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# Melanoma stem-like cells and melanoma cell lines: main molecular pathways and possible microRNA involvement

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# ABSTRACT

Cancer Stem Cells (CSCs) have been identified in several malignancies and indirect evidences support the existence of melanoma stem-like cells. Since microRNAs (miRs), an abundant class of small non-coding RNAs playing important roles in regulating gene expression, are involved in melanoma development and progression, we investigated their possible involvement also in melanoma stem-like cells. We identified a subset of melanoma cells, isolated from lung metastatic melanomas that, cultured in appropriate serum free conditions, were able to propagate as non adherent spheres (SC), whereas in the presence of serum they adhere to the plastic and acquire the typical morphologic features of differentiated cells (PC). These melanoma spheres are also capable of self-renewing and tumorigenesis in vivo. Here we analyzed a group of selected miRs by qReal Time PCR and among them miR-221/-222 had the most consistent and significant increased expression in PC cells. In these cells we confirmed the inverse correlation between microRNA-221/-222 and p27Kip1 and c-KIT receptor, already reported in melanoma development and progression. Moreover, looking for new miR-221/-222-dependent target genes, we selected the proto-oncogene ETS-1 because its functional role in melanoma development was not completely elucidated. In fact ETS-1 has been reported either as a valuable diagnostic/prognostic marker or as molecule without a clear association with clinical outcome. Here we demonstrated that in melanoma stem cells as well as in melanoma cell lines ETS-1 is a direct target of miR-222. Furthermore we showed the existence of an ETS1-mediated repression of miR-222 in melanoma cell lines, thus we suggest that in putative melanoma stem cells proliferating as spheres (Mel SC) in comparison to their adherent counterpart (Mel PC) may be active an ETS-1↔miR-222 circuitry whose deregulation could be relevant for melanoma progression. Further studies are in progress to understand the function of miRNAs in the context of gene networks controlling cell differentiation and tumorigenicity in melanoma stem-like cells.

# INTRODUCTION

## A molecular model of melanoma progression

Malignant melanoma is the most aggressive form of skin cancer and its incidence is continuously rising during the last decades at an alarming rate (Miller and Mihm, 2006). Although there is a good chance for the recovery of patients suffering from melanoma if the primary lesion is detected very early, prognosis of 5-year survival for more advanced melanomas is extremely poor (Chudnovsky *et al*., 2005). Several melanoma biomarkers have been evaluated for their prognostic utility with promising early results, such as the mitotic and the Ki-67 marker expression indexes, shown to be associated with a significant prognostic value (Vereecken *et al.,* 2007); however, to date, none has been proven to be clinically useful in large-scale studies (Larson *et al.,* 2009).

The main clinical and histopathological prognostic factors that are currently in use for melanoma include tumor depth (i.e. Breslow thickness), diameter, ulceration, anatomic site (i.e. acral, mucosal, cutaneous) and sentinel lymph-node status (Balch *et al*. 2001).

Although several molecular abnormalities have been associated with melanoma progression, as the loss of AP-2 transcription factor (Huang *et al.*, 1998) or the high mutation rate of the B-RAF oncogene (Dhomen *et al.,* 2009), the mechanisms underlying the differential gene expression are still largely unknown and the conventional histological classification remains the best prognostic factor (Clark *et al*., 1984). The Clark model describes the histological changes that accompany the progression from normal melanocytes to malignant melanoma. In this model, five distinct steps of melanoma development and progression are distinguished: a mature

melanocyte acquires mutations that lead from benign (Step 1) to dysplastic nevi (Step 2). The subsequent radial growth phase (RGP) primary melanoma (Step 3) is the first recognizable malignant stage in which cells do not possess metastatic potential but are already locally invasive. RGP is followed by the vertical growth phase (VGP) (Step 4), in which melanoma cells infiltrate and invade the dermis and show metastatic potential. This process finally results in metastases to distant organs by an overgrowth of disseminated tumor cells at these sites (Step 5) (Fig.1) (Gray-Schopfer *et al*., 2007).

However, clinically, only 26% of melanomas observed evolve from nevi, and less than 50% are associated with dysplastic nevi (Bevona *et al.*, 2003). This indicates that the majority of melanomas arise from normal-appearing skin and not from dysplastic nevi, suggesting that melanoma development may not follow the classical linear model of progression.

Actually an alternative theory suggests that melanocytic neoplasias derive from immature melanocytic cells: this theory follows the cancer stem cell hypothesis.

# Cancer Stem Cells (CSCs)

## 1.2 The cancer stem cell hypothesis: identification and isolation of CSCs

Tumor is regarded as an abnormal tissue descending from a single cell through the continuous accumulation of genetic errors and epigenetic changes. Accordingly tumor tissues have long been known to be composed of heterogeneous populations of cancer cells showing various differentiation levels.

The classical model of carcinogenesis suggests that all neoplastic cells within a tumor have tumorigenic capacity. However, recently the identification of Cancer Stem Cells (CSCs) has introduced a new concept in cancer therapy since this small subpopulation of cells, being theoretically able to sustain the tumor cell growth, might represent the actual therapeutic target (Reya *et al.,* 2001; Massard *et al.,* 2006; Schulenburg *et al.,* 2010). The small subset of CSCs is capable either of self-renewing or giving rise to all the components of a differentiated heterogeneous tumor (O'Brien *et al.,* 2010; Bomken *et al.,* 2010). Specifically, if mutations occur in stem cells or in early stem cell progenitors is not yet clear. In addition, the numerous factors present in the host microenvironment might trigger the initial steps of tumor formation (La Barge, 2010) (Fig.2). CSCs have been identified on the basis of their functional abilities experimentally demonstrated by serial transplantations into animal models where CSCs are able to reproduce the same heterogeneous phenotype observed in the primary tumor (Eisterer *et al.,* 2005; Gu *et al.,* 2007). According to the reported cancer stem cell hypothesis, this cell subset, able to grow as as non-adherent spheroid, is responsible for tumor progression (Dalerba *et al.,* 2007), recurrence, metastasis (Wicha *et al.,* 2006) and resistance to therapy (Dalerba *et al.,* 2007). In order to study CSCs, it is important

that these cells are accurately characterized and prospectively isolated from the rest of the tumor cell population in a consistent manner.

One of the methods frequently used for the enrichment and isolation of the poorly represented population of cancer cells with stem cell-like properties is the fluorescence-activated cell sorting (FACS), based on the use of one or more antibodies specifically directed against the cell surface markers. A number of stem cell-like surface markers, as CD133, CD20, ABCG2, ABCB5, CD24, CD44 and CD90, have been demonstrated on CSCs of several solid tumors, including glioblastoma, colon, lung and liver cancer, head and neck squamous carcinoma, breast carcinoma and melanoma (Ricci-Vitiani *et al.,* 2007; Eramo *et al.,* 2008; Al-Hajj *et al.,* 2003; Prince *et al.,* 2007; Yang *et al.,* 2008; Fang *et al.,* 2005; Monzani *et al.,* 2007; Schatton *et al.,* 2008). Hence, the isolated small sub-population of cancer stem cells may be subsequently expanded ex vivo in serum-free medium and further characterized by the non-adherent spheroid generation and clonogenicity assays for establishing their self-renewal and multilineage capacities *in vitro*. The implantation and serial transplantations assays may also be carried out with the isolated cancer stem cells in animal models for estimating tumorigenic potential and self-renewal ability in vivo. For example in 2003, Al-Haji *et al.* reported that in breast cancer, only a small population of tumor cells, expressing a CD44+/CD24 low cell surface phenotype, was able to induce new tumor formation into immune-deficient NOD/SCID mice. These data demonstrated the presence of a hierarchy of cells within breast tumors. However, whether in melanoma a similar hierarchy for tumorigenic potential among cancer cells does exist or every single cell in the tumor has the same capabilities, still requires a clearing up.

## 1.3 Melanoma stem-like cells

Melanoma stem cells are currently defined according to their ability to grow in immunodeficient mice. Although stem cell markers and subsets of stem-like cells have recently been described in melanoma, a definite molecular characterization is still pending (Fang *et al.,* 2005; Monzani *et al.,* 2007; Schatton *et al.,* 2008; Boiko *et al.,* 2010). Conflicting observations about the nature and the number of cells with tumor-propagating capability have been reported in melanoma. In particular, Schatton and co-workers showed that human melanoma-initiating cells (0.000001% of the tumor mass), that are able to produce tumors in immunodeficient mice, represent a biologically distinct population with stem-cell-like properties characterized by the expression of ABCB5, a member of the ABC cassette family of trasporter proteins (Schatton *et al.,* 2008). However, in the same year, the Morrison's group found that modification of the xenotransplantation assays, including the use of more highly immunocompromised mice, dramatically increased the frequency of cells with tumorigenic potential (up to 25% of the tumor mass). Furthermore, they did not observe a substantial difference in the tumorigenic potential between marker-positive and marker-negative cells (Quintana *et al.,* 2008). These last results are in agreement with those of Held *et al.* (2010), showing that tumorigenicity is a common property of melanoma cells in different mouse models. All these findings suggest that melanoma does not probably follow a cancer stem cell model. Conversely a recent publication from Weissman and colleagues, showing a CD271/NGFR/p75$^{+}$ subfraction of human melanoma cells enriched in tumorigenic capacity (Boiko *et al.,* 2010), encourages melanoma biologists to re-evaluate the existence of a melanoma-cell functional hierarchy. Experiments from the Herlyn's lab, reporting the identification of a low frequency sub-population of slow-cycling melanoma cells expressing high levels of JARID1B, showed that JARID1B

expression is dynamic and does not follow a rigid hierarchical model (Roesch *et al.*, 2010). This supports the notion that most melanoma cells have the potential to adopt a transient stem cell fate in response to appropriate signals suggesting the capability of melanoma cells to display cell plasticity.

The reasons for all these contrasting results are not clear. Differences in tissue digestion methods may result in variations in the viability of cells thus affecting the tumorigenic potential. Additionally, as the dermis is composed of much denser connective tissue elements than the subcutaneous tissue, it is conceivable that intradermal injection may inhibit tumor formation by melanoma cells that are tumorigenic after subcutaneous injection. Also, it is possible that the host environment in more highly immunocompromised mice inhibits melanoma cell tumorigenicity in unexpected ways, despite these mice lack B-, T- and NK cell function. These and possibly other methodological differences in the tumorigenesis assays may also contribute to these controversial data.

The field of melanoma stem-like cells is still young and the lack of markers by which to identify CSCs in melanoma makes the task far from being straightforward. In this view, it remains essential to understand the molecular pathways active in these cells aiming at improving the clinical outcome of patients with advanced melanoma.

# MicroRNAs

## 1.4 Signatures of MicroRNAs

MicroRNAs (MiRs) are a family of small non coding RNAs (21-25 nucleotides) that bind to partially complementary sites in the 3' untranslated regions of target genes and regulate gene expression mostly at post-transcriptional level (Calin and Croce, 2006). Interestingly, several miRs have been also reported to recognize sequences present in the 5' untraslated region (5'UTR) (Andersson Ørom *et al.*, 2008) and in the coding sequence (CDS) (Tay *et al.*, 2008).

Complex interconnected networks of different miRs seem to be expressed in different cell types to coordinately regulate cell-specific target genes. Although relatively few miR targets have been experimentally validated, growing evidence indicates that miRs play important roles in major cellular processes (e.g., proliferation and differentiation, apoptosis, angiogenesis). As a consequence, microRNA abnormal expressions appear to contribute to cancer development/progression (Inui *et al.*, 2010).

## 1.5 MicroRNA biogenesis

MicroRNAs are synthesized from a primary transcript (pri-miR). Pri-miRs may have independent transcription units or may originate from an intron of a specific gene. The pri-miR is recognized in the nucleus by the complex composed by the ribonuclease III (RNase III) Drosha and DGCR8 proteins. DGCR8 binds the RNA, while Drosha cuts the pri-miR to produce a precursor (pre-miR) about 70nt long. The resulting pre-miRs are exported into the cytoplasm by the Exportin-5 complex involving RanGTP hydrolysis. When microRNAs are in the cytoplasm, the pre-microRNA processing complex, containing the endonuclease Dicer and TRBP, cuts the pre-miRs to produce a

double strand mature miR about 22 nt long. Only one strand, actually representing the correct mature miR, remains stable on the RISC (RNA-induced silencing complex) playing its role by completely or partially pairing to the 3' untranslated region (3'UTR) of target mRNAs , thus determining their degradation or translation inhibition (Inui *et al.*, 2010) (Fig. 3).

## 1.6 MicroRNA function

Hundreds of microRNA genes have been found in plants and animals. Tissue-specific or organ-specific expression of microRNAs has been detected (Lagos-Quintana *et al.*, 2003; Bartel, 2004), suggesting the miR participation in tissue and organ-specific functions. An example concerns miR-133 and miR-1, that have specific skeletal muscle and cardiac myocytes expression and are downregulated in cardiac hypertrophy (Carè *et al.*, 2007). Comprehensive analysis of microRNA expression is helpful for understanding the complex miR-dependent regulatory effects and necessary for the characterization of microRNAs. The possibility that different microRNAs might target common genes and that multiple mRNAs might be recognized by the same microRNA complicates predictions of how isolated microRNA–target pairings might ultimately affect a biological pathway.

Since their initial discovery, miRNAs have emerged as regulators of essential biological functions, including apoptosis (Brennecke *et al.*, 2003), haematopoietic differentiation (Poy *et al.*, 2004), metabolism (Chen *et al.*, 2004) and skin morphogenesis (Yi *et al.*, 2006). It is estimated that vertebrate genomes encode up to 1,000 unique microRNAs, which are predicted to regulate expression of at least 60% of genes. More than 50% of annotated human microRNA genes are located in fragile chromosomal regions that are susceptible to amplification, deletion, or translocation during the course of tumor

development. On this basis, microRNAs themselves have been primarily implicated in cancer (Croce, 2009) and their expression profiles associated with human malignancies.

# MicroRNAs in Cancer and in Cancer Stem Cells

Recent evidence indicates that some microRNAs can function either as oncogenes or tumor suppressors, and expression profiling analyses have revealed characteristic microRNA signatures in several human tumors (Calin and Croce 2006). It has been reported that microRNAs play a crucial role in the initiation and progression of human cancer. Among a series of potentially interesting modulated microRNAs, we and other groups have focused our attention on miR-221 and -222 demonstrating that these two microRNAs are deeply involved in cancer (le Sage *et al*., 2007; Medina *et al.*, 2008; Felicetti *et al*., 2008). Indeed, they enhance tumorigenicity in non small cell lung cancer (NSCLC) and hepatocarcinoma cells (HCC) by targeting PTEN and TIMP3 tumor suppressors and, in turn, they are activated by c-Met through the AP-1 transcription complex (Garofalo *et al*., 2009). In addition we reported that miR-221 and -222 act on melanoma progression through multiple oncogenic pathways down-regulating p27Kip1 and c-KIT receptor, thus leading to enhanced proliferation and differentiation blockade of melanoma cells (Felicetti *et al*., 2008).

An emerging theme from several different works is that microRNAs may be involved both in maintaining the CSC phenotype and in the invasive and metastatic abilities of the tumor cells (Xia, 2008; Nicoloso *et al.,* 2009). There are accumulating evidences that miRNA-driven pathways are fundamental for cell stemness (Hatfield and Baker, 2008; Mallanna and Rizzino, 2010). Embryonic stem cells are highly enriched with a specific repertoire of miRNAs (Calabrese *et al.,* 2007), which on one hand have power over embryonic stem cell gene regulation and on the other are under the control of self-renewal and pluripotency transcription factors (Marson *et al.,* 2008). Strikingly, the majority of miRNAs, that are important in embryonic stem cells, are also involved in

cell cycle regulation and oncogenesis.

MiRs have been also shown by several groups to modulate cell cycle progression via the G1/S checkpoint in cancer cells. MicroRNA-221/-222 promote cell cycle progression by repressing the cyclin-dependent kinase (CDK) inhibitors p27 and p57 (Felicetti *et al.*, 2008; Galardi *et al.*, 2007; Medina *et al.*, 2008) and a recent work also reported microRNA-92b control of the G1/S checkpoint gene p57 in human Embryonic Stem Cells (Sengupta *et al.*, 2009). All together these data suggest that microRNAs influences the activity of cell cycle regulatory proteins that control competency for cell proliferation and that they are required for cells to bypass the G1/checkpoint, including stem cells.

# MATERIALS AND METHODS

## 2.1 Cell lines culture and transduction

Most of the human melanoma cell lines used in the current study was stabilized from surgical specimens obtained from primary or metastatic tumors at the Istituto Nazionale Tumori in Milan (Italy). Cell lines were characterized for growth in soft agar and, whenever possible, their metastatic potential was evaluated into athymic nude mice. The A375 cell line was from the American Type Tissue Collection (Rockville, MD) and its metastatic variant A375M was kindly provided by Dr. R. Giavazzi (Ist. M. Negri, Bergamo). Table 3 lists the analyzed cell lines. Normal human epidermal melanocytes from the foreskin were obtained from Promocell (Heidelberg, Germany). The ETS-1 cDNA, encompassing its complete coding sequence, and the ETS-1 transdominant negative mutant TM were cloned into the retroviral Pinco and into the lentiviral Tween vectors, respectively (Pourtier-Manzanedo *et al.*, 2003, Nakano *et al.*, 2000). Overexpression of miR-221 and -222 was obtained in melanoma cells by using a lentiviral vector system, as reported (Felicetti *et al.*, 2008). "Controls" are always intended as empty vector-transduced cell lines. ETS-1 was specifically silenced by using a validated small interfering RNA (IDT). Briefly, 24 h after plating, cells were transfected using Lipofectamine 2000 (Invitrogen) either with DsiETS-1 (HSC.RNAI.N001143820.10.1) or with a DsiRNA scrambled control (f.c. 200 nM). The level of ETS-1 mRNA was analyzed 48 h after transfection by qReal-time PCR (Applied Biosystem assay#Hs00428287) and the functional properties of the cells evaluated up to day 5.

### 2.2 Isolation and culture of melanoma cancer spheres

Tumors samples were obtained in accordance with consent procedures approved by the Internal Review Board of Department of Laboratory Medicine and Pathology, Sant'Andrea Hospital, University La Sapienza, Rome. Surgical specimens were washed several times and left overnight in DMEM–F12 medium supplemented with high doses of penicillin/streptomycin and amphotericin B to avoid contamination. Tissue dissociation was carried out by enzymatic digestion (1.5 mg/ml collagenase II, Gibco-Invitrogen, Carlsbad, CA) for 2 h at 37°C. Recovered cells were cultured at clonal in serum-free medium containing 50 μg/ml insulin, 100 μg/ml apo-transferrin, 10 μg/ml putrescine, 0.03μM sodium selenite, 2 μM progesterone, 0.6% glucose, 5mM HEPES, 0.1% sodium bicarbonate, 0.4% BSA, glutamine and antibiotics, dissolved in DMEM–F12 medium (Gibco-Invitrogen) and supplemented with 20 ng/ml EGF and 10 ng /ml bFGF. Flasks non-treated for tissue culture were used to reduce cell adherence and support growth as undifferentiated tumor spheres. The medium was replaced or supplemented with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures were expanded by mechanical dissociation of spheres, followed by re-plating of both single cells and residual small aggregates in complete fresh medium.

### 2.3 Differentiation of stem cell progeny

To obtain differentiation of melanoma cancer sphere-forming cells, stem cell medium was replaced with Melanocyte Growth Medium (Promocell) in tissue culture-treated flasks, to allow cell attachment and differentiation. The acquisition of differentiation markers and loss of stem cell markers were evaluated by flow cytometry.

### 2.4 QReal Time RT-PCR

In order to accurately detect mature miR-181b, miR-21, miR-99b, miR-221 and -222, a Real time quantification method was performed according to the TaqMan MicroRNA Assays from Applied Biosystems (miR-181b #4373116; miR21 #4373090; miR-99b #4373007; miR-221 #4373077; miR-222 #4373076). Samples were normalized by evaluating RNU6 expression (#4373381).

### 2.5 Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed according to standard procedures. The sequences of primers and the annealing conditions were:

- ETS-1 dir 5'-ACTTACCCCTCGGTCATTCTCCG-3' and rev 5'-CCACCTCATCTGGGTCAGAAAG-3' and the annealing temperature was 58° C;
- ABCB5 dir 5'-ATGTACAGTGGCTCCGTTCC-3' and rev 5'-ACACGGCTGTTGTCA CCA TA -3' and the annealing temperature was 54° C;
- GAPDH dir 5'-ATGTACAGTGGCTCCGTTCC-3' and rev 5'-ACACGGCTGTTGTCACCA TA -3' and the annealing temperature was 56° C;

When required, the amplified fragments were hybridized to an internal 30 base end-labelled oligonucleotide according to Southern blot standard procedures.

### 2.6 Western blot

Western blot was performed according to standard procedures. Cell lysates were separated by the pre-cast NuPAGE polyacrilamide gel system (Invitrogen). Antibodies against c-ETS-1 (anti-rabbit from Santa Cruz Biotechnology and anti-mouse from Novocastra) and c-JUN (Santa Cruz Biotechnology) were used in accordance to the manufacturer's instructions. ACTIN (Oncogene Research) was used as a loading control and for subsequent quantification. The expression levels were evaluated by the Scion Image Software (www.scioncorp.com).

### 2.7 Flow cytometric analysis.

For flow cytometry, tumor spheres were dissociated as single cells, washed and incubated with the appropriate dilution of control or specific antibody. Antibodies used were anti-CD133 (Miltenyi), anti-CD20 (Dako) anti-CD24 (Dako), anti-CD146 (BD), anti-CD90 (BD), anti-ABCG2 (Chemicon), anti-CD44 (Dako) or anti-CD44v6 (R&D). After 45 min incubation, cells were washed or, where necessary, incubated with FITC- or PE-conjugated secondary antibodies for 30 min and washed again before analysis using an LSRII flow cytometer (Becton Dickinson).

### 2.8 Target analysis

Bioinformatic analysis was performed by using these specific programs:

TargetScan (http://www.targETScan.org/), PicTar (http://pictar.bio.nyu.edu/) and RNAhybrid (http://bibiservice.techfak.uni-bielefeld.de/).

**2.9 pGL-3'UTR plasmid**

For luciferase reporter experiments, on the basis of bioinformatic analyses, we identified one conserved putative binding site predicted to interact with miR-221 and -222 in the ETS-1 3'UTR. This region was amplified by PCR (AccuPrime) from normal human genomic DNA using a Taq DNA polymerase high fidelity (Invitrogen). After sequence analysis, the construct was subcloned into the pGL3 promoter vector (Promega), immediately downstream from the stop codon of luciferase gene.

The putative ETS-1 seed (nt 3403) and its mutated version were 5'TTAGAG**ATGTAGC**GATGTA 3' and 5**'**TTAcAG**c TGccaC**GATGTA 3', respectively. The conserved core is indicated in bold while lower case letters represent the mutated nucleotides. 293FT cells ($5x10^4$ cells per well) were transfected with: (a) 20 ng of pGL3-3' UTR plasmid, (b) 15 pmol of either a stability-enhanced 2'-O-Methyl non targeting RNA control or miR-221 and/or miR-222 oligonucleotides (Dharmacon Inc.), (c) Lipofectamine 2000 (Invitrogen) and (d) 50 ng of Renilla. At 48 h cells were lysed and their luciferase activity measured by using the FemtomasterFB 12 (Zylux). Ratios between Firefly and Renilla luciferase activities were measured with a dual luciferase assay (Promega). The wt pGL3-3'UTR cotransfected with the control non targeting oligonucleotide was considered as 100%.

**2.10 Promoter Luciferase Assay**

In order to analyze the functional roles of the two putative ETS binding sites (indicated as BS1 and BS2 in Figure 10A), a DNA fragment containing the putative regulatory region upstream to miR-222/-221 (from -555 to +1nt) was amplified and cloned in pGL3 basic (Promega). Moreover a shorter construct (from -400 to +1 nt) containing the sole BS1 was also made. 293FT cell line was transfected with Lipofectamine 2000

(Invitrogen) and: (a) 200 ng of pGL3 basic or pGL3 containing the above genomic fragments, (b) 150 ng of empty or ETS-1-expressing plasmid and (c) 30 ng of Renilla. At 48 h cells were lysed and their luciferase activity measured by using the FemtomasterFB 12 (Zylux). The wt pGL3 plasmids cotransfected with the control vector were considered as 100%. As controls of specificity, point mutations were inserted in the wild type core binding sequence for ETS-1 by using the QuickChange site-directed mutagenesis kit (Stratagene). Mutated nucleotides are shown in lower case letters. BS1: 5'-TGCTGC**gaGc**TCTCCAG -3'; BS2: 5'-TGACt**GcAg**GCAACA -3'.

**2.11 Chromatin Immunoprecipitation (ChIP) Assay**

Five x$10^6$ cells from A375M melanoma cell line were fixed in 1% formaldehyde for 10 minutes at room temperature. Cells were washed with ice cold 1xPBS, scraped in 1xPBS plus protease inhibitors and collected by centrifugation. Cell pellets, resuspended in cell lysis buffer (50 mM Tris-HCl pH 8.0, 10mM EDTA, 1% SDS) plus protease inhibitors, were then sonicated. DNA-protein complexes were immunoprecipitated using 3 µg of anti-ETS-1 or, as an internal control, the unrelated anti-DVL-1 (Santa Cruz). DNA-protein crosslinks were reversed by heating at 65°C overnight. The recovered DNAs were then PCR-amplified with the following primer set: DIR(+47) 5'-GGATCTACACTGGCTACTGAG-3' and REV(-262) 5'-GTCACAAGGAATCATGTATGC-3', corresponding to ETS- binding site at -203 (BS1); DIR(-260) 5'-CAGCATACATGATTCCTTGTGA-3' and REV(-514) 5'-CTTTGGTGTTTGAGATGTTTGG-3', ETS-1 binding site at -407 (BS2). Control amplification was carried out on input chromatin (preserved before immunoprecipitation) and on DVL-1 (mock)-immunoprecipitated chromatin. To

confirm the specificity of the immunoprecipitated products, GAPDH PCR were also run.

### 2.12 Immunohistochemistry on tumor sections

Immunohistochemistry was performed on formalin-fixed paraffin-embedded or frozen tissue. Paraffin sections (5 mm) were dewaxed in xylene and rehydrated with distilled water.

Sections were treated with heat-induced epitope retrieval technique using a citrate buffer (pH 6). After peroxidase inhibition with 3% H2O2 for 20 min, the slides were incubated with the following antibodies: anti-MART1 (Dako) and anti S100 (Dako). The reaction was performed using Elite Vector Stain ABC systems (Vector Laboratories) and DAB substrate chromogen (DakoCytomation), followed by counterstaining with haematoxylin.

### 2.13 *In vitro* growth assay

Spheres were plated at 10000 cells/ml in growth medium supplemented with growth factors and after mechanical dissociation of culture aliquots, single cells were counted by Trypan blue exclusion once a week. Adherent differentiated cells were plated in six-well plates (10000 cells/well) and one well every week was used for cell count. To determine their self-renewal ability, melanoma cancer cells were seeded in 96-well plates containing a single cell per well. Shortly after seeding, single cell-containing wells were identified and analyzed for the ability to generate long-term-growing secondary spheres whose expansion was stable for more than 5 months.

The proliferative rate of melanoma cell lines was evaluated by an XTT-based colorimetric assay (Roche Molecular Biochemicals). Cells, grown for different times in

a 96-well tissue culture plate, were incubated with the XTT solution for 2 h. After this incubation period, orange formazan solution is formed, which is spectrophotometrically quantified using an ELISA plate reader (VICTOR2, Wallac).

### 2.14 *In vitro* invasion assay

Invasion was assayed, as previously described (Felicetti *et al.,* 2008), using cell culture inserts (Corning Costar Corporation) with 8μm pores coated with 100μg/cm$^2$ of Matrigel growth factor reduced (Becton Dickinson) as a barrier. Cells ($10^5$) were placed in the upper compartment in 100 μl of DMEM serum-free, while 600 μl of DMEM supplemented with 10% FBS were placed into the lower compartment of the chamber. Assays were incubated at 37° C in 5% CO2. After 24 or 48h the cells attached to the upper side of the membrane were removed with a cotton swab; each membrane was fixed and stained with crystal violet solution. Invasiveness was evaluated, as relative number of cells on the undersurface of the membrane, by a colorimetric assay at 595 nm in a microplate reader (VICTOR2, Wallac). The data were expressed as the mean absorbance ± SE for triplicate wells.

### 2.15 Growth in semisolid medium

Base layers of complete DME medium containing 0.5% agar were set in 60 mm plastic dishes. The bottom agar was overlaid with 1.5 ml of 0.33% agar containing suspensions ranging from $10^3$ to $10^4$ cells. Cultures were incubated for 3-4 weeks at 37°C and the colonies counted using an inverted microscope. Two experiments were performed for each cell line and results were calculated as the average ± SE of three dishes for each condition.

### 2.16 *In vivo* assay

For the *in vivo* assays, empty vector- or TM-transduced A375M cells in exponential growth phase were injected s.c. and i.v at doses of $10^6$ cells into adult athymic nude mice (Charles River, Calco, Italy) pretreated with rat anti-mouse mAb to IL-2Rb (TMb1, kind gift from Daniela Mannel, Regensburg, Germany) to deplete NK cells. Tumor growths were monitored twice a week. Six wks after the injection, mice were sacrificed for necropsy, and lung metastases were counted under a stereomicroscope after staining with India ink. All the in vivo experiments were performed according to institutional guidelines. In some of them performed in collaboration with Dr. MP Colombo, mice were treated and maintained at the Istituto Nazionale Tumori (Milan, Italy).

### 2.17 Generation of subcutaneous melanoma cancer xenografts into *nude* mice

For mice xenografts, cells were mechanically dissociated to obtain single cell suspensions, diluted in growth factor-containing medium alone or mixed with matrigel before subcutaneous injection. Similar results were obtained in both cell culture conditions. Serial dilutions of cells (down to as low as $5x10^2$ cells) were injected to evaluate the tumorigenic activity of melanoma cancer cells. Mice were monitored to check for the appearance of signs of disease, such as subcutaneous tumors or weight loss due to potential tumor growth at internal sites. When tumor volume reached at least 1.2 $cm^3$, mice were killed and tumor tissues collected, fixed in buffered formalin and subsequently analyzed by immunohistochemistry. Hematoxylin and eosin staining followed by immunohistochemical analysis were performed to analyze tumor histology and compare mouse xenografts with patient tumors.

**2.18 Statistical analysis.** Statistical and frequency distribution analysis was performed by Excel. Data represent mean values and error bars indicate standard deviations. Differences between two or three groups were compared with Student's t-test. A value of $p<0.05$ or less was considered statistically significant.

# RESULTS

## 3.1 Melanoma contains stem-like cells

During the last few years CSCs have been isolated from solid tumors of different origins, including colon cancer and glioblastoma, in our Department (Eramo *et al.*, 2006; Ricci-Vitiani *et al.*, 2007).

We have then searched for the purification and characterization of putative melanoma stem cells. Undifferentiated melanoma stem-like cells were capable of surviving and proliferating in the appropriate medium and grew as floating non adherent "melanoma spheres" (Mel SC). All the established stem-like cell lines are listed in table 1.

## 3.2 Evaluation of the percentage of spheres with unlimited growth potential

Mel SC activity was evaluated through their potential to prove both self-renewal and tumor propagation. To determine the percentage of putative cancer stem cells in melanoma spheres, we evaluated the ability of single cells to auto-replicate and generate new spheres endowed with unlimited growth potential in secondary cultures. To ensure the purity of cell populations, we separated melanoma spheres into single cells and reseeded them at a clonal density obtaining new spheres from individual seeded cells. These melanoma stem-like cells appear to be limitless expanded and maintained in culture as tumor spheres containing a variable, but considerable percentage of tumorigenic cells. Among our stem-like cell lines (n= 5), we found a variety in the number of self renewing cells ranging from 50% of Mel 1 to 35% of Mel 4 spheres (Fig.4A and not shown).

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### 3.3 Melanoma spheres are tumorigenic in vivo and reproduce the human tumor

We then evaluated the tumorigenic potential of melanoma spheres *in vivo* through subcutaneous cell injections into athymic nude mice.

The injection of as low as $5x10^2$ cells consistently resulted in the growth of tumor xenografts (Fig.4B) with morphological features closely resembling the original tumor, as shown by haematoxylin/eosin staining (Fig.4C).

### 3.4 Melanoma spheres generate a differentiated progeny with phenotypic features of melanoma cancer cells

The *in vitro* differentiation potential of melanoma cancer spheres was then analyzed. In the presence of serum or in melanocyte-specific medium, melanoma cancer spheres adhered to the plastic acquiring the typical morphologic features of differentiated cells (Mel PC) (Fig.5A). Furthermore, upon differentiation, melanoma spheres cells showed a decreased expression of BMI-1, a critical promoter of stem cell self-renewal (Park *et al*., 2003); western blot analysis of Mel SC compared with Mel PC confirmed a direct correlation between BMI-1 expression and the stemness potential, with lower BMI-1 levels in the more differentiated progeny (Fig. 5B).

Trying to substantiate that melanoma is initiated by a population of stem-like cells, we compared the growth potential of undifferentiated and differentiating cells. While melanoma spheres displayed a stable exponential growth, *in vitro* differentiating tumor cells were able to proliferate for about 2 weeks before declining in number, thus suggesting that the high proliferation potential of melanoma-stem like cells was lost during differentiation (data not shown). To rule out the possibility that such a limited in vitro growth resulted from unfavourable culture conditions, we compared the tumorigenic potential of undifferentiated and differentiated cells into athymic nude

mice. We found that the subcutaneous injection of $5x10^4$ undifferentiated cells in nude mice produced exponentially growing tumor xenografts detectable as early as 3 wk after injection. On the contrary, the same number of more differentiated cells gave rise to barely palpable nodules up to 15 wk (Fig.5C).

**3.5 Surface markers expression profile of melanoma stem-like cells**

Cells cultured in stem cell medium were characterized by flow cytometric analysis and RT-PCR. Unexpectedly, melanoma stem-like cells were found not to express CD133, CD20 and ABCG2 surface markers, already reported as cancer stem cells markers (Clark *et al.*, 1984; Fang *et al*., 2005; Monzani *et al*., 2007; Schatton *et al*., 2008). Conversely they were positive for melanocytic markers such as Mart1, Tyr and HMB45 and for CD44, CD44v6 and C-KIT (Table 2 and Fig.5D). Interestingly, whereas flow cytometric analysis of total CD44 revealed a high (~100%), not modulated, level in Mel SC compared with Mel PC (not shown), the expression of the CD44v6-containing isoforms, known to be associated with tumor progression and metastasis in many types of cancer, was detectable in undifferentiated stem-like cells, but not in normal melanocytes and in differentiated adherent cells (not shown). Looking for CD44v6 as a putative stem-cell marker, we decided to isolate the CD44v6 positive and negative subsets by fluorescence-activated cell sorting (FACS). However, 14 days after sorting, the CD44v6-negative cells gave rise to a heterogeneous progeny which included CD44v6-positive cells. We therefore applied to investigate the influence of CD44v6 knockdown on the *in vivo* tumor growth. Results showed that CD44v6 knocked-down cells produced slowly growing tumors respect to CD44v6-expressing cells. On the basis of this study we argued that CD44v6 does not appear as a good marker to identify the cancer stem cell population within a melanoma. Therefore the identification of more

specific and less dynamic biological markers is required to selectively and properly isolate melanoma stem-like cells, excluding the more differentiated progeny.

### 3.6 MicroRNA expression in melanoma stem like-cells: microRNA-221/-222

Growing evidences indicate microRNAs as important regulators of the main biological processes, including cancer, where they can act as tumor suppressors or oncogenes (Calin and Croce, 2006). Here we searched for any possible difference in miRNAs expression which might distinguish melanoma spheres from their more differentiated progeny. Total RNA, enriched for low molecular weights, was isolated according to standard procedures and microRNA profiling performed. By comparing the expression profiles we found a differential miRNAs expression in melanoma stem-like cells respect to differentiated cells. Five selected miRs (Fig.6A,B) were then analyzed by qReal Time PCR (Fig.6C,D) in order to validate the overall results obtained through microRNA expression arrays (not shown). Among them microRNA-221/-222 had the most consistent and significant increase of expression during differentiation (Fig.6D). According to this up-regulation in differentiated melanoma stem-like cells, we searched for the involvement of miR-221/-222 as at least one of the cause underlying proliferation of the differentiated progeny. We therefore focused our attention on the cell cycle inhibitor p27Kip/CDKN1B, already reported as a target of miR-221 and miR-222 in the context of melanoma, thyroid papillary carcinoma, glioblastoma and prostate carcinoma (Felicetti *et al*., 2008; Visone *et al*., 2007; Gillies and Lorimer, 2007, Galardi *et al.*, 2007). As possibly expected, the analysis of p27Kip protein levels in melanoma stem-like cells versus their more differentiated progeny, confirmed p27Kip decrease during the differention process (Fig.7A). In this view it is important to consider the incompleteness of differentiation in these melanoma cells, characterized by the lack of

terminal maturation.

Accordingly, microRNA-221/-222 were also reported to target c-KIT receptor in normal human erythropoietic (Felli *et al.*, 2005), endothelial (Poliseno *et al.*, 2006) and melanoma cells (Felicetti *et al.*, 2008). Flow cytometric analysis showed c-KIT expression higher in melanoma stem like cells compared with the more differentiated cancer cells, confirming the inverse correlation between these two miRs and c-KIT (Fig. 7B).

### 3.7 ETS-1 expression in melanoma stem like-cells and melanoma cell lines

Looking for new miR-221/-222-dependent target genes and basing on bioinformatics analyses (TargetScan, RNAHybrid), we found the presence of one conserved binding site in the 3'UTR of the ETS-1 transcription factor.

ETS-1 is a transcription factor that plays an important role in various physiological and pathological processes, such as development, angiogenesis, apoptosis and tumor invasion (Ditmer *et al.*, 2003). The proto-oncogene ETS-1 has been associated with tumor progression in various carcinomas, but its role in melanoma is still controversial. We therefore decided to examine ETS-1 expression in a panel of Mel SC (Table 1): interestingly, by western blot analysis, we found ETS-1 protein expression higher in Mel SC respect to differentiating Mel PC (Fig.7C). This result confirmed the inverse correlation with both miR-221 and miR-222 (Fig.7D).

In order to better define its expression and functional role, we evaluated ETS-1 level also in a panel of melanoma cell lines at different stages of progression, including primary vertical growth phase (VGP) melanomas, as well as subcutaneous and lymph-node metastases (Table 3). Traditional and qRT-PCR as well as western blot analyses showed that all these melanoma cell lines expressed ETS-1 and that its level was

inversely related to melanoma malignancy. In particular metastatic and primary melanomas expressed low and high amount of ETS-1 at mRNA (Fig.8A) and protein levels (Fig.8B), respectively. To rule out any possible artifactual result due to in vitro cell culture, this expression pattern was confirmed in early passages cells obtained from melanoma bioptic samples (Fig.8C). Of note a high level of ETS-1 mRNA and protein was detected in normal human melanocytes (Fig.8A,B). These experiments revealed that both miR-221 and miR-222 expressions are inversely correlated with ETS-1 nuclear level in melanoma progression (Fig.8D).

**3.8 ETS-1 level is down- regulated by miR-222**

Based on the inverse correlation between ETS-1 and miR-221/-222 expression patterns (Fig.7D, 8D), we decided to test whether miR-221 and -222 directly target ETS-1 using a luciferase assay. The ETS-1 3'UTR encompassing the seed sequence (recognized by miR-221 and -222) was cloned downstream to the luciferase open reading frame in a modified pGL3 promoter vector and cotransfection experiments were performed in the 293FT cell line. As shown in Fig. 9A, transfections of miR-222 in presence of ETS-1 3'UTR induced a significant decrease of luciferase activity (roughly 50% less than non targeted control sequence). Surprisingly, negligible was the effect induced by miR-221 co-transfection, suggesting that the coordinated function reported for miR-221/-222 in the regulation of p27Kip, c-Kit (Felicetti *et al*., 2008) and other target genes (Garofalo *et al*., 2009) can be uncoupled. As control, cotransfection of ETS-1 3'UTR with a non-targeting sequence as well as of ETS-1 mutated 3'UTR with miR-221/-222 did not show any repression in luciferase activity confirming the specificity of such interactions (Fig. 9A). We avoided the use of the whole 3.6 Kb 3'UTR in the transfection assays because this sequence is, per se, very unstable due to the presence of a series of AU rich

repeats (Nakano *et al*., 2000; Pourtier-Manzanedo *et al*., 2003). ETS-1 downregulation was confirmed at both mRNA and protein levels in miR-222 transduced Me1402/R melanoma cell line (Felicetti *et al*., 2008). Also, in agreement with luciferase results, overexpression of miR-221 was without significant effect on ETS-1. These data indicate that miR-222 is a true regulator of ETS-1 expression (Fig. 9B).

**3.9 ETS-1 directly regulates miR-222 transcription**

Considering the possibility of a miR222-ETS-1 loop, we looked for ETS-1 binding sites (EBS) into the ~0.6 Kb sequence upstream to pre-miR-222/-221 (MatInspector software- http://www.genomatix.de) (Fig.10A). To test whether such putative EBS were truly functional, we performed a series of promoter luciferase assays. The sequence from -555 to +1 nt (containing -203 and -407 ETS-1 BS, named BS1 and BS2, respectively) as well as the shorter fragment from -400 to +1 nt (containing -203 ETS-1 BS1) were cloned in a promoter-less pGL vector and co-transfected with empty or ETS-1 containing vectors into 293FT cells (Fig.10B). In the 293FT cells, a 30-45% ETS-1-dependent reduction of the luciferase activity was likely due to ETS-1 binding to the miR-222 regulatory sequences. Accordingly, the introduction of point mutations in each core-binding site restored the luciferase levels (Fig.10B left) either totally or partially when mutations were into the BS1 (-203) or BS2 (-407) sites, respectively. This suggests a different role/hierarchy for these two binding sites and/or the presence of interacting activators in the region -555/-400 (Fig.10B left). To close the loop, ETS-1 should regulate miR-222 and perhaps-221. To test that, 293FT cells were transiently transfected with the ETS-1 expressing vector (Fig.10C left) and the level of both endogenous miR-221 and -222 evaluated by qReal-time PCR at early as well as late time points (from 4 to 48 h) (Fig.10C right). A significant stepwise decrease of miR-222

followed ETS-1 up-regulation. On the contrary, no clear modulation of miR-221 was observed confirming the fidelity of the miR-222-ETS-1 liaison (Fig. 10C right).

To confirm that ETS-1 nuclear protein binds directly BS1 and BS2 upstream to miR-221/-222, we performed a ChIP assay in the A375M metastatic melanoma cell line. PCR amplifications of unsheared input genomic DNA and anti-ETS-1 antibody-mediated reaction gave PCR products of the expected sizes, whereas the same reactions, immunoprecipitated with an irrelevant antibody, did not show any detectable amplified product (Fig.10D).

### 3.10 ETS-1 functional role

To assess the functional significance of ETS-1 in melanoma cells, we investigated the effects of either a stable trans-dominant negative form (TM) or a transient specific Dsi-ETS-1. The TM truncated form of ETS-1 contains only its binding domain and has been already shown to compete with the wild type protein for the DNA binding sites and the underlying functions (Fig. 11A, left) (Pourtier-Manzanedo *et al.*, 2003, Nakano *et al*., 2000). Six separate gene transductions (named from TM1 to TM6) were performed in the metastatic melanoma A375M, and the A375M/TM4 melanoma cell line, selected for the highest amount of the truncated protein, was successively utilized in most of the functional analyses (Fig. 11A, right). In vitro biological assays showed that TM-transduced cells proliferate faster (20-30% increase) than empty vector-transduced counterpart (not shown) and that they increased the invasive capacity, evaluated by a Boyden chamber assay, of 2.5-fold (Fig. 11B left). The invasive activity was dependent on the amount of TM expression as shown comparing high and low expressing A375M/TM4 and /TM5 cells, respectively (Fig.11B left). Accordingly, a significant enhancement (of approx. 3-fold) of melanoma capacities of forming foci in agar

semisolid medium was also observed in A375M/TM4 cells compared to vector-transduced cells (Fig. 11B middle).

Finally, the behavior of control Tween- and TM4-transduced A375M cells were tested in vivo following subcutaneous or intravenous injection into NK-depleted athymic nude mice. No size differences were observed in s.c. nodules despite the presence of the truncated TM protein was confirmed by western blot (not shown). Differently, i.v. injection generated a significant difference in lung metastasis between TM4- and Tween-transduced cells. Moreover, only TM4 cells showed abdominal metastasis dissemination to liver and kidney. (Fig.11B right and not shown).

As a final point, we considered the possibility that this ETS-dominant negative protein might interfere with other members of the ETS family, although in our melanoma samples we did not observe any significant modulation of ETS-2, the closest member to ETS-1 (data not shown). To confirm its specificity, ETS-1 expression was repressed using a DsiRNA. Silenced A375M melanoma cells showed 75% downregulation of ETS-1 by qRT-PCR (Fig. 12A left). The data confirmed the main role for ETS-1 among all the ETS family genes, possibly targeted by the TM dominant negative, although the effect of ETS-1 knockdown on cellular invasion (Fig. 12A right) was lower than that of TM (Fig.11B left). This result might possibly derive from the contribution of other ETS family members or from the presence of the residual 25% of untransfected cells. Furthermore, the abrogation of ETS-1 mRNA and even more the expression of ETS-TM dominant negative were paralleled by a significant increase of miR-222, suggesting that both the DsiETS and the TM protein were able to unblock miR-222 regulatory region by abrogating or competing with the ETS-1 (Fig.12B).

### 3.11 c-JUN expression in melanoma cell line and melanoma stem-like cells

ETS-1 protein was reported to transcriptionally repress c-JUN in an in vitro cellular system (Goldberg *et al.,* 1994). In melanoma c-JUN is known to be affected by the constitutively active MAPK signaling (Lopez-Bergami *et al.*, 2010) and was recently reported to bind and transactivate miR-221/-222 (Garofalo *et al*., 2009). Considering that ETS-1 silencing in A375M melanoma cells increased miR-221/-222 expression (Fig. 12B), we analyzed the expression level of c- JUN in the same cells looking for its up-regulation. As expected, in view of a possible ETS-1-c-JUN-miR-222 regulatory way, we found an increase of c-JUN protein (Fig. 13A). To substantiate the inverse correlation between ETS-1 and c-JUN, we examine by western blotting c-JUN expression in a representative Mel SC founding c-JUN expression lower in Mel SC respect to differentiating Mel PC (Fig.13B). These data confirm an ETS-1→c-JUN→miR-221/-222 signal transduction pathway in melanoma (Fig.13C).

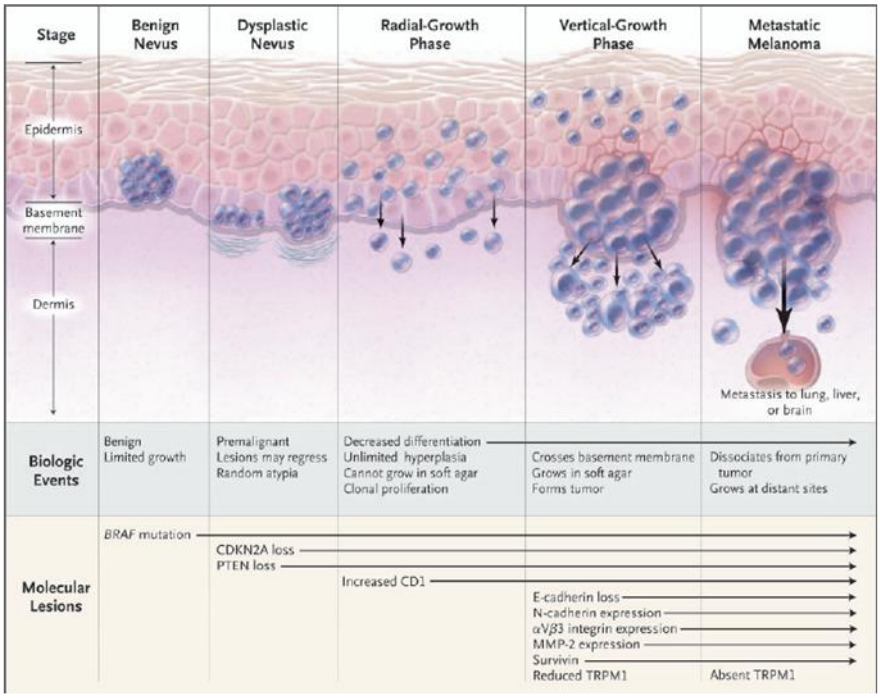


**Figure 1. Biologic Events and Molecular Changes in the Progression of Melanoma.**
Arlo J. Miller, N Engl J Med 2006

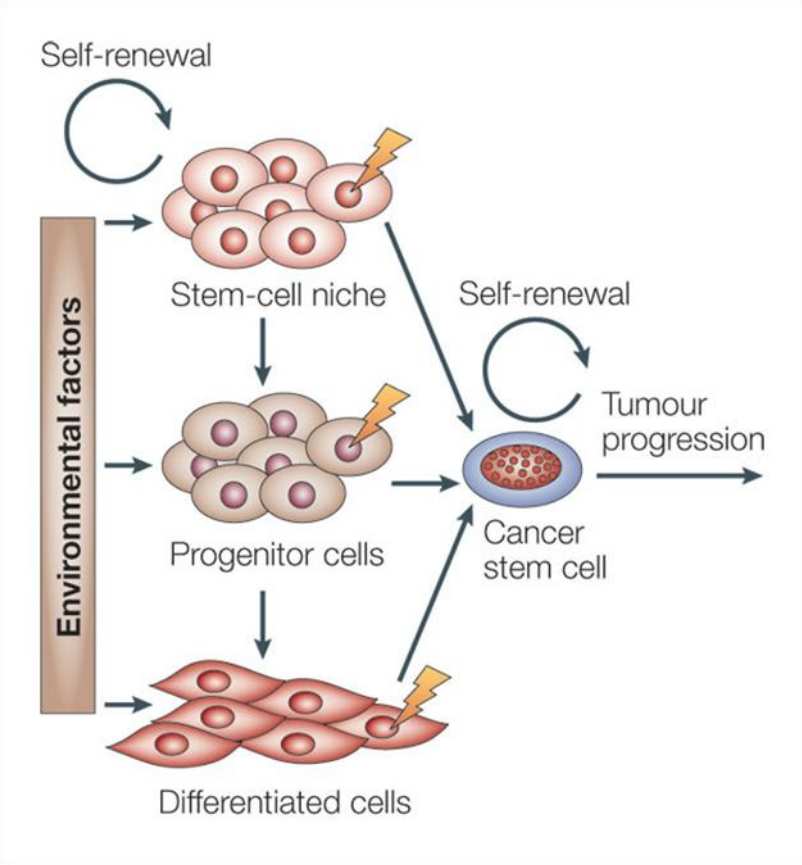


**Figure 2. Mutations in stem cells and/orprogenitor cells might give rise to cancer stem cells.** Bjerkvig R, Nat Rev Cancer 2005

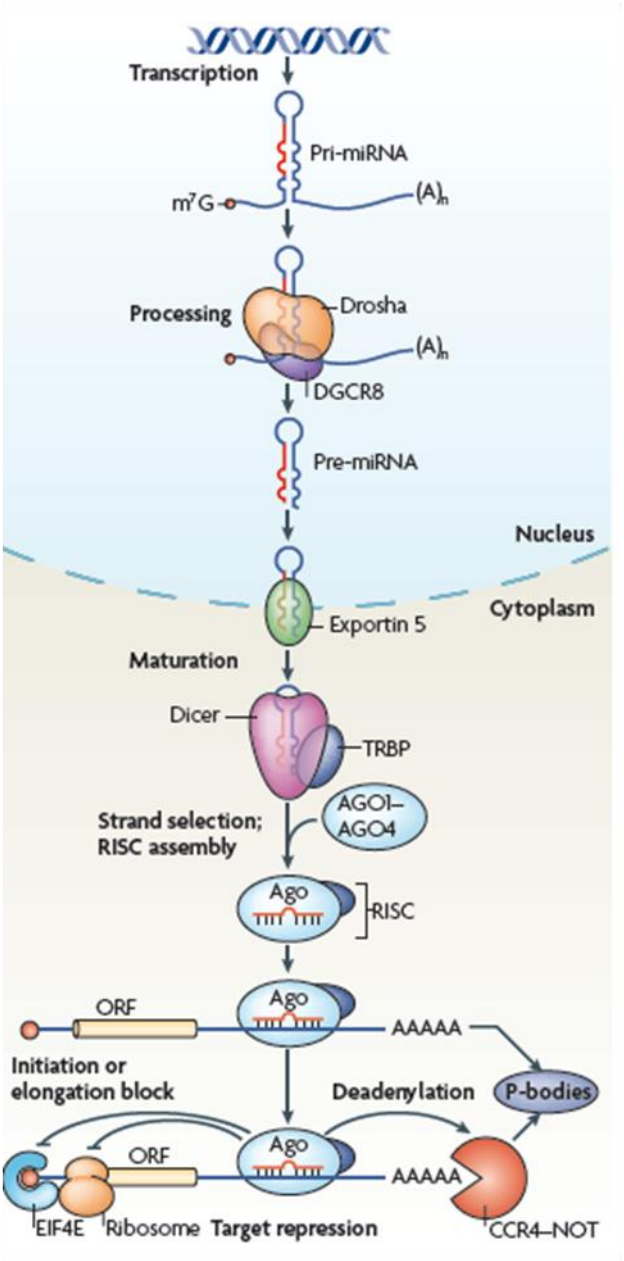


**Figure 3. RNA biogenesis and mechanisms of action.** Inui M, Mol Cell Biol 2010

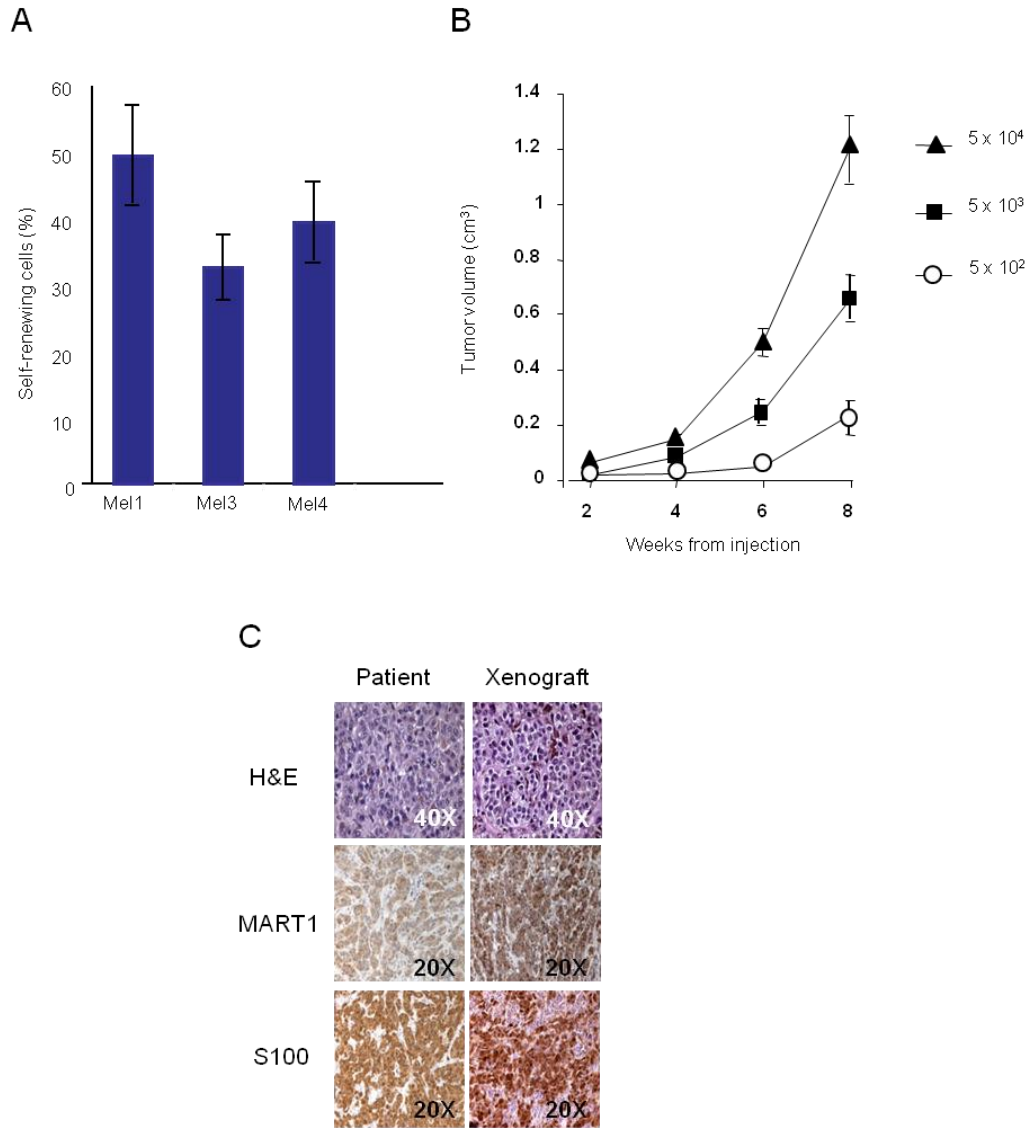


**Figure 4** **A**: Evaluation of the percentage of secondary spheres with unlimited growth potential. **B**: In vivo growth of one representative melanoma stem like-cell line. **C**: Histology panels represent H&E staining and immunohistochemical analysis of MART1 and S100 of xenografted tumors to confirm a melanoma phenotype.

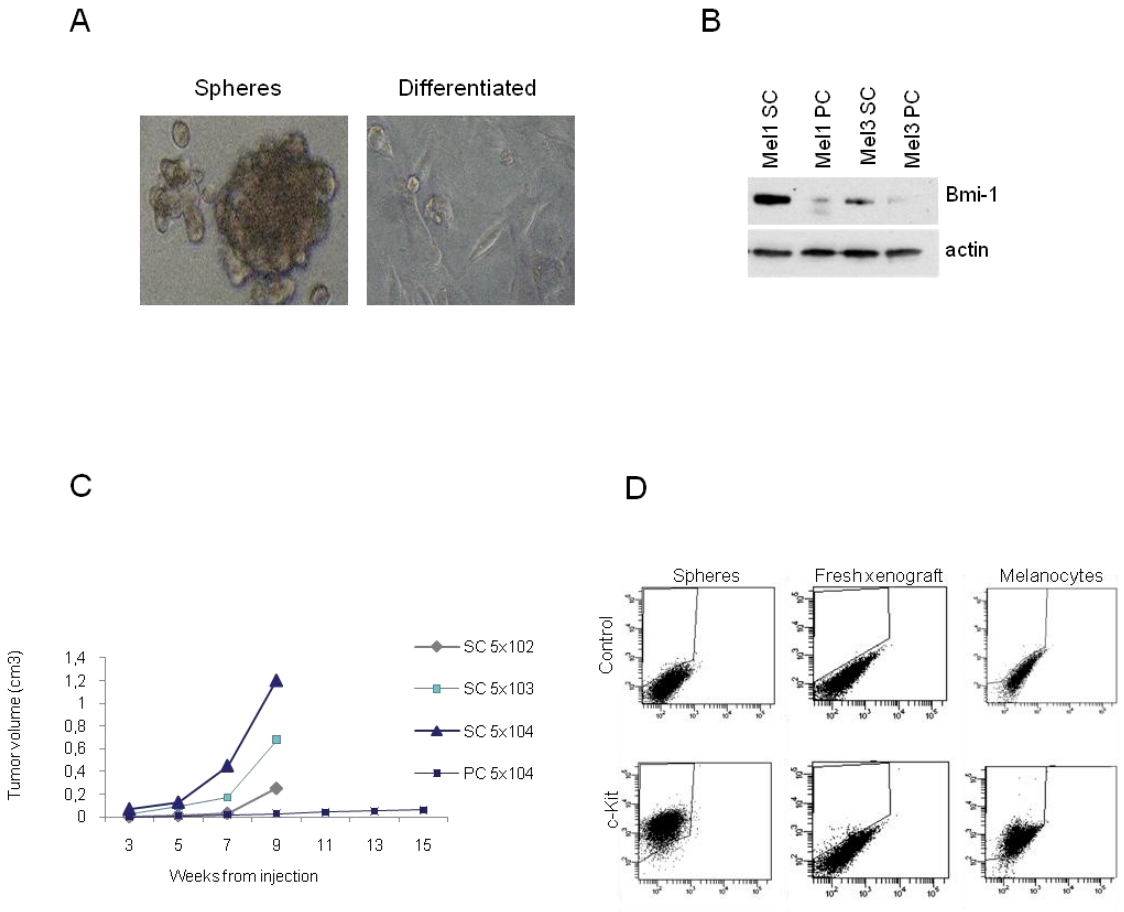


**Figure 5. A**: In vitro differentiation potential of melanoma spheres. **B**: Western Blotting of BMI-1 in melanoma stem like-cells (SC) in comparison with their differentiated progeny (PC). Actin is the internal control. **C**: In vivo growth of Mel1 SC vs Mel1 PC. **D**:Flow cytometry analysis of c-KIT in one representative case of control melanoma tissue, in one cases of freshly dissociated melanoma cancer sample and in normal human melanocytes.

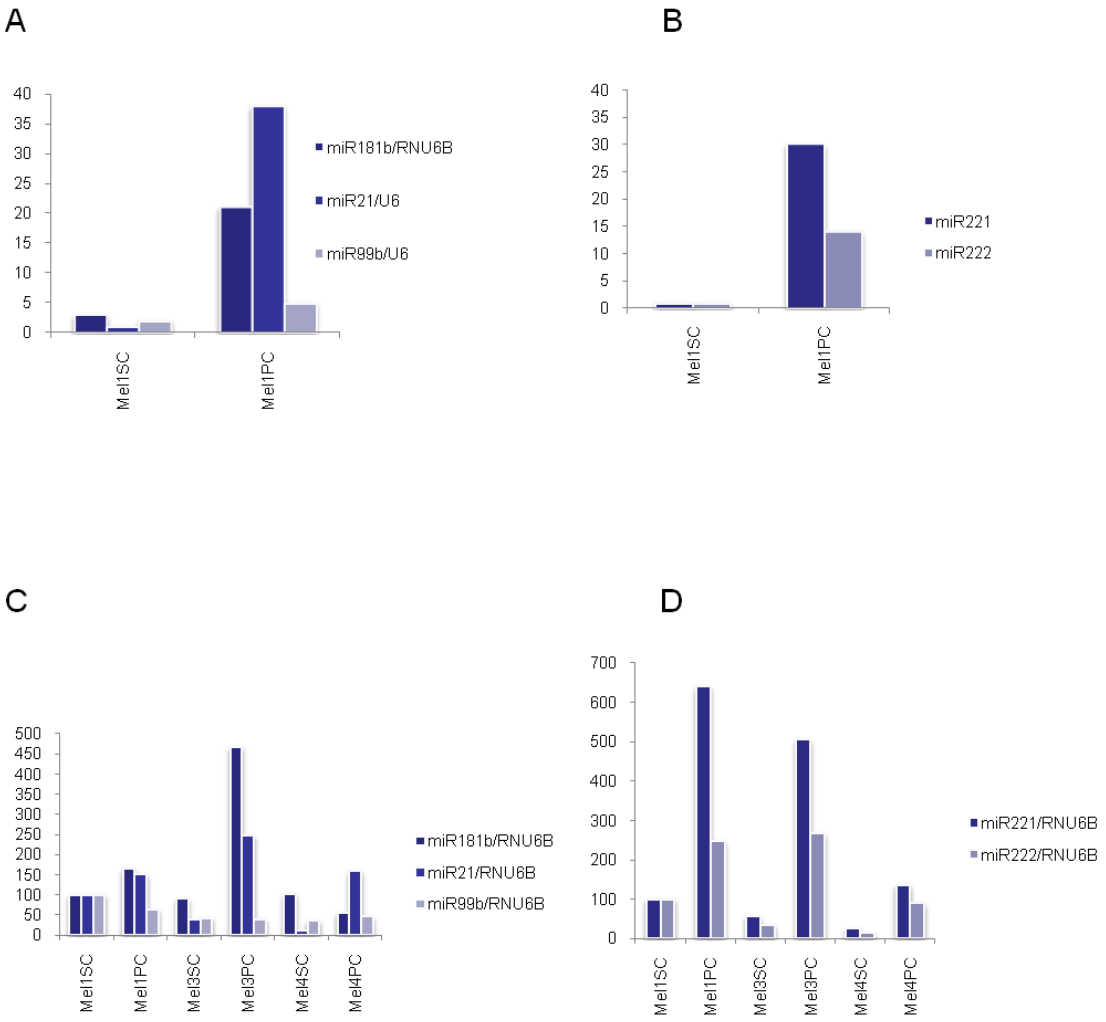


**Figure 6 A**: Array data showing microRNA-181b/-21/-99b expression in Mel1 SC in comparison with Mel1 PC. **B**: Array data showing microRNA-221/-222 expression in Mel1 SC in comparison with Mel1 PC. **C,D**: qRT-PCR analyses in Mel1 Mel 3 and Mel 4 melanoma stem-like cell lines (SC) and in their more differentiated progeny (PC).RNU6 was use to normalize.

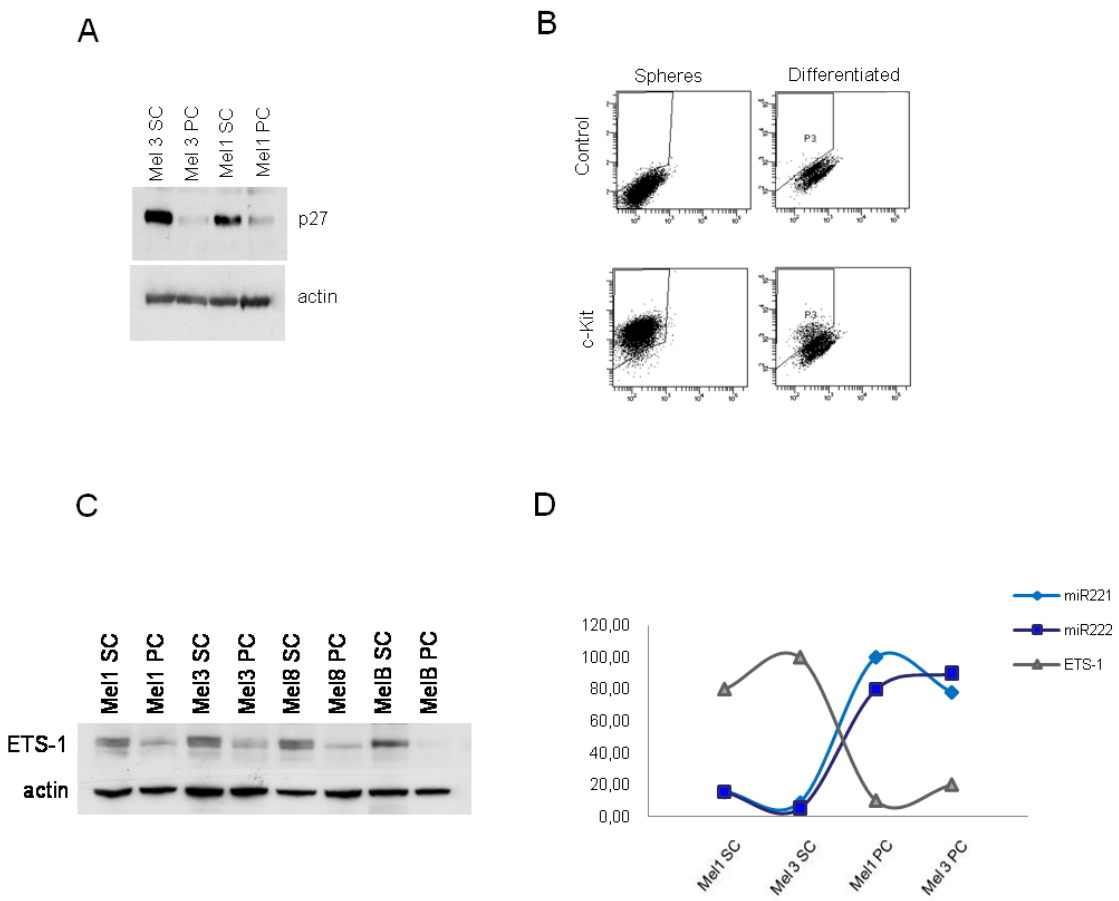


**Figure 7 A**: Western Blotting analyses of p27 in Mel1 and Mel3 melanoma stem-like cell lines vs their more differentiated progeny. Actin is the internal control. **B**: Flow cytometric of c-KIT expression in Mel1 SC in comparison with Mel1 PC. **C**: Western Blotting analysis of ETS-1 in a panel of melanoma stem-like cell lines vs their more differentiated progeny .Actin is the internal control. D: miR-221 and miR-222 expressions are inversely correlated with ETS-1 protein level in Mel1 and Mel3 melanoma stem-like cell lines vs their more differentiated progeny.

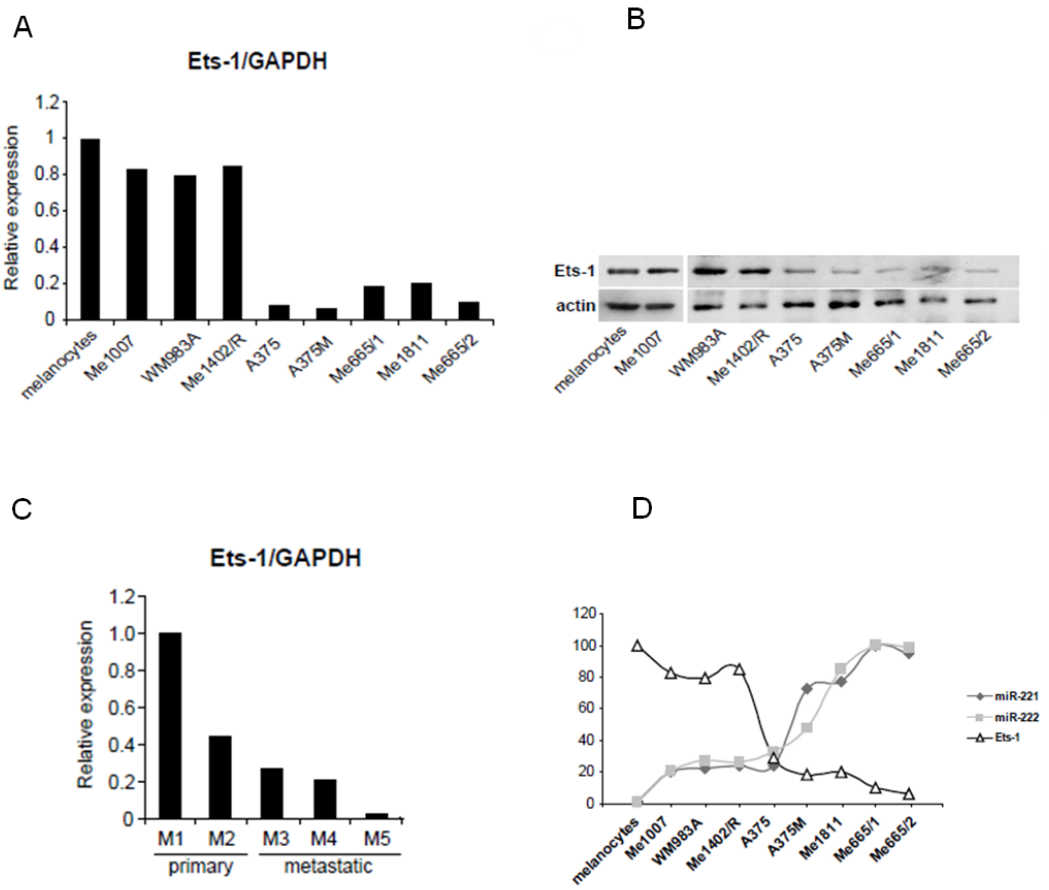


**Figure 8. A:** qRT-PCR and **B**, Western blot analyses of ETS-1 expression in normal human melanocytes and stabilized melanoma. cell lines. **C**, qRT-PCR analysis of cells at early in vitro passages. **D**: miR-221 and miR-222 expressions are inversely correlated with ETS-1 protein level in melanoma progression.

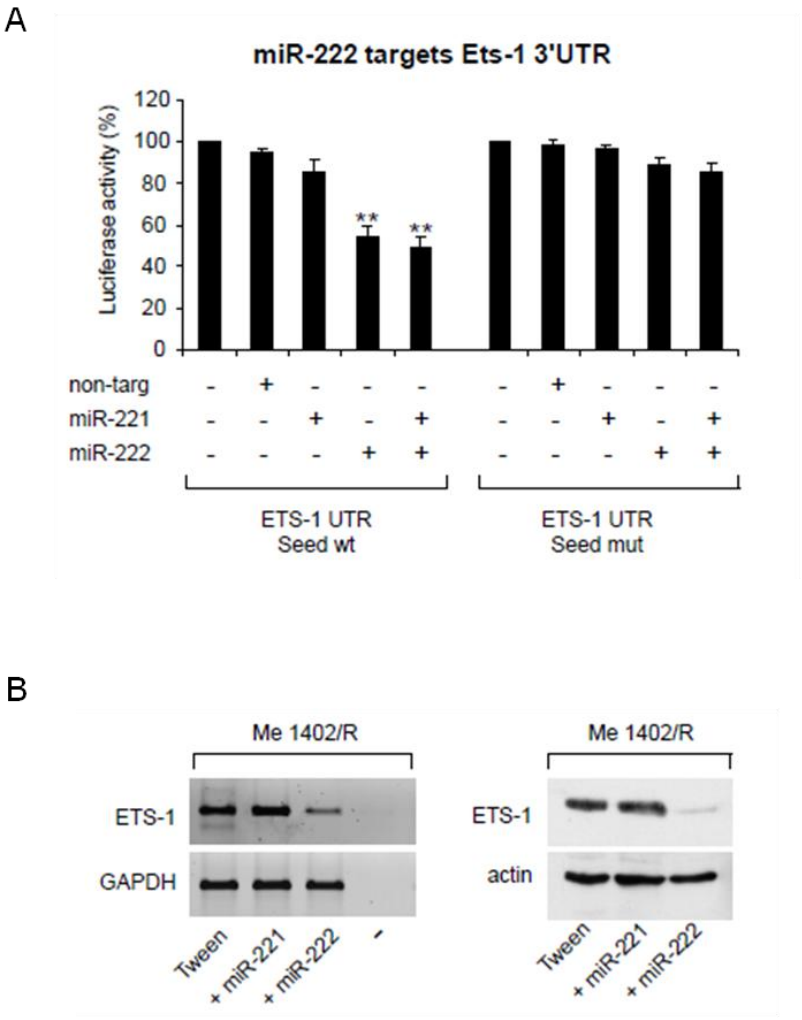


**Figure 9 A:** Luciferase (LUC) reporter assay (columns of minimum 3 exps, bars, SD) performed by cotransfecting miR-221 and/or miR-222 in presence of the LUC reporter gene linked to the ETS-1 3'UTR. As controls, mutated 3'UTR sequence and a non targeting oligomer were also included. ** $p<0.01$. **B**: Evaluation by RT-PCR (left) and WB (right) of ETS-1 expression in Me1402/R melanoma transduced with miR-221, miR-222 or control Tween vector.

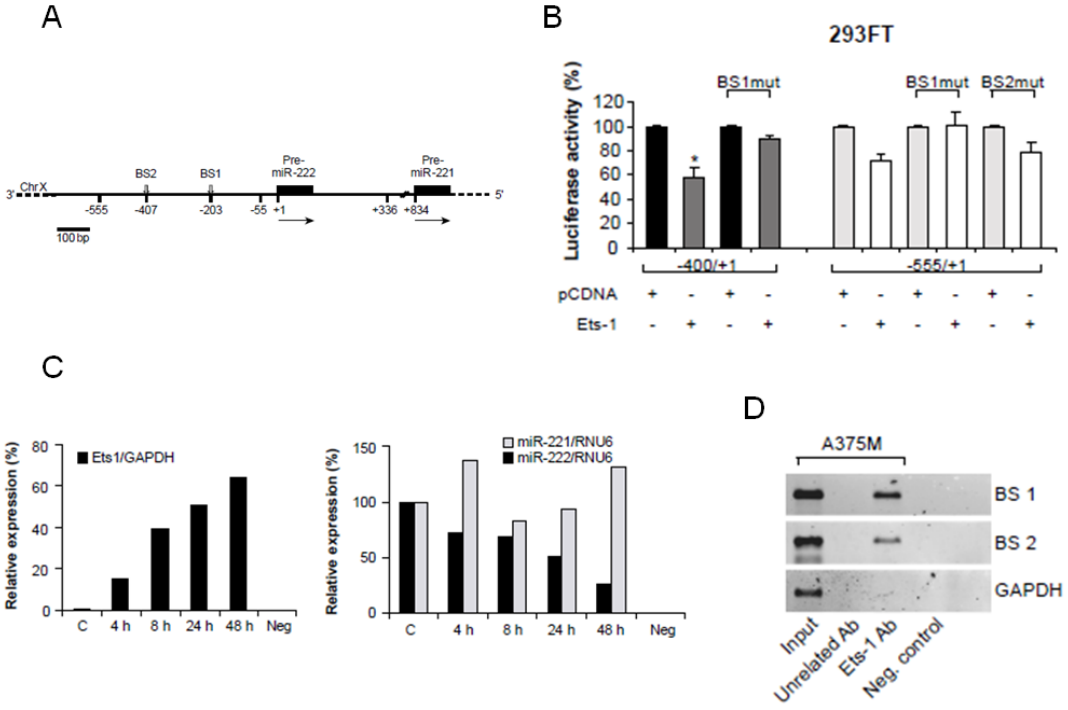


**Figure 10. A:** Schematic depict of the genomic region upstream to pre-miR-222. BS1 and BS2 indicate ETS-1 putative binding sites and horizontal arrows the direction of miR transcription. **B**: Luciferase assays obtained by cotransfecting different genomic fragments (as indicated in A) in 293FT cells in presence or not of ETS-1. As controls, mutations of core nucleotides have been included. *p<0.05. **C:** left, qRT-PCR analysis at different time points of ETS-1 mRNA (left) and miR-222 (right) levels in 293FT ETS-1-transfected cells. **D:** ChIP assay performed in A375M cells.

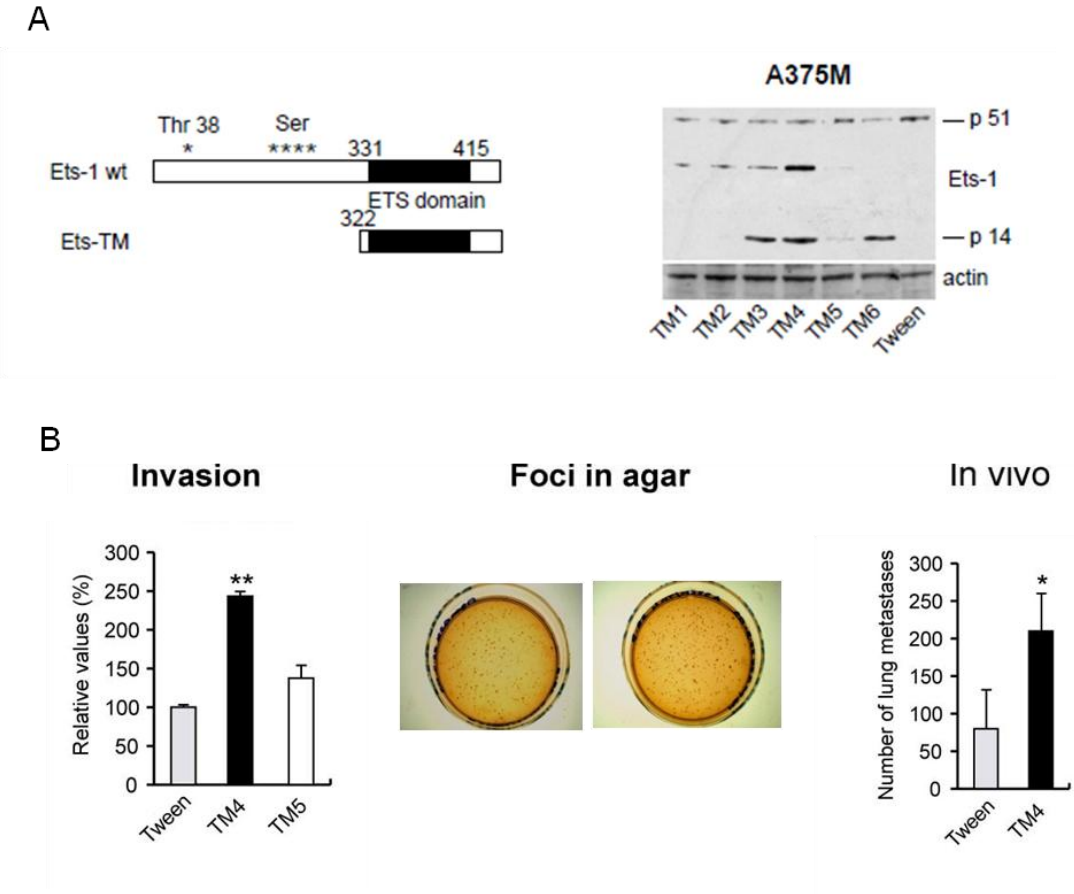


**Figure 11. A:** Schematic picture of ETS-1 protein and its dominant negative (TM) truncated form; right, WB analysis of TM-transduced A375M cell line (TM1 to TM6 represent six independent lentiviral infections). **B**: Invasion (left), number of foci in semisolid medium (middle), in vivo effects of i.v. injected athymic nude mice evaluated as number of lung metastases (right). *p<0.05, ** p<0.01.

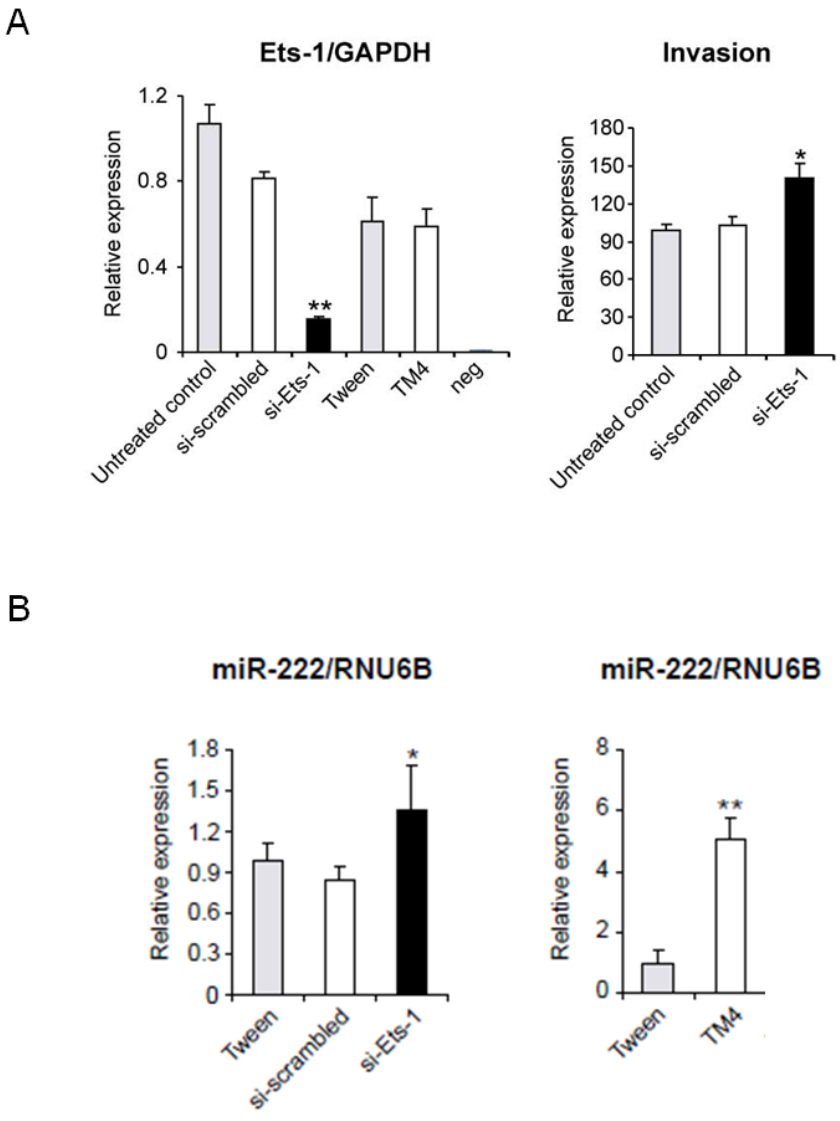


**Figure 12. A:** left, qRT-PCR evaluation of ETS-1 in si-Ets-1 A375M transfected cells; right, invasion assay. **B**: qRT-PCR of miR-222 in si-ETS-1 transiently transfected (left) and TM- stably infected A375M cells (right). RNU6 was use to normalize. *p<0.05, ** p<0.01.

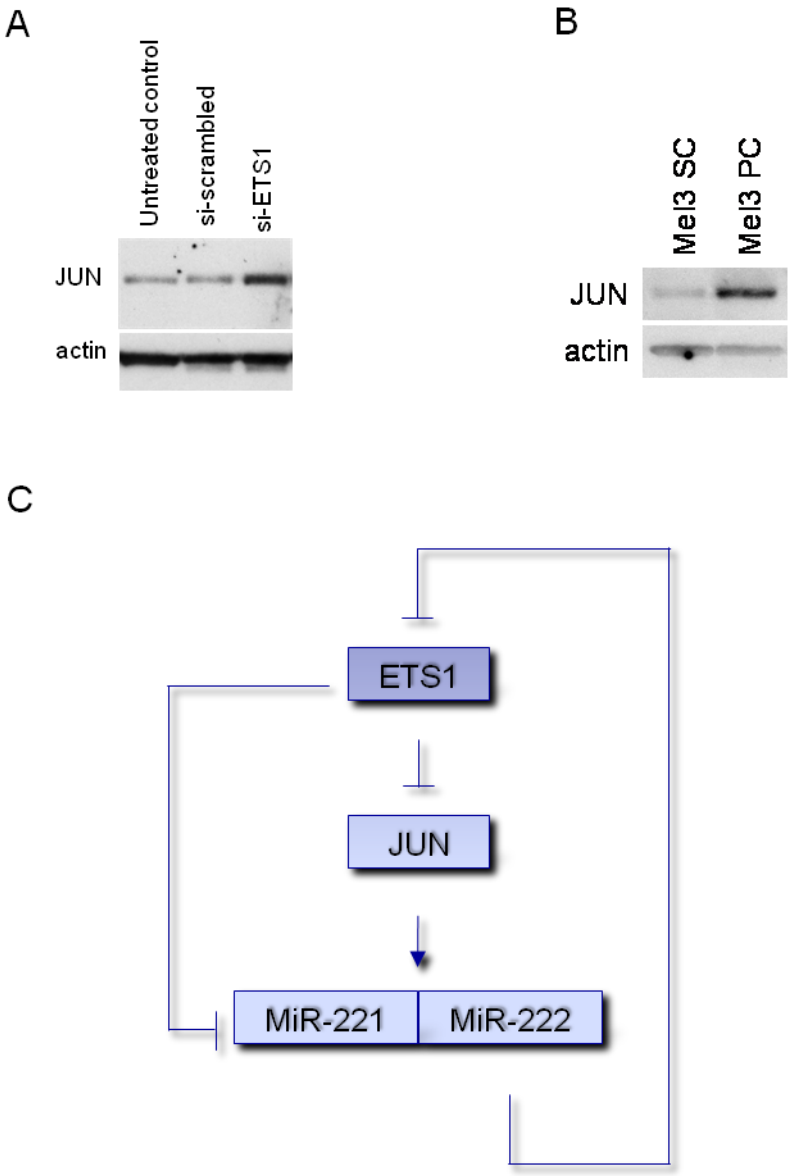


**Figure 13. A:** Western Blot analysis of c-JUN in A375M melanoma cell line. Actin is the internal control **B**: Western Blot analysis of c-JUN in Mel3SC and in the more differentiated progeny Mel1PC. Actin is the internal control **C**:Schematic of the ETS-1→JUN→miR-221/-222 signal transduction pathway in melanoma.

**Table 1.** Melanoma spheres

| | |
|---|---|
| Mel1 | Lung metastasis |
| Mel3 | Lung metastasis |
| Mel4 | Lung metastasis |
| Mel8 | Lymph node metastasis |
| MelB | Subcutaneous metastasis |

**Table 2.** Expression of markers in Mel1 and Mel3 melanoma spheres

| | Mel1SC | Mel3C |
|---|---|---|
| mart1/melan1 | + | + |
| Tyr | + | + |
| HMB45 | + | + |
| ABCB5 | - | - |
| ABCG2 | - | - |
| CD133 | - | - |
| CD20 | - | - |
| CD24 | - | - |
| CD146 | + | + |
| CD90 | + | + |
| cKIT | + | + |
| CD44 | + | + |
| CD44v6 | + | + |

**Table 3.** Melanoma cell lines

| | |
|---|---|
| Melanocytes | Normal human neonatal melanocytes |
| Me1007 | Primary tumor VGP |
| WM983A | Primary tumor VGP |
| Me1402/R | Recurrence of primary tumor |
| A375 | Metastatic melanoma |
| A375M | Melanoma variant metastatic in nu/nu mice |
| Me665/1 | Recurrence of primary tumor |
| Me1811 | Lymph node metastasis |
| Me665/2 | Lymph node metastasis |

# DISCUSSION

A number of recent studies have demonstrated in solid tumors the presence of CSCs, which share many characteristics with tissue stem cells, such as self-renewal and differentiation, and are primarily responsible for sustaining the growth of tumors (O'Brien *et al.,* 2010; Bomken *et al.,* 2010). In many tumor types, as glioblastoma, colon, lung, breast carcinoma and melanoma, the isolation of CSCs has been accomplished using cells surface markers and their characterization was mostly performed by xentransplantation into immunodeficient mice (Ricci-Vitiani *et al.,* 2007; Eramo *et al.,* 2008; Al-Hajj *et al.,* 2003; Schatton *et al.,* 2008, Boiko *et al.,* 2010). Importantly, the phenotypic distribution of these cells in tumor xenograft closely resembled that of the original tumor. However, it is not clear whether melanoma, one of the most aggressive human cancers with metastatic lesions resistant to conventional therapies, follows a cancer stem cell model in that several point of weakness are to take in consideration (Shackleton and Quintana 2010; Rambow and Larue, 2010). First of all, recent studies have shown that melanoma stem-like cells (Mel SC) are not necessarily rare. In addition, another limitation of the current melanoma stem-like cells hypothesis is that the markers used to purify the Mel SCs do not seem highly specific (Quintana *et al.,* 2008).

This study shows that melanoma contains putative stem cells proliferating as spheres. These spheres, after isolation from heterogeneous populations, are capable of *in vitro* self-renewing capacity, forming tumors *in vivo* and self renewing after transplantation. Moreover, the human melanoma xenografts, generated in immunocompromised mice by the injection of these melanospheres, recapitulate the original heterogeneity of the patients' tumors. A relevant feature of the stem-like cells is their differentiating

potential. In this regard, we found that melanoma spheres are able to grow as adherent cells in melanocyte-specific medium, at the same time acquiring a decreased self renewal capacity, as shown by the downregulation of the BMI-1 protein. Consequently, after the injection of both melanoma spheres and their more mature adherent counterpart into athymic nude mice, we observed a delayed growth in the group of tumors derived from adherent melanoma when compared with the corresponding melanospheres.

Currently no definitive consensus has been reached on the CSC phenotype for melanoma and there are contrasting results about the identification of markers that distinguish tumorigenic from non tumorigenic melanocytes (Quintana *et al*., 2008). Accordingly, our findings indicate that some of the potential cell surface markers we examined (CD133, CD20 ABCG2 and ABCB5) show no specific correlation with melanoma stem-like cells (Fang *et al.*, 2005; Monzani *et al.*, 2007; Schatton *et al.*, 2008). Our data parallel the complexity of characterizing CSC markers and raise the difficulty to identify the actual molecular drivers of tumorigenesis in rare and unselected malignant cells.

An emerging theme from several different works is that microRNAs (miRNA) may be involved in the maintenance of the cancer stem cell (CSCs) phenotype as well as in the induction of the invasive and metastatic properties of tumor cells (Nicoloso *et al.*, 2009). MicroRNAs are non coding small sequences that regulate gene expression by binding to the 3'UTRs of their target mRNAs (Calin and Croce, 2006). Growing evidences indicate that miRs are abnormally expressed in all types of cancer where they act either as oncogenes or as tumor suppressors (Voorhoeve, 2010). In addition microRNA signatures appear as a new important parameter which will hopefully allow, in a not too far future, the selection of a truly tailored therapy based on the predicted

risk (Galasso *et al.*, 2010).

There are accumulating evidences that miR-driven pathways are fundamental for cell stemness (Hatfield and Ruohola, 2008). Embryonic stem cells are highly enriched with a specific repertoire of microRNAs (Calabrese *et al.*, 2007), which on one hand have power over embryonic stem cell gene regulation and on the other are under the control of self-renewal and pluripotency transcription factors (Marson *et al.*, 2007). Strikingly, the majority of miRs, that are important in embryonic stem cells, are also involved in cell cycle regulation and oncogenesis. This emerging role of miRs spurred us to investigate whether they are involved in maintaining the cancer stem cell phenotype. Studies from others and our laboratory demonstrated that microRNA-221/-222 are among the microRNAs most implicated in cancer according to their up-regulation in a number of solid tumors (Galardi *et al.*, 2007; le Sage *et al.*, 2007; Medina *et al.*, 2008; Gillies and Lorimer, 2009). In addition Croce and his group described an increase of miR-221 and -222 in NSCLC and HCC through AP-1 induction and their targeting of PTEN and TIMP3 tumor suppressors (Garofalo *et al.*, 2009).

Our data show a reduced expression of miR-221/-222 in melanoma stem-like cells respect to their more differentiated progeny, thus suggesting a possible functional role of these two microRNAs in controlling melanoma stemness. Considering that neurons and melanocytes are derived from a common pluripotent progenitor cell, this result is consistent with the report of Wu demonstrating the upregulation of miR-222 in the differentiation process of undifferentiated hES cells to neural progenitor cells and its decline upon further differentiation (Wu *et al.*, 2007).

Deregulated miR-221 and -222 promote cancer cell proliferation by regulating the G1/S checkpoint cyclin dependent kinase, p27Kip (le Sage *et al.*, 2007; Medina *et al.*, 2008; Felicetti *et al.*, 2008), which specifically exerts its function by binding to

CDK/CYCLIN complexes. Although p27Kip is a recognized tumor suppressor (Li *et al.*, 2006), inactivating point mutations are rare and protein levels mostly regulated at posttranscriptional/posttranslational levels. The principal p27Kip regulatory mechanisms include ubiquitin-dependent degradation by the proteasome (Kotoshiba *et al.*, 2005), functional inactivation by mislocalization into the cytoplasm, or phosphorylation events controlling p27Kip binding to its cellular targets (Delmas *et al.*, 2003; Koff *et al.*, 2006). MiR-221/-222–based mechanism, blocking p27Kip translation, represents an additional oncogenic mechanism underlying the abnormal cell cycle rate of advanced melanoma and of many other tumors. In addition Fornari and co-workers have shown that the down-modulation of another member of CDKIs, p57, was associated with miR-221 upregulation and increased aggressiveness in human hepatocarcinoma (Fornari *et al.*, 2008), thus further substantiating the biological significance of miR-221 and -222 as cell cycle regulators. According to the reported inverse correlation between miR-221/-222 and p27Kip, we found both these miRNAs down-regulated in melanoma stem-like cells in comparison with their adherent counterparts; conversely, a higher amount of p27Kip protein was detected in Mel SC, suggesting an involvement of these miRNAs also in cell-cycle progression and proliferation of melanoma stem-like cells. A plausible reason to explain the high level of p27 protein in the melanoma stem-like cells might be its involvement in sustaining self-renewal, as reported for p21. Actually, Viale has demonstrated that the expression of leukaemia-associated oncogenes in mouse haematopoietic stem cells (HSCs) induces DNA damage and activates a p21-dependent cellular response, which leads to reversible cell-cycle arrest and DNA repair (Viale *et al.*, 2009).

We have also reported that miR-221/-222 exert their function by repressing the c-KIT receptor, a melanocytic multifunctional player regulating melanogenesis, cell growth,

migration, and survival (Alexeev *et al.*, 2006). In the progression of human melanoma, the loss of c-KIT represents a crucial event: up to 70% of metastases lack the receptor acquiring, as a consequence, the possibility to escape SCF/c-KIT–triggered apoptosis (Huang *et al*., 1998). The direct targeting of c-KIT by miR-221/-222 has been reported also in normal erythropoiesis (Felli *et al*., 2005), neoangiogenesis (Suarez *et al.*, 2007), as well as in papillary thyroid carcinoma (He *et al*., 2005). Here we have also shown that melanoma spheres express higher level of c-Kit protein in comparison to their adherent counterparts, confirming again the inverse correlation with the microRNA-221/-222. This finding seems to concord with the role played by c-KIT in maintenance and survival of the hematopoietic stem cells (Kent *et al.*, 2008).

In order to further dissect the main molecular pathways associated with melanoma stem-like cells as well as melanoma cell lines, we have recently focused our attention on the ETS-1 transcription factor.

ETS-1 is the founding member of the ETS gene superfamily, encoding a class of phosphoproteins characterized by a conserved domain that recognizes and binds to a GGAA/T DNA core sequence. ETS-1 is involved in an array of cellular functions, acting either as a positive or a negative regulator (Nakayama *et al*., 1999; He *et al.*, 2007).

Although ETS-1 gene expression correlates with progression of tumors like thyroid, pancreas, liver, lung and breast carcinomas (Seth and Watson, 2005), controversial data exist for cutaneous and non cutaneous melanoma, where ETS-1 has been described either directly involved in determining the malignant phenotype (Rothhammer *et al*., 2004) or as a useless marker unable to distinguish between benign and malignant melanocytic lesions (Torlakovic *et al.*, 2004).

Torlakovic and collaborators have analyzed for ETS-1 expression a large collection of

nevi, primary and metastatic melanomas randomly collected from their archives. They have shown ETS-1 downregulation associated with tumor progression, with an inverse correlation between its expression and melanoma dimension, but no relationship with the tumor thicknesses. Further studies on post-transcriptional and/or post-translational modifications as well as ETS-1 topographic cell distribution might clarify this issue.

Here we have shown that ETS-1 expression is inversely related to melanoma malignancy. Although the presence of ETS-1 in tissues derived from the neural crest, as melanocytes, might suggest the association of ETS-1 downregulation with the loss of the neural crest associated phenotype along with melanoma progression, our data address in favor of its role in tumorigenesis. In addition, we have identified ETS-1 as another gene directly regulated by miR-222, but not by miR-221, demonstrating the existence of miR-221/-222 common, but also independent functionalities, as recently reported for miR-222 in inflammation-mediated neovascularization through STAT5A (Dentelli *et al.*, 2010). We have also found ETS-1 protein expression to be higher in Mel SC respect to differentiating Mel PC, revealing again that ETS-1 protein is inversely correlated with both miR-221 and miR-222. The high amount of ETS-1 protein in melanoma spheres is probably related to neural crest associated phenotype. Of interest is the similarity between ETS-1 and c-KIT.

This study also demonstrates that in normal cells or melanomas at early stages, ETS-1 binds to and transcriptionally inhibits miR-221 and miR-222. Thus, the lack of ETS-1 in advanced melanoma unblocks both these two oncomiRs.

Considering that ETS-1 protein has been reported to repress c-JUN (Goldberg *et al.*, 1994 ) and that in non-small cell lung cancer and hepatocarcinoma cells c-JUN has been involved in miR-221/-222 activation (Garofalo *et al*., 2009), our data suggest a complex ETS-1/JUN/miR-221/-222 regulatory loop in melanoma cell line (Fig.13C). Likely,

miR-222 mediated translational repression/degradation of ETS-1 remains steady until miR elevation does not reach a fixed boundary starting to reduce ETS-1. This theoretical threshold should be different in normal vs. neoplastic cells and could vary along tumor progression, leading to a different balance miR-222/ ETS-1. Although we assume that ETS-1 negatively regulates miR-221 and miR-222 also in melanoma spheres, further studies are essential to confirm this hypothesis.

In the young and emerging context of the cancer-stem-cell research, still slowed down by a great number of limitation, a better understanding of the functional roles played by miR-221/-222 in the survival and maintenance of the subpopulation of cancer stem cells might add a new small piece in the elucidation of the complex mechanism regulating the balance between self-renewal and differentiation.

# REFERENCES

Alexeev V, Yoon K. (2006). Distinctive role of the c-kit receptor tyrosine kinase signaling in mammalian melanocytes. *J Invest Dermatol* **126**:1102–1110.

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. (2003). Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* **100**:3983-3988.

Balch CM, Soong SJ, Gershenwald JE, Thompson JF, Reintgen DS, Cascinelli N, et al. (2001). Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system. *J Clin Oncol* **19**: 3622–3634.

Bartel DP. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**: 281-97.

Bevona C, Goggins W, Quinn T, Fullerton J, Tsao H. (2003). Cutaneous melanomas associated with nevi. *Arch Dermatol* **139**:1620-1624.

Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. (2005). Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* **5**:899-904.

Boiko AD, Razorenova OV, van de Rijn M, Swetter SM, Johnson DL, Ly DP, et al. (2010). Human melanoma-initiating cells express neural crest nerve growth factor receptor CD271. *Nature* **466**:133-7.

Bomken S, Fiser K, Heidenreich O, Vormoor J. (2010). Understanding the cancer stem cell. *Br J Cancer* **103**:439-45.

Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM. (2003). bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the

proapoptotic gene hid in Drosophila. *Cell* **113**:25–36.

Calabrese JM, Seila AC, Yeo GW, Sharp PA. (2007). RNA sequence analysis defines Dicer's role in mouse embryonic stem cells. *Proc Natl Acad Sci USA* **104:**18097-18102.

Calin GA and Croce CM. (2006). MicroRNA signatures in human cancers. *Nat Rev Cancer* **6**:857-866.

Carè A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, et al. (2007). MicroRNA-133 controls cardiac hypertrophy. *Nat Med* **13**:613-8.

Chen CZ, Li L, Lodish HF, Bartel DP. (2004). MicroRNAs modulate hematopoietic lineage differentiation. *Science* **303**:83–86.

Chudnovsky Y, Khavari PA, Adams AE. (2005). Melanoma genetics and the development of rational therapeutics. *J Clin Invest* **115**:813-24.

Clark WH, Elder DE, Guerry D 4th, Epstein MN, Greene MH, Van Horn M. (1984). A study of tumor progression: the precursor lesion of superficial spreading and nodular melanoma. *Hum Pathol* **15**:1147-65.

Croce CM. (2009). Causes and consequences of microRNA dysregulation in cancer. *Nat Rev Genet* **10**: 704-714.

Dalerba P, Cho RW, Clarke MF. (2007). Cancer stem cells: models and concepts. *Annu Rev Med* **58**:267-84.

Dalerba M. (2007). Cancer stem cells and oncology therapeutics. *Curr Opin Oncol* **19**:61-4.

Delmas C, Aragou N, Poussard S, Cottin P, Darbon JM, Manenti S. (2003). MAP kinase-dependent degradation of p27Kip1 by calpains in choroidal melanoma cells. Requirement of p27Kip1 nuclear export. *J Biol Chem* **278**:12443-1251.

Dentelli P, Rosso A, Orso F, Olgasi C, Taverna D, Brizzi MF. (2010). microRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. *Arterioscler Thromb Vasc Biol* **30**:1562-1568.

Dhomen N, Marais R. (2009). BRAF signaling and targeted therapies in melanoma. *Hematol Oncol Clin North Am* **23**:529-45.

Dittmer J. (2003). The biology of the Ets-1 proto-oncogene. *Mol Cancer* **2**:29.

Eisterer W, Jiang X, Christ O, Glimm H, Lee KH, Pang E, et al. (2005). Different subsets of primary chronic myeloid leukemia stem cells engraft immunodeficient mice and produce a model of the human disease. *Leukemia* **19**:435-441.

Eramo A, Ricci-Vitiani L, Zeuner A, Pallini R, Lotti F, Sette G et al. (2006). Chemotherapy resistance of glioblastoma stem cells. *Cell Death Differ* **13**:1238-41.

Eramo A, Lotti F, Sette G, Pilozzi E, Biffoni M, Di Virgilio A et al. (2008). Identification and expansion of the tumorigenic lung cancer stem cell population. *Cell Death Differ* **15**:504-514.

Fang D, Nguyen TK, Leishear K, Finko R, Kulp AN, Hotz S et al. (2005). A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res* **65**:9328-37.

Felicetti F, Errico MC, Bottero L, Segnalini P, Stoppacciaro A, Biffoni M et al. (2008). The Promyelocytic Zinc Finger-MicroRNA-221/-222 Pathway Controls Melanoma Progression through Multiple Oncongenic Mechanisms. *Cancer Res* **68**:2745-2754.

Felli N, Fontana L, Pelosi E, Botta R, Bonci D, Facchiano F et al. (2005). MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation. *Proc Natl Acad Sci USA* **102**:18081-6.

Fornari F, Gramantieri L, Ferracin M, Veronese A, Sabbioni S, Calin GA et al. (2008). miR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma. *Oncogene* **27**:5651-5661.

Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafrè SA, et al. (2007). miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1. *J Biol Chem* **282**:23716-23724.

Galasso M, Elena Sana M, Volinia S. (2010). Non-coding RNAs: a key to future personalized molecular therapy?. *Genome Med* **2**:12.

Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A et al. (2009). MiR-221&222 Regulate TRAIL Resistance and Enhance Tumorigenicity through PTEN and TIMP3 Downregulation. *Cancer Cell* **16**: 498–509.

Gillies JK and Lorimer IA. (2007). Regulation of p27Kip1 by miRNA 221/222 in glioblastoma. *Cell Cycle* **6**:2005-2009.

Goldberg Y, Treier M, Ghysdael J, Bohmann DJ. (1994). Repression of AP-1-stimulated transcription by c-Ets-1. *Biol Chem* **269**:16566-73.

Gray-Schopfer V, Wellbrock C, Marais R. (2007). Melanoma biology and new target therapy. *Nature* **445**:851-7.

Gu G, Yuan J, Wills M, Kasper S. (2007). Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo. *Cancer Res* **67**:4807-4815.

Hatfield S and Ruohola-Baker H. (2008). microRNA and stem cell function. *Cell Tissue Res* **331**:57-66.

He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S et al. (2005). The role of microRNAgenes in papillary thyroid carcinoma. *Proc Natl Acad Sci U S A* **102**:19075–80.

He J, Pan Y, Hu J, Albarracin C, Wu Y, Dai JL. (2007). Profile of Ets gene expression in human breast carcinoma. *Cancer Biol Ther* **6**:76-82.

Held MA, Curley DP, Dankort D, McMahon M, Muthusamy V, Bosenberg MW. (2010). Characterization of melanoma cells capable of propagating tumors from a single cell. *Cancer Res* **70**:388-97.

Huang S, Jean D, Luca M, Tainsky MA, Bar-Eli M. (1998). Loss of AP-2 results in downregulation of c-KIT and enhancement of melanoma tumorigenicity and metastasis. *EMBO J* **17**:4358-69.

Inui M, Martello G, Piccolo S. (2010). MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol* **11**:252-263.

Kent D, Copley M, Benz C, Dykstra B, Bowie M, Eaves C. (2008). Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin Cancer Res* 14:1926-30.

Koff A. (2006). How to decrease p27Kip1 levels during tumor development. *Cancer Cell* **9**:75-76.

Kotoshiba S, Kamura T, Hara T, Ishida N, Nakayama KI. (2005). Molecular dissection of the interaction between p27 and Kip1 ubiquitylation-promoting complex, the ubiquitin ligase that regulates proteolysis of p27 in G1 phase. *J Biol Chem* **280**:17694-17700.

La Barge MA. (2010). The difficulty of targeting cancer stem cell niches. *Clin Cancer* Res **16**:3121-9.

Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T. (2003). New microRNAs from mouse and human. *RNA* **9**: 175-9.

Larson AR, Konat E, Alani RM. (2009). Melanoma biomarkers: current status and vision for the future. *Nat Clin Pract Oncol* **6**:105-17.

le Sage C, Nagel R, Egan DA, Schrier M, Mesman E, Mangiola A et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. *EMBO J* **26**:3699-3708.

Li W, Sanki A, Karim RZ, Thompson JF, Soon Lee C, Zhuang L, et al. (2006). The role of cell cycle regulatory proteins in the pathogenesis of melanoma. *Pathology* **38**:287-301.

Lopez-Bergami P, Kim H, Dewing A, Goydos J, Aaronson S, Ronai Z. (2010). c-Jun regulates phosphoinositide-dependent kinase 1 transcription: implication for Akt and protein kinase C activities and melanoma tumorigenesis. *J Biol Chem* **285**:903-13.

Mallanna SK, Rizzino A. (2010). Emerging roles of microRNAs in the control of embryonic stem cells and the generation of induced pluripotent stem cells. *Dev Biol* **344**:16-25.

Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, et al. (2008). Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* **34**:521-533.

Massard C, Deutsch E, Soria JC. (2006). Tumor stem cell-targeted treatment: elimination or differentiation. *Ann Oncol* **17**:1620-4.

Medina R, Zaidi SK, Liu CG, Stein JL, Van Wijnen AJ, Croce CM et al. (2008). MicroRNAs 221 and 222 bypass quiescence and compromise cell survival. *Cancer Res* **68**:2773-2780.

Miller AJ, Mihm MC Jr. (2007). Melanoma. *N Engl J Med* **355**:51-65.

Monzani E, Facchetti F, Galmozzi E, Corsini E, Benetti A, Cavazzin C et al. (2007). Melanoma contains CD133 and ABCG2 positive cells with enhanced tumorigenic potential. *Eur J Cancer* **43**:935-46.

Nakano T, Abe M, Tanaka K, Shineha R, Satomi S, Sato Y. (2000). Angiogenesis Inhibition by transdominant mutant Ets-1. *J Cell Physiol* **184**:255-262.

Nakayama T, Ito M, Ohtsuru A, Naito S, Nakashima M, Sekine I. (1999). Expression of the ets-1 proto-oncogene in human thyroid tumor. *Mod Pathol* **12**:61-68.

Nicoloso MS, Spizzo R, Shimizu M, Rossi S, Calin GA. (2009). MicroRNAs--the micro steering wheel of tumor metastases. *Nat Rev Cancer* **9**:293-302.

O'Brien CA, Kreso A, Jamieson CH. (2010). Cancer stem cells and self-renewal. *Clin Cancer Res* **16**:3113-20.

Ørom UA, Nielsen FC, Lund AH. (2008). MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell* **30**:460-71.

Park IK, Qian D, Kiel M, Becker MW, Pihalja M, Weissman IL et al. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**(6937):302-5.

Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, et al. (2006). MicroRNAs modulate the angiogenic properties of HUVECs. *Blood* **108**:3068-71.

Pourtier-Manzanedo A, Vercamer C, Van Belle E, Mattot V, Mouquet F, Vandenbunder B. (2003). Expression of an Ets-1 dominant-negative mutant perturbs normal and tumor angiogenesis in a mouse ear model. *Oncogene* **22**:1795-1806.

Poy MN, Eliasson L, Krutzfeldt J, Kuwajima S, Ma X, Macdonald PE, (2004). A pancreatic islet-specific microRNA regulates insulin secretion. *Nature* **432**: 226–230.

Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P et al. (2007). Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci USA* **104**:973-978.

Quintana E, Shackleton M, Sabel MS, Fullen DR, Johnson TM, Morrison SJ. (2008). Efficient tumor formation by single human melanoma cells. *Nature* **456**:593-598.

Rambow F, Larue L. (2010). The quest for the melanoma stem cell: still more questions than answers. *Pigment Cell Melanoma Res* **23**:307-9.

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**:105–11.

Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C et al. (2007). Identification and expansion of human colon-cancer-initiating cells. *Nature* **445**:111-5.

Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A et al. (2010). A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. *Cell* **141**:583-94.

Rothhammer T, Hahne JC, Florin A, Poser I, Soncin F, Wernert N et al. (2004). The Ets-1 transcription factor is involved in the development and invasion of malignant melanoma. *Cell Mol Life Sci* **61**:118-128.

Schulenburg A, Ulrich-Pur H, Thurnher D, Erovic B, Florian S, Sperr WR, et al. (2006). Neoplastic stem cells: a novel therapeutic target in clinical oncology. *Cancer* **107**:2512-20.

Sengupta S, Nie J, Wagner RJ, Yang C, Stewart R, Thomson JA. (2009). MicroRNA 92b controls the G1/S checkpoint gene p57 in human embryonic stem cells. *Stem Cells* **27**:1524-8.

Seth A, Watson DK. (2005). ETS transcription factors and their emerging roles in human cancer. *Eur J Cancer* **41**:2462-1478.

Shackleton M, Quintana E. (2010). Progress in understanding melanoma propagation. *Mol Oncol*. [Epub ahead of print].

Schatton T, Murphy GF, Frank NY, Yamaura K, Waaga-Gasser AM, Gasser M (2008). Identification of cells initiating human melanomas. *Nature* **451**:345-9.

Suarez Y, Fernandez-Hernando C, Pober JS, Sessa WC. (2007). Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circ Res* **100**:1164–73.

Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I. (2008). MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. *Nature* **455**:1124-8.

Torlakovic EE, Bilalovic N, Nesland JM, Torlakovic G, Flørenes VA. (2004). Ets-1 transcription factor is widely expressed in benign and malignant melanocytes and its expression has no significant association with prognosis. *Mod Pathol* **17**:1400-1406.

Vereecken P, Laporte M, Heenen M. (2007) Significance of cell kinetic parameters in the prognosis of malignant melanoma:a review. *J Cutan Pathol* **34**: 139-145.

Viale A, De Franco F, Orleth A, Cambiaghi V, Giuliani V, Bossi D et al. (2009).Cell-cycle restriction limits DNA damage and maintains self-renewal of leukaemia stem cells. *Nature* **457**:51-6.

Visone R, Russo L, Pallante P, De Martino I, Ferraro A, Leone V, et al. (2007). MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27Kip1 protein levels and cell cycle. *Endocr Relat Cancer* **14**:791-798.

Voorhoeve PM. (2010). MicroRNAs: Oncogenes, tumor suppressors or master regulators of cancer heterogeneity?. *Biochim Biophys Acta* **1805**:72-86.

Wu H, Xu J, Pang ZP, Ge W, Kim KJ, Blanchi B, Chen C, Südhof TC, Sun YE. (2007). Integrative genomic and functional analyses reveal neuronal subtype differentiation bias in human embryonic stem cell lines. *Proc Natl Acad Sci U S A.* **104**:13821-6.

Wicha MS. (2006). Cancer stem cells and metastasis: lethal seeds. *Clin Cancer Res*

**12**:5606-7.

Xia H. P. (2008). Great Potential of MicroRNA in Cancer Stem Cell. *J Cancer Mol* **4**:79-89.

Yang ZF, Ho DW, Ng MN, Lau CK, Yu WC, Ngai P et al. (2008). Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell* **13**:153-16.

Yi R, O'Carroll D, Pasolli HA, Zhang Z, Dietrich FS, Tarakhovsky A, et al. (2006). Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nature Genet* **38**:356–362.