenesis (Qamsari E, 2016).

Also, melanoma is characterized by a unique expression of some gangliosides, very different from that of healthy melanocytes. Ganglioside GD3 is considered a melanoma-associated antigen. Its content is very low in melanocytes but it becomes abundant in melanoma (Furukawa K, 2008). GD3 was found also in cells characterized by high proliferation rate such as fetal brain tissues and mitogen-activated T lymphocytes (Furukawa K, 2008). GD3 has been demonstrated to increase the activation of p130Cas and paxillin, involved in the control of cell invasion and motility (Furukawa K, 2008). Other gangliosides abundant in melanoma cells are GM3, GM2, and GD2 (Furukawa K, 2008; Tringali C, 2014). A recent work performed in the laboratory where I performed my PhD demonstrated that the ganglioside profile is not equal in all melanomas. Primary melanoma cell lines can be clustered in at least three groups: the first enclosing melanomas displaying GM3, mainly in the unusual form carrying N-glycolyl GM3 instead of N-acetyl GM3 and GD3; the second enclosing melanomas displaying GM3 at low levels, GD3, GM1, GM2, GD1a, GD2, and GT1b; the third enclosing melanomas displaying an intermediate ganglioside profile (Tringali C, 2014). The ganglioside profile of normal melanocytes is constituted by GM3 and GD3 at very low levels, therefore, it is clear that their malignant transformation can activate the synthetic pathways leading to atypical gangliosides for the melanocytic lineage. Moreover, melanoma clustering based on ganglioside profiling showed an interesting correlation with the patients' survival despite the AJCC staging and the therapies. Patients whose tumors were grouped in cluster 1 have the shortest survival (some months after the diagnosis) whereas patients whose tumors were grouped in cluster 3 were still alive after 5 years from the diagnosis (Tringali C, 2014). Thus, gangliosides seem to be able to strongly affect the aggressiveness of melanoma, its progression and, then patients survival. Based on these results, it can be assumed that their characterization on melanomas could have an important diagnostic and prognostic significance.

In the past, the appearance of atypical gangliosides (GD3, GM3, N-glycolyl GM3) on melanoma cells was also used to develop vaccines against melanomas but their efficacy was low.

2. ABERRANT GLYCOSYLATION IN CANCER AND SIALIDASES

It has been demonstrated that modifications concerning cellular ganglioside profile arise from alterations of enzymes that synthesize or metabolize gangliosides, including sialidases (Hakomori S, 2002). Sialidases or neuraminidases (EC 3.2.1.18; *exo- α -sialidase*; *N-acetylneuraminosyl glycohydrolase*) are glycosidases catalyzing the removal of α -glycosidically linked sialic acid residues from carbohydrate groups of glycoproteins, glycolipids, and oligosaccharides (Miyagi T, 2012). Sialidases are widely distributed in nature, from microorganisms such as virus, bacteria, protozoa, and fungi to avian and mammalian species. Sialic acid is present in sialoglycoconjugates as a terminal non-reducing residue usually linked to galactose (α 2-3 or α 2-6), and to *N*-acetylgalactosamine or *N*-acetylglucosamine (α 2-6); moreover, sialic acid can be found linked to internal saccharide residues or it can be present in co-polymers (α 2-8; α 2-9). Sialic acid has been demonstrated to play a key role in cellular events mainly due to its hydrophilic and acidic features and to its ability to work as a recognition or masking site (Miyagi T, 2008). Thus, glycoconjugated carrying one or more sialic acid residues acquire unique characteristics and are often involved in cell-cell interactions, adhesion to extracellular matrix, signaling. Therefore, the removal of sialic acid and the catabolism of sialo-glycoconjugates catalyzed by sialidases strongly modulate many biological processes.

Aberrant glycosylation, above all aberrant sialylation, is known to occur during carcinogenesis. In the 1960's and 1970's, the sialylation profile of cancer cells was particularly studied (Miyagi T, 2008). Many studies showed that an increase in the content of sialic acid enhanced cell adhesion whereas the bacterial sialidase action reduced malignancy (Miyagi T, 2008). Currently, it has been confirmed that an increase in sialylation is often present in glycoproteins produced by cancer cells but there is no agreement on the precise role played by the content of sialic acid. Instead, many studies focused their attention on the qualitative changes connected to sialic acid carrying glycoconjugates. Sialidases preferentially recognize the link α 2-3 or α 2-6; internal residues are quite resistant to hydrolysis.

2.1. MAMMALIAN SIALIDASES

In mammals, sialidases have been proved to be involved in several cellular phenomena, including cell proliferation, differentiation, adhesion, cell-cell interaction, signal transduction, and immune response (Monti E, 2002). In mammals there are 4 genes encoding sialidases that differ based on their cellular localization and enzymatic characteristics. Sialidases can exist in the lysosome (NEU1), the cytosol (NEU2), the plasma membrane (NEU3), and the mitochondria/ER (NEU4) (Miyagi T, 2012). Among them, in particular, NEU3 plays a key role in cancer.

The lysosomal sialidase, NEU1, plays a key role in the lysosomal catabolism of molecules containing sialic acid but it is, also involved in cancer metastasis, elastin disposition, lymphocytic cell activation, and exocytosis. NEU1 preferentially removes sialic acid residues from gangliosides, oligosaccharides, and glycoproteins. In lysosomes, NEU1 sialidase is part of a heterotrimeric complex together with β -galactosidase and cathepsin (protective protein). When this multiproteic complex cannot be formed, due to gene mutations leading to the lack of cathepsin, both sialidase NEU1 and β -galactosidase are not active. In this case, a lysosomal storage disease called galactosialidosis occurs. Instead, gene mutations leading to the deficiency of NEU1 sialidase are the bases of the lysosomal storage disorder called sialidosis. Both galactosialidosis and sialidosis are lethal within few months/years after the birth. NEU1 negatively regulates the lysosomal exocytosis, in haematopoietic cells, by processing the sialic acid residues carried by the lysosomal membrane protein, Lamp-1. Increase in the lysosomal exocytosis is caused by the oversialylation of Lamp-1 whereas a decrease in the lysosomal exocytosis is due to the desialylation of Lamp-1 (Miyagi T, 2012). A recent study reported that the regulation of lysosomal exocytosis acted by NEU1 plays a significant role in tumor progression and chemoresistance (Machado E, 2015).

Apart from glycoconjugate catabolism in lysosomes, NEU1 is also involved in cellular signaling processes targeting β 4 integrin. Low levels of sialylated β 4 integrin due to NEU1 over-expression in human colon cancer cells induce the subsequent attenuation of FAK and ERK1/2 pathways and the down-regulation of the matrix metalloprotease 7, leading to the suppression of metastasis (Uemura T, 2009). Overall, in many tumors, NEU1 has been shown to be down-regulated.

Biochemical subcellular fractionization and immunostaining evidences shows that NEU2 sialidase is located in the cytosol (Miyagi T, 2012). The tridimensional structure of NEU2 has been determined by X ray: NEU2 is composed by six-blade beta propeller and its active site is located in a shallow crevice, as observed in viral and bacterial sialidases (Chavas LM, 2005). NEU2 sialidase is involved in the muscle, neuronal and erythrocyte cell differentiation. It works towards a wide range of oligosaccharides, glycoproteins, and glycolipids at both acidic and neutral pH. NEU2 is not expressed in all tissues; its high expression is seen in the skeletal muscles. The downregulation of NEU2 gene was associated with the impairment of muscle regeneration and differentiation (Miyagi T, 2012). NEU2 was also found to be involved in carcinogenesis. The high invasive and metastatic B16-BL6 melanoma mouse cell line gave rise to a lower number of pulmonary metastases, after NEU2 over-expression, due to a decreased content of ganglioside GM3 and an increased level of lactosylceramide (Miyagi T, 2012). Moreover, the over-expression of NEU2 in human leukemic cells, K562, decreased apoptosis resistance (Tringali C, 2007).

Sialidase NEU3 is associated with the plasma membrane and is mainly located in lipid rafts. NEU3 can also move to membrane ruffles together with Rac1 (Miyagi T, 2012) and in endosomes (Zanchetti G, 2007). NEU3 has a strict substrate specificity toward gangliosides. It has been found NEU3 is upregulated in various human cancer and promotes malignant phenotype regulating several cell properties including migration, invasiveness, and survival (Miyagi T, 2012). Indeed, NEU3 participates in the skeletal muscle differentiation, in neuronal cell differentiation, and in adaptive immune response (Miyagi T, 2012). Together NEU1 and NEU3 are upregulated during dendritic cell differentiation of monocytes (Miyagi T, 2012).

Sialidase NEU4 is a membrane-bound enzyme and, in humans, it is present as two forms, long and short (NEU4L and NEU4S), differing for 12 aminoacids at the N-term. Liver and colon mainly express NEU4S; brain, muscle, and liver express both NEU4L and NEU4S. At a subcellular level, the long form of NEU4 localizes in mitochondria, while the short form is mainly associated with the endoplasmic reticulum. Both forms exhibit substrate specificity to oligosaccharides, glycoproteins, and glycolipids. In mouse, NEU4 is expressed predominantly in the brain, whereas it is found very low in other tissues. NEU4 plays a major role in brain development by digesting polysialic acids on the surfaces of neuronal

cells (Miyagi T, 2012). Moreover, the long form of human NEU4 is involved in the regulation of neuronal cell apoptosis (Hasegawa T, 2007).

In contrast to NEU3, the human colon cancer cells showed decreased NEU4 mRNA levels (Miyagi T, 2012).

Some properties of human sialidases are resumed in Tab.1.

Table 1: Human sialidase (Miyagi T, 2008).

	NEU1	NEU2	NEU3	NEU4
Major subcellular localization	Lysosomes	Cytosol	Plasma membrane	Lysosomes, Mitochondria, intracellular membrane
Good substrates	Oligosaccharides, Glycopeptides	Oligosaccharides, Glycoproteins, Gangliosides	Gangliosides	Oligosaccharides, Glycoproteins, Gangliosides
Optimal pH	4.4 - 4.6	6.0 - 6.5	4.6 - 4.8	4.4 - 4.5
Total aminoacids				
(Human)	415	380	428	496 (484)
(Mouse)	409	379	418	478
Chromosome location				
(Human)	6p21.3	2q 37	11q13.5	2q37.3
(Mouse)	17	1	7	10
Frequent changes in cancer	Down	Down	Up	Down

2.2. GENE ORGANIZATION OF HUMAN SIALIDASES

The human gene, NEU1, was identified in the chromosome 6p21.3, consists of six exons that span in about 5kb (Milner CM, 1997) (Fig. 4). NEU1 protein has 415 aminoacids and a molecular weight of 45.5kDa. The predicted aminoacid sequence has homology to the mammalian cytosolic sialidase and to bacterial sialidases, including the F(Y)RIP sequence, the Asp boxes and a potential N-linked glycosylation site (Miyagi T, 2012).

The human gene, NEU2, is very short, has only two exons, and is located on chromosome 2q37.1 region (Figure 4). NEU2 protein consists of 380 aminoacids with a molecular weight of 42.8kDa (Monti E, 1999). The promoter region contains a classic TATA box and four E-boxes that are DNA binding motif recognized by several nuclear factors belonging to the basic helix-loop-helix family of DNA binding protein (Monti E, 1999). Among other mammalian sialidases, NEU2 cDNA was first cloned (Miyagi T, 1993).

The human gene, NEU3, comprises of four exons encompassing over 22kb, located in the chromosome region 11q13.5 (Fig. 4) (Monti E, 2000). The nucleotide sequence of NEU3 in human, mouse and rat shows higher homology in the open reading frame and, in turn, exhibits higher identity in the aminoacid sequences. (Miyagi T, 2012). In humans, the deduced sequence of NEU3 comprises 428 aminoacids, that contains one F(Y)RIP-box and three Asp boxes in the N-terminal and in the center of the aminoacid sequence. Sequence analysis of the 58 untranslated regions reveals the presence of several Sp1-binding sites and the absence of TATA and CAAT box sequences, in agreement with the Northern blot analysis that shows a ubiquitous expression pattern.

The human gene, NEU4, identified by searching sequence databases for entries showing homologies to the human cytosolic sialidase NEU2, maps in the telomeric region of the long arm of chromosome 2 (2q37.3), and is organized in four exons (Figure 4). The NEU4 gene encodes a 484-residue protein; the predicted NEU4 protein contains all the typical sialidase amino acid motifs and, apart from an amino acid stretch that appears unique among mammalian sialidases, shows high sequence homology with the cytosolic (NEU2) and the plasma membrane-associated (NEU3) enzymes.

Summing up, all the mammalian sialidases cloned so far show high degree of homology and share amino acid blocks of highly conserved residues, F(Y)RIP motif and Asp boxes in topologically equivalent positions throughout the primary structure (Monti E, 2002). In addition, 8 or 9 of 12 of the amino acid residues that form the catalytic site of *S. thypimurium* enzyme are conserved (Crennell SJ, 1993).

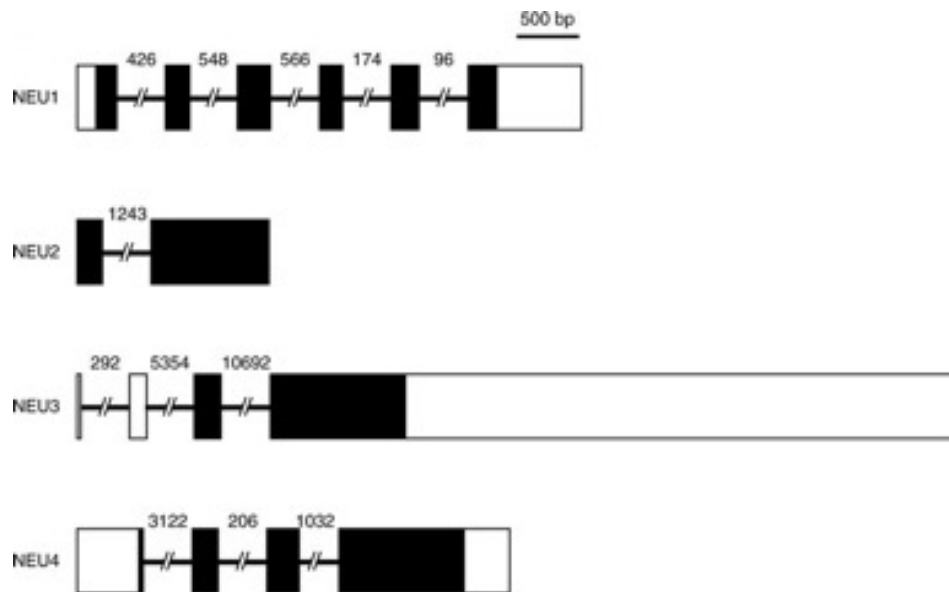


Figure 4: Structure of human NEU1, NEU2, NEU3 and NEU4 genes (Miyagi T, 2012).

In 1993, the first mammalian sialidase was cloned: it was the rat cytosolic sialidase. Its first analysis demonstrated a significant homology with those of bacteria and virus: in particular, the domain F(Y)RIP at the amino-terminus of the protein, and the so-called Asp-boxes (SxDxGxxT/W) (Roggentin P, 1989). The sequence F(Y)RIP is involved in the catalytic site of sialidases, giving an arginine that can interact with sialic acid carboxyl group during the stages of substrate binding and catalysis. Instead, it is believed that Asp boxes are involved in the folding of sialidases (Roggentin P, 1993).

At the end of 1990s, the human lysosomal sialidase NEU1 was cloned and, in 1999, the human cytosolic sialidase NEU2 was also identified and cloned (Monti E, 1999); after one year, the sialidase NEU3 was discovered (Monti E, 2000). In 2004, NEU4 was identified (Monti E, 2004).

Overall, the level of similarity between sialidases from different species reaches 75%; for these reasons, it was hypothesized that a common ancestral gene could exist (Schauer R, 1984).

2.3. NEU3 SIALIDASE AND CANCER

As previously outlined, several reports indicate that NEU3 is upregulated in various cancers including colon, renal, ovarian and prostate. In contrast, in acute lymphoblastic leukemia, NEU3 appeared to be down-regulated (Mandal C, 2010). Moreover, many evidences suggest that it can be responsible for the appearance of many alterations among sialoglycoconjugates in cancer cells (Miyagi T, 2012). Many data concur to classify NEU3 as a novel oncogene (Yamaguchi K, 2006).

In particular, in human colon cancer cells, NEU3 sialidase is 3 to 100 fold increased, as mRNA level, compared to non-tumor mucosa. Instead, during sodium butyrate-induced apoptosis, colon cancer cells showed to undergo to the down-regulation of NEU3 expression. On the other hand, NEU3 gene transfection into colon cancer cells inhibited sodium butyrate-induced apoptosis, by increasing the expression of the anti-apoptotic protein, Bcl2. It has been suggested that lactosylceramide, a product of NEU3 action on cellular gangliosides, could be responsible for the anti-apoptotic and pro-survival effects elicited by NEU3 over-expression.

Moreover, in colon cancer cells, NEU3 regulates cell proliferation modulating the integrin mediated signaling triggered by adhesion to the proteins constituting the extracellular matrix, in particular laminin. Triggered by laminin, NEU3 enhanced tyrosine phosphorylation of integrin β 4 together with the recruitment of Shc and Grb-2 and the stimulation of FAK and ERK1/2 phosphorylation (Miyagi T, 2012).

In colon cancer cells, the silencing of NEU3 induced the down-regulation of the Wnt pathway (Wnt1, Wnt3a and EGFR) (Takahashi K, 2015).

Also, in renal carcinoma cells, NEU3 has been demonstrated to be up-regulated. Here, NEU3 up-regulation was closely interconnected with IL-6 signalling. NEU3 enhanced IL-6

signalling promoting the PI3K/AKT signaling pathway and thus contributing to suppression of apoptosis and migration (Miyagi T, 2012). Downregulation of NEU3 decreased AKT phosphorylation and inhibited Rho activation (Ueno S, 2006).

In renal carcinoma CA-TC cells, NEU3 silencing increased the content of ganglioside GD1a leading to the up-regulation of RAB25 and to the downregulation of the chloride intracellular protein 3 (CLIC3). These events strongly affected the trafficking and recycling of β 1 integrin to the plasma membrane leading to a higher degradation and to a minor content of β 1 integrin at the plasma membrane (Tringali C, 2012).

Upregulation of NEU3 is also detected in prostate cancer. In the prostate cancer cell line, PC-3, the silencing of NEU3 caused a reduction in cell growth both in vitro and in vivo and the decrease of cell migration and invasion. The studies suggested the role of NEU3 in the progression of prostate cancer through androgen receptor signaling (Miyagi T, 2012).

In chronic myeloid leukemia cells, K562, NEU3 silencing reduced proliferation rate and apoptosis resistance, triggering megakaryocytic differentiation (Tringali C, 2009).

Many effects above described and triggered by the up-regulation of NEU3 are caused by the modulation acted by this sialidase on signalling molecules, such as the epidermal growth factor receptor (EGFR), FAK, ILK, and integrins. Regarding EGFR, many studies indicated a link between NEU3 and the activation of EGFR downstream pathways. It was shown that human sialidase NEU3 co-immunoprecipitate with EGFR and can modulate its activation modifying the surrounding gangliosides or the level of sialylation of EGFR itself (Mozzi A, 2015). Moreover, EGF is able to stimulate NEU3 activating the phospholipase D (PLD1) and the formation of phosphatidic acid. Phosphatidic acid was demonstrated to be a strong inducer of NEU3 translocation to the plasma membrane (Shiozaki K, 2016).

NEU3 has been demonstrated to be over-expressed also in melanoma. NEU3 silencing in the melanoma cell line MeWo reduced cell growth similarly to that observed in other tumours (Miyata M, 2011).

All these data demonstrated that the NEU3 plays a key role in tumorigenesis and that it could be involved also in melanoma development. These evidences constitute the basis of the present thesis.

3. AIMS OF THE THESIS

Among many other alterations regarding oncogenes, tumour suppressors and signalling pathways, melanoma cells have been demonstrated to be characterized also by the up-regulation of the plasma membrane sialidase NEU3 (Miyata M, 2011; Tringali C, 2014).

Sialidase NEU3 is also known as the “ganglioside sialidase” due to its strict substrate preference. As discussed in the Introduction paragraph, melanoma cells are marked by the presence of atypical gangliosides, not usually present in normal melanocytes. This evidence induced also the employment of anti-ganglioside vaccines. Moreover, it has been demonstrated that gangliosides can significantly influence the aggressiveness of melanoma cells. Thus, it can be hypothesized that NEU3 alterations could be involved in the modulation of melanoma cell ganglioside profile and, therefore, in its malignancy.

A previous paper demonstrated that NEU3 silencing/over-expression modified melanoma cell survival capability (Miyata M, 2011). Moreover, preliminary results obtained in my laboratory demonstrated that NEU3 silencing significantly reduced the growth of primary melanoma cells in soft agar and in vivo, along to their migration potential. Genome-wide expression profiling and GO enrichment analysis demonstrated that many genes altered by NEU3 silencing are related to the control of migration, differentiation, and survival.

Therefore, based on these data, the aims of this thesis were:

- to validate by Real Time PCR the expression of genes emerged by microarray analysis and related to the control of migration and motility;
- to analyse the expression as proteins of markers of epithelial-mesenchymal transition, as this process is related to both differentiation and altered adhesion and motility, in melanoma cells;
- to identify the signalling pathways changed by NEU3 silencing and possibly related to the control of epithelial-mesenchymal transition and motility.

To pursue this aims, two primary melanoma cell lines, named L3 and L6, and previously established NEU3 silenced clones were employed.

4. MATERIALS AND METHODS

4.1. CELL CULTURE

Primary human melanoma called 3 and 6 and established by Fondazione IRCCS Istituto Nazionale dei Tumori at Milan were grown at 37°C, in the presence of 5% CO₂. Cells were grown in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 1% glutamine and 1% penicillin, and streptomycin. Cells were cryopreserved in a medium constituted by 90% FBS and 10% DMSO, in liquid nitrogen.

Stable NEU3 silenced clones were previously obtained through a lenti-viral approach, in the laboratory where I worked for this thesis: for melanoma cell line 3, NEU3 silenced clone 3C was obtained; for melanoma cell line 6, NEU3 silenced clones 6A and 6B were obtained.

4.2. CELL LYSIS

7.5×10^5 mock and NEU3 silenced cells from cell line 3 and 6.5×10^5 mock and NEU3 silenced cells from cell line 6 were usually plated in 100 mm plates and cultivated for 48 hours, at 37°C. Then, cells were harvested by scraping and by centrifugation (300xg, 5 minutes, 4°C). Cell pellets were resuspended using phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4) and adding EDTA free protease inhibitor cocktail and 1 mM sodium orthovanadate (Roche). Then, cells were homogenized by sonication (2 pulses, 10 sec). Total cell lysate were employed for Western blot analysis.

For sialidase activity assay, total cell lysates were centrifuged at 800xg, for 10 minutes, at 4°C to remove unbroken cells and nuclei from the crude homogenate. Subsequently, the crude homogenates were again centrifuged at 70,000 x g, for 20 minutes, at 4°C to separate cellular membranes from cytosol. The pellets containing plasma membranes were resuspended in PBS with protease inhibitors and 1 mM sodium orthovanadate.

4.3. BRADFORD PROTEIN ASSAY

The protein assay was performed using Bradford method. The concentration of proteins in samples was measured by comparing it with standard calibration curve prepared using bovine serum albumin (BSA) at three concentrations (1 µg/ml, 2 µg/ml, 3 µg/ml). Coomassie blue diluted in water with 1:4 ratio and 1 ml of this solution was added to samples and blank containing only water. Absorbance was measured at 595 nm using JASCO spectrophotometer.

4.4. SIALIDASE ACTIVITY ASSAY

Sialidase activity present in mock and NEU3 silenced clones obtained from melanoma cell lines 3 and 6 was assayed by using the artificial fluorescent substrate 4-methylumbelliferyl-N-acetyl-D-neuraminic acid (MU-NeuAc) and membranes prepared as described in paragraph 4.2.

A reaction mixture was prepared as follows:

- Membrane preparations - 10µg
- Bovine serum albumin (BSA) - 600µg
- MU-NeuAc - 0.1mM (10µl)
- Phosphate/Citrate buffer - 0.5M, pH 3.8 (10µl)
- Standard MU-Na - 0.5nM

Distilled water was added to make the total volume 100µl.

The samples were incubated in water bath at 37°C, for 30 minutes and subsequently, 0.2M glycine pH 10.8 was added to the samples and enzyme reaction was stopped.

0.5 mole standard MU-Na (methylumbelliferone-Na) was added in a total volume of 100 µl, in water. The fluorescence was measured at excitation λ of 365 and emission λ of 448, using the Nanodrop 3300 fluorospectrometer, 4MU program set up. Glycine was used as blank.

The enzyme activity was expressed as U/mg protein: U equals to 1µmole substrate released in 1 minute.

4.5. SDS-PAGE

The discontinuous polyacrylamide gel electrophoresis was used to separate the proteins.

20-30 µg of cell lysate were diluted with sample buffer (62.5 mM Tris/HCl pH 6.8, 2% SDS, 10% Glycerol, 5% 2-mercaptoethanol, 0.01% Bromophenol blue), and boiled for 5 minutes, at 100°C. Samples were loaded onto SDS-PAGE gel and electrophoresis was performed in Tris/glycine running buffer (25 mM Tris, 19.5 mM Glycine, 0.1% SDS, pH 8.3), at 20-30 mA constant.

The gel was constituted by a Running gel and a Stacking gel.

Running Gel Composition (10% Acrylamide)

- 1.5 mM Tris/HCl, pH 8.8
- 10-12% Acrylamide
- 0.1% w/v SDS
- 0.05% w/v ammonium persulphate (APS)
- TEMED
- H₂O

Stacking Gel Composition (4% Acrylamide)

- 0.5 mM Tris/HCl, pH 6.8
- 4% Acrylamide
- 0.1% w/v SDS
- 0.05% w/v ammonium persulphate (APS)
- TEMED
- H₂O

After the running, proteins were transferred onto PVDF membrane for western blot analysis.

4.6. WESTERN BLOT

Proteins were transferred onto PVDF membrane (25 mM Tris, 190 mM glycine, pH 8.3 plus 20% methanol), at 100V, for 2 hours, at 4°C. The membrane was incubated with blocking buffer for 1 hour in shaker at room temperature in order to saturate nonspecific binding of antibodies. The blocking buffer was constituted by 5% non-fat milk powder in TBS (50 mM Tris/HCl, 0.15 M NaCl, pH 7.4) plus 0.1% (w/v) Tween 20. After blocking, the membrane was washed 3 times for 5 minutes, in TBS-Tween 20 (wash buffer). Followed, the primary antibodies diluted in blocking buffer were added onto the membrane and incubated overnight, at 4°C.

After incubated with primary antibody (Tab.2), membrane was washed with wash buffer (TBS-Tween 20) 3 times for 5 minutes. Subsequently, the peroxidase conjugated secondary antibody diluted in blocking buffer was added to the membrane and incubated in shaker for 45 minutes, at room temperature. After that, the membrane was washed 3 times with TBS-Tween 20 for 5 minutes. The Chemiluminescent substrate (Bio-Rad and Westar η ultra2 Pico/Dura Kit) was added to the membrane and incubated under dark condition for 5 minutes. The membrane was placed in UVITEC; image was captured and quantitative protein expression was analysed.

Table 2: Antibodies used for western blot.

Antibody	Host	Company
N-Cadherin	Rabbit	Santa Cruz Biotechnology
E-Cadherin	Rabbit	Cell Signaling
Vimentin	Rabbit	Cell Signaling
ZO-1	Rabbit	Cell Signaling
MITF	Rabbit	Sigma Aldrich
Claudin1	Rabbit	Cell Signaling
β -Actin	Mouse	Cell Signaling

4.7. STRIPPING OF MEMBRANES

After detecting the phosphorylated form of some proteins, the membranes were stripped to detect total proteins. To this end, membranes were put in stripping buffer (50 mM Tris/HCl pH 6.8, 2% SDS, 100 mM β -mercaptoethanol) for 20 minutes, at 50°C in water bath.

After stripping, the membranes were washed using wash buffer (TBS-Tween 20) in order to eliminate β -mercaptoethanol for proper binding of antibodies to the target protein. After washing, the membranes were blocked with 5% milk in TBS-Tween 20 for 1 hour and then treated again with the primary antibody.

4.8. PROTEIN ARRAY

The PathScan® Intracellular signaling array kit (Chemiluminescent readout) (Cell Signaling) is a slide based antibody array used to detect several cellular proteins and signaling nodes only when phosphorylated or cleaved at the specified residues (ERK 1/2, Stat1, Stat3, AKT, AMPK alpha, S6 ribosomal protein, mTOR, HSP27, BAD, p70 kinase, PRAS40, p53, p38, SAPK/JNK, PARP, Caspase-3, GSK-3 beta).

7.5×10^5 mock and NEU3 silenced cells from cell line 3 and 6.5×10^5 mock and NEU3 silenced cells from cell line 6 were plated in 100 mm plates and cultivated for 48 hours. After, the medium was aspirated. The cell plates were kept on ice tray under cold condition. 4 ml of cold 1X PBS was added to the plates and aspirated immediately. 500 μ l of lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM disodium EDTA, 1 mM EGTA, 1% Triton X-100, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 μ g/ml leupeptin) containing EDTA free protease cocktail inhibitor was added to the cells and incubated on ice for 2 minutes. Then, cells were harvested by scraping, transferred into the eppendorf tubes and centrifuged for 2 minutes, at 14.000xg, at 4°C. The supernatants containing cell lysates were collected.

The protein assay was performed using Bradford method as described in paragraph 4.3 with minor modifications. Because Triton X-100 interferes with this assay at high

concentrations, it was added to blank and to the standard calibration curve, at the same concentration present in samples.

Cell lysates were diluted to reach a final concentration of 0.3 mg/ml and 0.6 mg/ml from total protein samples by using the array diluent buffer provided by the kit.

Then, the procedure described by manufacturer was followed. In details, the glass slide was placed carefully on top of the multiwell gasket with the nitrocellulose pads facing down. The metal clips were inserted on both sides into the groove in the gasket and ensured that the clips were on the same side as the orientation line on the slide followed by rotating the clips into the locked position, assembled array is done.

100 µl of the array-blocking buffer (provided by the manufacturer) was added to all the wells of the multiwell signaling array glass slide. Then it was covered with sealing tape, kept in shaker for 15 minutes. After discarding the blocking buffer from each well completely, 75 µl of the diluted protein samples were added to their corresponding well and covered. Incubation was carried out overnight in shaker at 4°C. After incubation, the contents were discarded and washed with 150 µl of the array wash buffer 4 times for every 5 minutes. After washing, 75 µl of 1X detection antibody cocktail (provided by the manufacturer) was added to each well. The array was covered by sealing tap and incubated for 1 hour in shaker. After incubation, the wells were washed with 100 µl of 1X array wash buffer for 4 times with each duration of 5 minutes. Followed, 75µl of 1X HRP-streptavidin (secondary antibody) was added to wells and incubated in shaker for 30 minutes. After incubation, the wells were washed 4 times for every 5 minutes with 100 µl of 1X array wash buffer. The glass slide was removed from the gasket and put inside the box filled with 10 ml of 1X array wash buffer and washed briefly. After decanting array wash buffer, the glass slide was covered with 10 ml of LumiGLO® and peroxide chemiluminescent reagents. The glass slide was transferred into UVITEC and images were captured by detecting chemiluminescent signals immediately. Spot intensity was quantified by using array image analysis software.

4.9. RNA EXTRACTION

Total RNA was extracted from 7.5×10^5 mock and NEU3 silenced cells from cell line 3 and from 6.5×10^5 mock and NEU3 silenced cells from cell line 6 cultivated for 48 hours, at 37°C, using RNeasy mini kit (Qiagen). Plated cells were detached by using trypsin. Cell pellets were obtained by centrifuging at 300xg, for 5 minutes. The cell pellets were lysed by adding 350 µl RLT buffer (cell lysis buffer). Followed, 350 µl of 70% ethanol mix was added, vortex and finally transferred into the column filter. Centrifugation was done at 8000xg for 30 seconds and supernatant was removed. 350 µl of RW1 (wash buffer) was added to the column filter and again centrifuged at 8000xg for 30 seconds, supernatant was removed. In order to remove DNases contamination, 70 µl of digest buffer and 10 µl of DNases were mixed together in a separate tube and added into the column filter, incubated for 20 minutes in room temperature. Soon after, 350 µl of RW1 (wash buffer) buffer was added to column filter and incubated for 5 minutes, at room temperature. Centrifugation was done at 8000xg for 30 seconds and supernatant was removed. Followed, 500 µl RPE (wash buffer) was added and centrifuged at 8000xg for 30 seconds. After the removal of supernatants, again 500 µl RPE (wash buffer) was added and centrifuged at 8000xg for 2 minutes. The column filter was transferred into another eppendorf tube in order to dry the filter completely, centrifuged to the maximum speed for 30 seconds. 40 µl of RNase free water was added to the column filter and incubated for 1 minute at room temperature, and centrifuged at 8000xg for 1 minute. In this way, the total RNA was isolated.

4.10. RNA CONCENTRATION ASSAY

The RNA assay was done by using Ribogreen RNA reagent (Life Technologies), which is one of the most sensitive detection dye for analysing linear fluorescence for the quantitation of RNA. The Ribogreen reagent bound to RNA has a maximum absorbance at 500 nm and a maximum emission at 525 nm. A standard calibration curve with a mixture of rRNA (16S and 23S) was prepared, at four concentrations. The Ribogreen was diluted 1:200 in TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5). 100 µl of aqueous Ribogreen was added to the RNA samples and to standards and incubated at room temperature, for 5 minutes.

Fluorescence was detected by using Nanodrop 3300 fluorospectrometer at the above reported wavelengths.

4.11. REVERSE - TRANSCRIPTION OF RNA

Quantitative gene expression was carried out through the creation of complementary DNA (cDNA, transcripts from RNA) using reverse transcriptase enzyme.

The reverse transcription process was carried out by using 800 ng from total RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories).

Reaction mix was made as follows:

- 4 µl of 5X iScript reaction mix (containing oligo (dT) and random hexamer primers)
- 1 µl of iScript reverse transcriptase
- 800 ng of RNA

Nuclease free water was added to make the final volume to 20 µl.

The complete reaction mix was incubated in a thermal cycler (Eppendorf) using the following protocol:

Annealing - 5 minutes at 25°C

Reverse transcription - 25 minutes at 42°C

Reverse transcriptase inactivation - 5 minutes at 85°C

Hold at 4°C

A negative control containing only RNA and water was prepared to verify the absence of genomic DNA contamination.

4.12. REAL TIME PCR

Real Time PCR was performed using the fluorescent dye SYBR green.

The reaction mix contained:

- 15-25 ng cDNA;
- 200 nM primers (reported in Table 3);
- 10 µl of SYBR Green super mix (Bio-Rad Laboratories) containing hot-start iTaq DNA Polymerase, dNTPs, SYBR Green I, fluorescein and ROX normalization dye.

Distilled water was added to make the final volume 20µl.

To check a possible contamination in the reaction, blanks were prepared without cDNA.

By using iCycler Bio-Rad, we followed this protocol:

Denaturation - 95°C for 1 minute and 30 seconds

Denaturation - 95°C for 10 seconds	}	repeat 45 cycles
Annealing		
Extension - 58°C for 30 seconds		
Detection of Fluorescence		

After amplification process, melting-curve analysis was done to exclude the presence of non-specific products.

Data were analysed using the software distributed by the manufacturer (Bio-Rad Laboratories).

Table 3: Primers used for REAL -TIME PCR.

Gene	Forward	Reverse
SEMA 3B	5'-AGGACATTGGTACTGAGTG-3'	5'-CATCCTCTATCCTTCCTGG-3'
SEMA 3C	5'-ATCAATGAGGAGCTTTTCTC-3'	5'-TTCCTCTTGGTTAAACTTCG-3'
SEMA 5A	5'-CTATAAAGAAATTGGCCCTG-3'	5'-ACAACAAGTTCTTTCTGTCC-3'
NEU3	5'-GGCTTGTTTGGGTGTTTGTT-3'	5'-TTTTGAATTGGCTTGGGTTC-3'
β -ACTIN	5'-CGACAGGATGCAGAAGGAG-3'	5'-ACATCTGCTCCAAGGTGGA-3'

5. RESULTS

5.1. NEU3 SIALIDASE mRNA EXPRESSION AND ACTIVITY IN NEU3 SILENCED CLONES ESTABLISHED FROM PRIMARY MELANOMA CELL LINES

In order to understand the role of NEU3 in melanoma malignancy, NEU3 was stably silenced employing a lentiviral approach, in two primary melanoma cell lines named, L3 and L6. These cell lines are characterized by a similar cellular ganglioside profile composed by GM3 and GD3 but are quite different regarding some biological features; in particular, cell line L3 retains the capability to produce melanin on the contrary of cell line L6.

NEU3 silencing was verified in terms of both NEU3 mRNA expression (by Real Time PCR) and membrane sialidase activity (toward 4-MUNeuAc).

We recorded the decrease of NEU3 mRNA expression in clone 3C compared to L3 mock cells (-35%) (Fig. 5A) and in clone 6A (-30%) (Fig. 5A), and in clone 6B (-31%) (Fig. 5A) compared to L6 mock cells.

In parallel, we recorded the decrease of membrane sialidase activity in clone 3C compared to L3 mock (-29.15%) (Fig. 5B), and in clone 6A (-10.25%) (Fig. 5B) and in clone 6B (-39.40%) (Fig. 5B) compared to L6 mock cells. It should be noted that membrane sialidase activity consists of both NEU1 and NEU3 activity; NEU1 expression did not change among the clones belonging to both L3 and L6 cell lines.

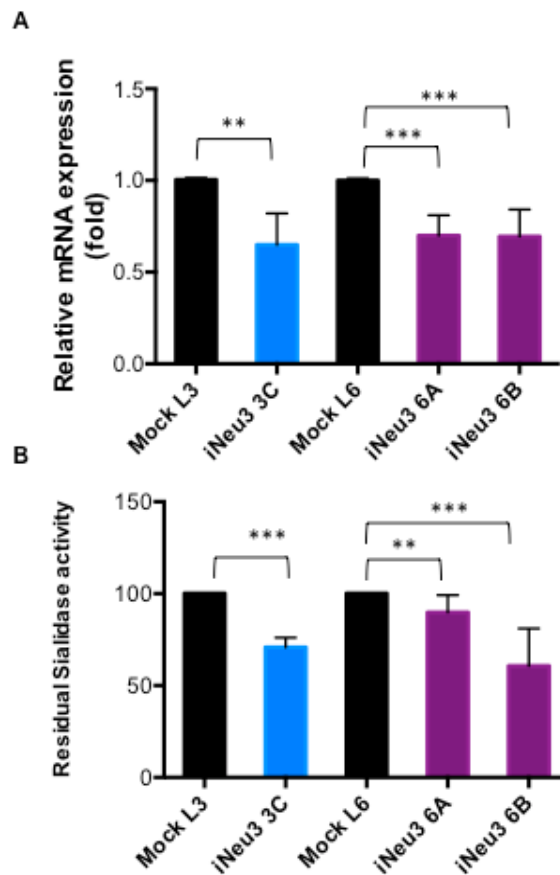


Figure 5: (A) Expression of NEU3 gene assessed by Real Time PCR. (B) Membrane sialidase activity toward 4MU-NeuAc, in mock L3 and its NEU3 silenced clone iNeu3 3C, and in mock L6 and its NEU3 silenced clones, iNeu3 6A and iNeu3 6B. Significance was calculated by unpaired t-Student test. *: P<0.05; **: P<0.01; ***: P<0.001.

5.2. THE EXPRESSION OF SOME GENES INVOLVED IN MELANOMA MALIGNANCY ARE ALTERED BY NEU3 SILENCING

Microarray analyses performed in the laboratory where I worked for PhD revealed that NEU3 silencing altered the expression of some genes related to cell motility and cell survival.

We validated some results by Real Time PCR.

In particular, we confirmed that the gene MAL was up-regulated in both NEU3 silenced clones of cell line 3 (11.6 - fold) and cell line 6 (148 - fold in clone 6A and 21 - fold in clone 6B) (Fig. 6A). MAL encodes an integral protein, which plays a key role in the

formation and maintenance of glycosphingolipid membrane domains (Magal LG, 2009). It is significantly down-regulated in many epithelial tumours (Mimori K, 2003; Cao W, 2010).

The expression of the semaphorin 3B (SEMA 3B) was up-regulated in both NEU3 silenced clones of cell line 3 (0.53 - fold) and cell line 6 (2.63 - fold in clone 6A and 1.93 - fold in clone 6B) (Fig. 6B). Semaphorin 3B is an angiogenesis inhibitor (Varshavsky A, 2008) and inhibits the PI3K-AKT pathway in breast and lung tumours (Castro-Riviera E, 2008). It is known as tumour suppressor (Capparuccia L, 2009).

Semaphorin 3C (SEMA 3C) was up-regulated only in silenced clones of cell line 6 (4 - fold in clone 6A and 1.3 - fold in clone 6B) (Fig. 6C). SEMA 3C has oncogenic effects in some cancers but it is also an inhibitor of tumour lymphangiogenesis and metastasis (Mumblat Y, 2015).

Semaphorin 5A (SEMA 5A) was down-regulated in both NEU3 silenced clones of cell line 3 (-0.47 - fold) and cell line 6 (-0.37 - fold in clone 6B) (Fig. 6D). SEMA 5A demonstrated oncogenic effects in gastric and pancreatic cancers (Lu TP, 2012).

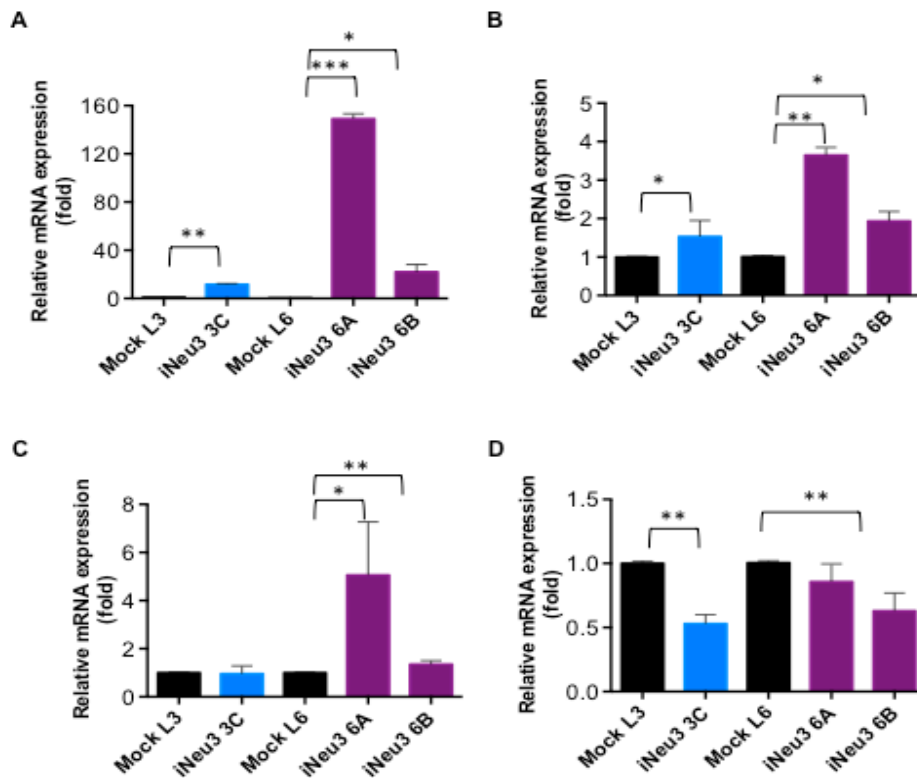


Figure 6: Real time PCR analyses of MAL (A), SEMA 3B (B), SEMA 3C (C), and SEMA 5A (D). Significance was calculated by unpaired t-Student test. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

5.3. THE EXPRESSION OF SOME PROTEIN RELATED TO EPITHELIAL-MESENCHYMAL TRANSITION ARE ALTERED BY NEU3 SILENCING

Epithelial-mesenchymal transition (EMT) is a significant process responsible for the switching of cellular phenotype toward an invasive behaviour, in many tumours (Larue L, 2005). During this event, epithelial cells acquire a mesenchymal behaviour, increasing their motility and altering adhesion properties (Larue L, 2005). It has been demonstrated that EMT occurs also in melanoma even if, in this case, it is more correct to define the process a pseudo-EMT. Many markers identified for EMT in carcinomas were detected also in melanoma cell lines, whereas others are typical of melanomas (Kim JE, 2013).

We recorded the following changes (Fig. 7):

- N cadherin expression decreased in clone 6A (-0.7 - fold) and in clone 6B (-0.32 - fold), compared to mock L6 cells. No significant changes have been detected in clone 3C compared to mock L3 cells;

- E cadherin expression increased in clone 3C compared to mock L3 cells (+1.81 - fold); E cadherin was not expressed by mock and NEU3 silenced L6 cells;

- Vimentin expression decreased in clone 6A (-0.48 - fold) and in clone 6B (-0.29 - fold), compared to mock L6 cells. The decrease of vimentin expression was not statistically significant in clone 3C compared to mock L3 cells, but we recorded its tendency toward the reduction;

- Zo 1 expression increased in clone 6B (+0.38 - fold) compared to mock L6 cells;

- MITF expression increased in clone 6A (+0.52 - fold) and in clone 6B (+0.71 - fold), compared to mock L6 cells. No significant changes have been detected in clone 3C compared to mock L3 cells;

- Claudin 1 expression increased in clone 3C compared to mock L3 cells (+0.25 - fold), in clone 6A (+0.27 - fold) and in clone 6B (+1.13 - fold), compared to mock L6 cells.

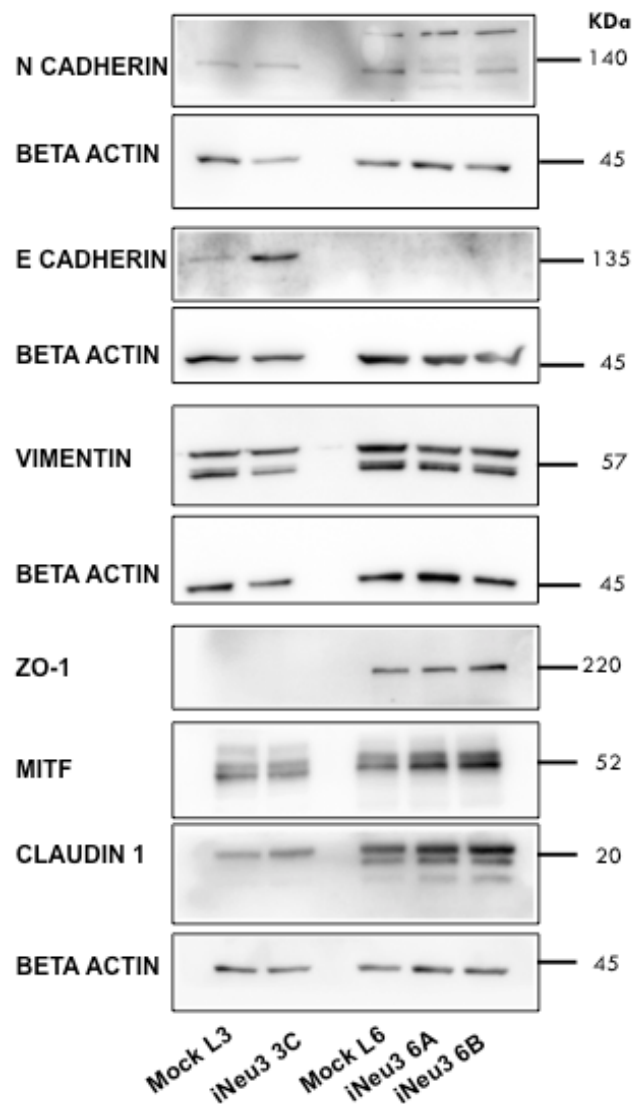


Figure 7: Western blot analysis of N, E cadherins, vimentin, Zo-1, MITF, and claudin 1 in mock L3, iNeu3 3C clone, mock L6, iNeu3 6A and 6B clones. Beta actin was employed as loading control.

5.4. ANALYSIS OF SIGNALING PATHWAYS BY PROTEIN ARRAY

As described in Material and Methods, in order to identify the signalling pathways altered by NEU3 silencing, we employed a protein array.

Thus, we revealed that, in both melanoma cell line 3 and 6, the substrates of the PI3K-AKT pathway (mTOR, BAD, PRAS40) and p38 kinase were less phosphorylated, thus active in NEU3 silenced clones than mock cells (Fig. 8).

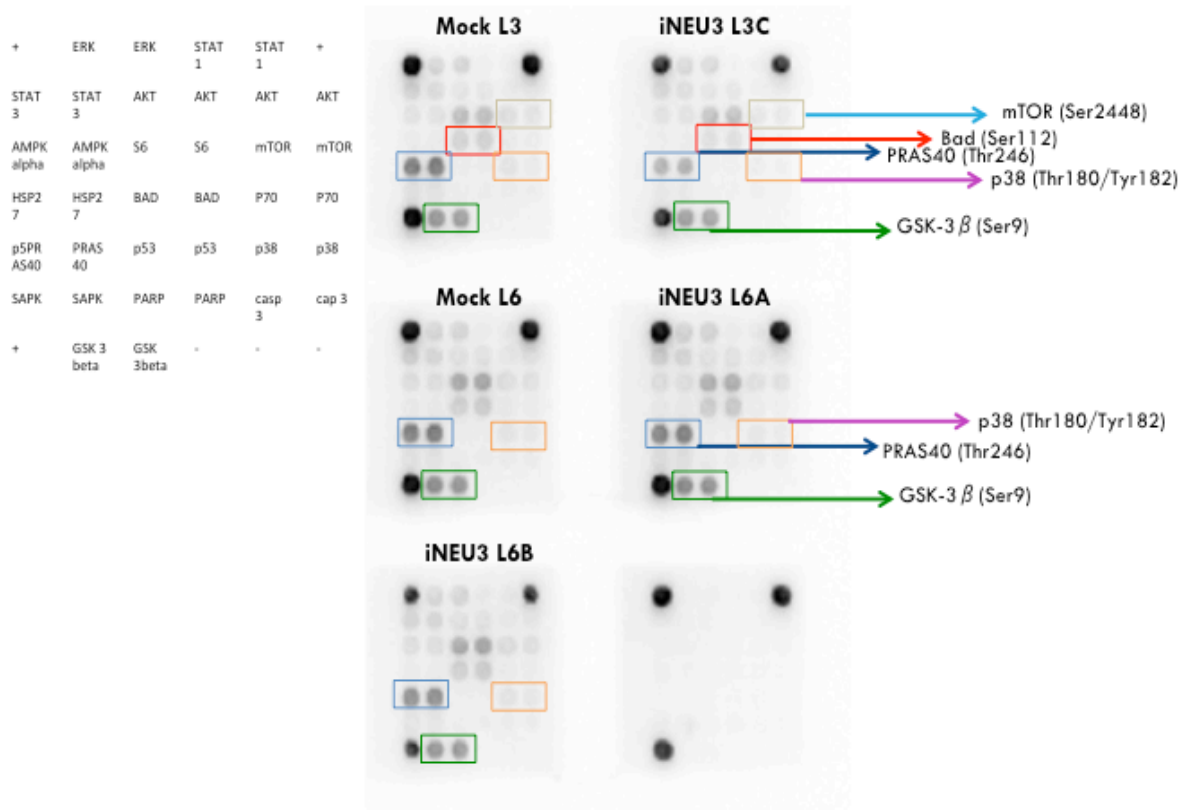


Figure 8: Protein Array

6. DISCUSSION

Aberrant glycosylation is a peculiar feature of cancer, reflecting specific changes in glycan biosynthesis and catabolism pathways (Meany DL, 2011). Alterations concerning the expression/functionality of several enzymes including glycosyltransferases and sialidases allow cancer cells to produce glycolipids and glycoproteins bearing modified glycan chains. These changes have been demonstrated to be largely functional to the malignant phenotype with particular reference to metastasis potential and invasiveness (Hakomori S, 2002). In this context, also modifications concerning the quantitative and qualitative expression of gangliosides play a key role. The type of gangliosides present on the plasma membrane can modulate key cellular processes including cell adhesion, proliferation, differentiation and oncogenic transformation. Therefore, in this view, both the synthesis and the catabolism pathways leading to ganglioside expression constitute fields of interest in cancer biology research.

Ganglioside catabolism can occur directly on the plasma membrane due to the plasma membrane sialidase NEU3. NEU3 was first cloned from bovine brain and it is also known as the “ganglioside sialidase” due to its strict substrate specificity (Miyagi T, 2012). NEU3 is markedly up-regulated in various human cancers, including colon, renal, ovarian and prostate and this event contributes to augmentation of malignant properties of cancer cells by causing disturbance of transmembrane signaling (Miyagi T, 2012). In particular, it has been demonstrated the involvement of NEU3 in association with gangliosides in processes such as adhesion, differentiation, proliferation, and apoptosis resistance (Rodriquez Walker M, 2015).

Melanoma cells summarize all these features, being characterized by an altered expression of gangliosides and the up-regulation of NEU3 (Miyata M, 2011; Tringali C, 2014). Previous results collected in my laboratory but not still published demonstrated that NEU3 silencing in primary melanoma cell lines established from patients reduced cell growth in soft agar and in vivo and migration.

Starting from these data, this research project was aimed to understand the role of NEU3 in melanoma malignancy, focusing on signaling pathways involved in the control of cell motility and differentiation.

Two different primary human melanoma cell lines were used in this study: they were characterized by similar cellular ganglioside profiles enriched by GM3 and GD3 and were established from patients with short survival. Nevertheless, they also were endowed with some different biological properties. In particular, cell line L3 had the capacity to produce melanin contrary to cell line L6.

A lentiviral approach was used to produce stable silencing of NEU3. NEU3 silencing was verified by assaying NEU3 mRNA expression by Real Time PCR and membrane sialidase activity towards 4-MUNeuAc.

We first identified a different expression of some genes related to cell motility in NEU3 silenced melanoma cells. In particular, MAL has been demonstrated to be deeply up-regulated in both NEU3 silenced clones established from cell line 3 and 6. Interestingly we observed a 148-fold increase in clone 6A of cell line L6 (Fig. 6A). Even if, MAL is also known as the T-cell differentiation protein, it is expressed also by other cell types and its down-regulation has been described in various epithelial malignancies (Cao W, 2010). MAL over-expression in esophageal cancer has been demonstrated to reduce migration in addition to modifying other properties (Mimori K, 2003).

Moreover, we recorded a different expression of some semaphorins. Semaphorins are proteins, which participate in many physiological and developmental functions. The semaphorin family includes more than 30 genes, which are divided into seven subfamilies, characterized by the presence of the SEMA domain (Neufeld G, 2016). The members of the SEMA family are differentially expressed in cancer and either promote or suppress cell proliferation, migration, angiogenesis and induction of drug resistance. Selected members of the semaphorin 3 family are involved in the suppression of tumor progression and are considered as potent tumor suppressors. SEMA3s are secreted by cells of multiple lineages, including epithelial cells, neurons, and specific tumor cells (Loginov VI, 2015). Significantly, we demonstrated that both NEU3 silenced clones of cell line L3 and cell line

L6 up-regulated semaphorin 3B (Fig. 6B). Instead,, the expression of semaphorin 3C was up-regulated only in NEU3 silenced clones of cell line L6 (Fig. 6C).

Semaphorin 5A seems to act as an oncogene in some tumors, i.e. gastric and pancreatic cancers (Lu TP, 2012). Its expression was down regulated in both NEU3 silenced clones of cell lines 3 and 6 (Fig. 6D).

In addition to these results, we recorded in NEU3 silenced clones a different expression of some markers of EMT. EMT is a process by which cells loose epithelial characteristic and gain a migratory mesenchymal phenotype (Gonzalez DM, 2014). we observed a decreased N-Cadherin expression in NEU3 silenced clones established from cell line 6, whereas increased expression of E-Cadherin was observed in clone 3C of cell line 3.

The mesenchymal marker vimentin decreased in clone 6A and 6B of cell line 6 when compared to mock cells; instead, no significant decrease of vimentin was observed in mock NEU3 silenced clone 3C (Fig. 7).

We also recorded an increased expression of ZO-1 and claudin 1 after NEU3 silencing further demonstrating changed cell-cell interactions and the increased expression of MITF.

MITF is a master regulator of melanocyte differentiation and stimulates melanin synthesis and acts as a marker of EMT in melanoma, decreasing during the acquisition of the mesenchymal phenotype. The increased expression revealed in NEU3 silenced clones demonstrated that a partial differentiation process occurred.

Summing up, these results prove the acquisition of a different molecular phenotype after NEU3 silencing that could explain the less motile properties of these cells.

Based on our results, the signalling pathway involved in these changes appeared to be the PI3K/AKT. In fact, we revealed that PRAS40, a substrate of AKT, mainly AKT3 (Madhunapantula SV, 2007) was less phosphorylated in NEU3 silenced clones. Significantly, PI3K/AKT is also involved in EMT induction (Xu W, 2015).

Thus, our results demonstrated that NEU3 silencing could reduce the PI3/AKT/PRAS40 signalling pathway and thus induce a less aggressive cell phenotype.

Therefore, NEU3 could constitute a novel target in melanoma.

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