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"**MiR-221 acts as stemness promoter in breast cancer cells by targeting DNMT3b**"

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1 Quintavalle C, Donnarumma E, Iaboni M, **Roscigno G**, Garofalo M, Romano G, Fiore D, De Marinis P, Croce CM, Condorelli G. Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells. Oncogene 2013;32(34):4001-8.

2 Quintavalle C, Mangani D, **Roscigno G**, Romano G, Diaz-Lagares A, Iaboni M, Donnarumma E, Fiore D, De Marinis P, Soini Y, Esteller M, Condorelli G. MiR-221/222 target the DNA methyltransferase MGMT in glioma cells. PLoS One 2013; 8(9):e74466.

LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
AML	Acute Myeloid Leukemia
BCSC	Breast Cancer Stem Cell
CALLA	Lymphoblastic Leukaemia Antigen
CLL	chronic lymhocitic leukaemia
CK	Cytokeratin
CSC	Cancer Stem Cell
DCIS	In situ Ductal Carcinoma
EGFR	Epidermal Growth Factor Receptor
ESC	Embryonal Stem Cells
EMT	Epithelial-Mesenchymal Transition
EpCAM	Epithelial Cell Adhesion Molecule
ER	Estrogen Receptor
EXP5	Exportin-5
FACS	Fluorescence-Activated Cell Sorting
HER2	Human Epidermal Receptor 2
HSA	Heat Stable Antigen
IDC	Infiltrating Ductal Carcinoma
LCIS	Lobular Carcinoma In Situ
MS	MammoSphere

MDR	Multidrug resistance
miR	microRNA
MUC	Mucin
NSC	Normal Stem Cell
PDGF	Platelet Derived Growth Factor
PR	Progesterone Receptor
SMA	Smooth Muscle Actin
Thy-1	Thymocyte Differentiation Antigen
TDLU	Terminal Duct Lobular Units
UTR	Untranslating Region
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

Current hypotheses suggest that tumor originates from cells that carry out a process of “malignant reprogramming” driven by genetic and epigenetic alterations. Multiples studies reported the existence of stem-cell-like cells that acquire the ability to self-renew and are able to generate the bulk of more differentiated cells that form the tumor. This population of cancer cells, called cancer stem cells (CSC), is responsible for sustaining the tumor growth and is able to disseminate and migrate giving metastases to distant organs. Furthermore, CSCs have shown to be more resistant to anti-tumor treatments than the differentiated cells, suggesting that surviving CSCs could be responsible for tumor relapse after therapy. Nevertheless, the cancer stem-like properties are not well characterized yet. MicroRNAs (miRs) are small, noncoding RNAs (20-25 nucleotidies) that play a crucial role in biological processes including development, proliferation, and apoptosis. Previous investigations have linked miRs to the control of self-renewal and differentiation of normal stem cells. The aim of this study was to test the functional role of miRs in human Breast Cancer Stem Cells (BCSCs) also named mammospheres. We analyzed, by miR-Array, the miRs differentially expressed in BCSCs and their differentiated counterpart. Among several miRs, we focused our attention on miR-221 that was found increased in mammospheres. In order to validate data achieved in primary cultures, we obtained mammospheres from T47D, an immortalized breast cancer cell line. Interestingly, like primary cultures mammospheres, also T47D mammospheres exhibited increased levels of miR-221 compared to T47D differentiated cells. Moreover, the overexpression of miR-221 by a miR mimic in T47D differentiated cells was able to increase the number of mammospheres and the expression of stem cell protein markers. Among miR-221 targets, we demonstrated, by luciferase-assay, that miR-221 targets the 3' untranslated region of DNMT3b, a DNA Methyl Transferase. Furthermore, our data showed that DNMT3b was able to repress the expression of some stemness genes, such as Nanog and Oct3/4, and mammospheres formation partially reverting miR-221 mediated effects on stemness properties. In conclusion, we hypothesize that miR-221 may contribute to breast cancer tumorigenicity regulating the stemness properties through DNMT3b expression.

1. BACKGROUNG

1.1 The cancer stem cell theory

1.1.1 Two models to describe cancer cells heterogeneity

Cancer is a group of diseases characterized by uncontrolled cell proliferation. Tumor cells are able to invade nearby parts of the body and/or metastasize to more distant tissue. It is well established that tumors are heterogeneous entities and that not all cells are identical within a single tumor. In fact, cancer cells exhibit different phenotype and proliferation potential, express several differentiated markers and only a subfraction of tumor cells is able to self-renew in vivo xenograft models.
Two theories have been proposed to explain the tumor growth and heterogeneity (Figure 1.1a). The "stochastic" theory suggests that all tumor cells are equipotent and that cancers arise from genetic and epigenetic mutations that affect the survival and reproduction of cells, providing a selective advantage and generating clonal heterogeneity. Differently, the "Cancer Stem Cell" theory holds that tumors are hierarchically organized like a normal tissue, with a subset of cells that possess an unlimited self-renewal potential (the ability to generate identical undifferentiated daughters) and give rise, through an altered differentiation process, to the "phenotypically different mature cells" (Weissman et al 2000). Owing to the analogy to tissue-specific stem cells, which are responsible for the maintenance and homeostasis of the adult tissue, these cells have been referred to as Cancer stem cells (CSCs) and have been proposed to be responsible for the maintenance and tumour progression. In contrast to the "stochastic" model of oncogenesis, where transformation results from random mutations and subsequent clonal selections, according to the CSC model, cancers would originate from the malignant transformation of a stem or progenitor cells through the deregulation of the normally tightly regulated self-renewal program or from transformation of committed cells that gain a self-renewal potential through dedifferentiation (Reya et al. 2001).

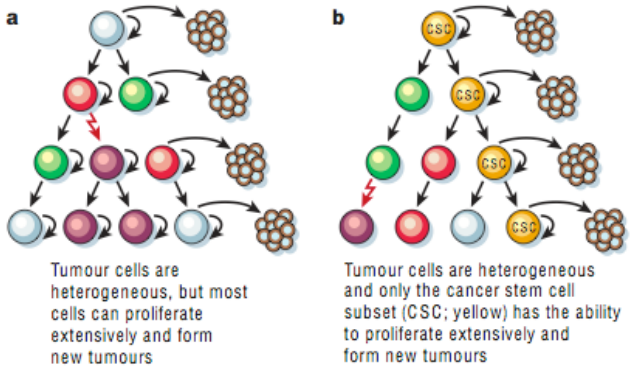

Figure 1. Two general models to explain the heterogeneity in solid cancers: A) The stochastic model; B) The cancer stem cells model.

1.1.2 Relation between normal and cancer stem cells

The CSC model assumes the tumours can be viewed as an "aberrant organ". On the basis of this consideration, the principles of normal stem cell biology can be applied to cancer stem cells to better understand how tumours develop. Therefore, it is important to define the unique properties of normal stem cells.
A normal adult stem cell is defined as an undifferentiated cell able to undergo unlimited cell division. It has the potential to give rise to both stem cells and daughter cells that differentiate into specialized cells, producing all cell types founded in mature tissue. A normal stem cell must possess two qualities to perform its natural function: self-renewal and differentiation. Self-renewal is a special cell property that enables a stem cell to produce another stem cell with essentially the same unspecialized phenotype and replication potential. Differentiation is the second function of a stem cell and involves the production of daughter cells that become tissue-specific specialized cells. The balance between self-renewal and differentiated cells maintains the tissue homeostasis by replacing senescent or damaged cells and ensures its correct function. It depends on systemic or local signals that are able to influence the cell division of stem cells (Morrison et al 2008). A stem cell can go through a symmetric division to generate two cells identical to itself. The ability to self-renew enables the expansion of the stem cell compartment and maintains a pool of undifferentiated stem cells. The stem cell might go through an asymmetric cell division to generate one cell that is identical

to itself and one cell that is distinct. The distinct cell goes through a series of cell divisions and differentiative steps to generate the ultimate terminally specialized cells. The cells that form the intermediates between stem cells and terminally differentiated cells are usually referred to as progenitor cells or transit amplifying cells. Differently to the stem cells, the progenitors are characterized by a reduction of self-renewal capacity and high rate of cell division. Therefore, in a tissue, cells are organised in a hierarchy, where only the stem cells present a long term self renewal and differentiation potential, whereas the more committed progenitors have a restrictive renewal potential (Morrison et al 1994, Morrison et al 1997).
In a similar way to the normal tissue, the CSCs are at the apex of hierarchy and though an alteration of signalling that regulate normal stem features are able to expand the number of cancer stem cells and form a tumour mass composed by "differentiated cells" (Morrison et al 1994).

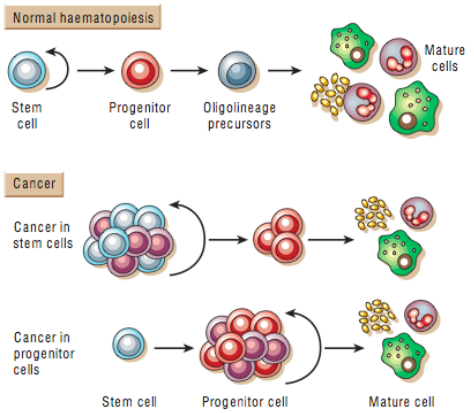

Figure 2. **Comparison of self-renewal during haematopoietic stem cell development and leukaemic transformation.** Unlike normal haematopoiesis, where signalling pathways that regulate self-renewal are tightly regulated (top), during transformation of stem cells, the same mechanisms may be dysregulated to allow uncontrolled self-renewal.

Normal stem cells (NSCs) and CSCs share a number of important phenotypic properties (Sell et al. 1994, Dontu et al. 2003). Particularly, they both have the capacity of unlimited self-renewal and extensive proliferation. In the case of normal stem cells, this is a tightly controlled process that occurs during the maintenance and repair of adult tissues and responds to environmental changes. In contrast, for the cancer stem cells this takes the form of self-sufficiency in growth signalling and uncontrolled cellular proliferation. NSCs and CSCs also share the

ability to be undifferentiated cells and to differentiate, giving rise to their progenies in tissue and to tumor heterogeneity, respectively. Moreover, both cell types exhibit a resistance to environmental toxins, chemotherapeutic and radiation agents, often as a result of multidrug resistance (MDR) via expression of ABC (ATP-Binding Cassette) family of transporter proteins. NSCs and CSCs also share the characteristic of being able to survive in anchorage independent conditions (resistance to anoikis), leading to migration for stem cells and potentially to metastatic disease for tumor. Finally, both cell types are long-lived with active telomerase activity and anti-apoptotic pathways.

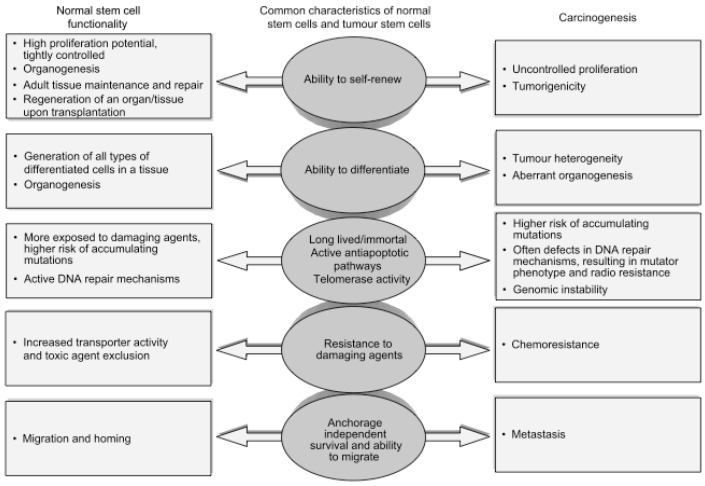

Figure 3. **Similarities between NSCs and CSCs and their impact on stem-cell functionality and carcinogenesis.**

1.1.3 Implication of the CSC theory in cancer treatment

The CSC theory has important implications in cancer treatment. Current anti cancer therapy is effective for debulking the tumor mass but often treatment effects are transient, with occurring tumor relapse and metastatic disease. A possible explanation for the failure of anti-cancer therapies is that they fail to kill CSCs. As mentioned before, NSCs from various tissues tend to be more resistant to chemotherapeutics than mature cell types from the same tissues (Harrison and Lerner 1991). One of the reasons that could explain this is that NSCs express high levels of anti-apoptotic proteins (Feuerhake et al. 2000) and ABC transporter proteins (Zhou et al., 2001). CSCs also seem to overexpress transporter proteins and this could explain why these cells are more resistant to chemotherapeutics than committed/differentiated tumor cells with limited proliferative potential. Even therapies that cause complete regression of tumors might spare enough CSCs to allow regrowth of the tumors. Targeting differentiated as well as tumor stem cells is a prerequisite for therapy to be efficient. On the other hand, it is noteworthy to consider the recent emerging possibility of the existence of equilibrium between CSCs and non-CSCs within tumors. In particular, whereas CSCs can differentiate into non-CSCs giving rise to the tumor heterogeneity, the reverse process would be possible: non-CSCs, could be reprogrammed into CSCs. This phenotypic plasticity has implications for cancer treatment: if non-CSCs can give rise to CSCs, therapeutic elimination of CSCs may be followed by their regeneration from residual non-CSCs, allowing tumor regrowth and clinical relapse.

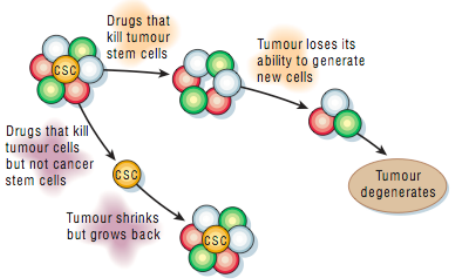

Figure 4. Comparison between conventional therapies and CSCs targeted therapies

1.1.4 Evidence for CSCs

CSCs were first discovered in Acute Myeloid Leukemia (AML). In this case, thanks to the markers $CD34^{+}CD38^{-}$ that are normally expressed on the surface of normal myeloid stem cells, Lapidot and colleagues were able to distinguish AML stem cells from the remaining AML cells with limited proliferative potential (Lapidot et al. 1994; Bonnet and Dick 1997). This cell population (approximately 0,2%) was found to be the only capable to propagate leukaemia in immunodeficient mice, providing evidence that not all AML cells are tumorigenic in vivo and that only the small subset of CSCs was capable of regenerating the cancer. The first solid CSCs were identified in breast tumors (Al-Hajj et al. 2003), and then CSCs were isolated from brain (Singh et al. 2003), colon (O'Brien et al. 2007), melanoma (Fang et al. 2005), pancreatic (Hermann et al. 2007), prostate (Collins et al. 2005), ovarian (Bapat et al. 2005), lung (Ho et al. 2007) and gastric (Fukuda et al. 2009) cancers.

Breast cancer was the first tumor in which a subpopulation with cancer stem characteristics has been isolated by Clarke and colleagues. In this study, in nine breast cancer samples, a minority of cells, expressing the surface marker phenotype $CD44^{+}CD24^{-}$, were able of generating tumors in NOD/SCID mice even when implanted in low numbers. By contrast, the other cancer cell populations, such as $CD44^{+}CD24^{+}$, failed to generate tumors even when implanted in high numbers (Al-Hajj et al. 2003).

Using a clonogenic sphere culture technique, CSCs were isolated from human brain tumors (glioblastomas) as spherical structure called neurospheres (Singh et al. 2003). These neurospoheres are highly enriched for cell surface marker CD133 and nestin (a neural stem cell marker), have a marked proliferation capacity, self-renewal and are able of in vitro differentiation into phenotypes identical to the tumor in situ. The original culture methodology employed by Singh has been used to isolate and characterize cells suspected of being stem or progenitor cells also in other cancer types.

1.1.5 Origin of CSCs

Initially the CSC theory considered the normal stem cells as the appropriate candidates for tumor initiating thanks to their longevity and possibility to accumulate mutation by the time. But recently there has been some controversy about the nature of the cells that serve as targets of transformation. A revisiting of the theory proposes that the cells of origin, the normal cell that acquires the first cancer promoting-mutation, is not necessary related to the CSCs, the cellular subset that uniquely sustain malignant growth. In fact, in a variety of malignancies, evidence for the clonal generation of tumors that display markers of multiple lineages has confirmed the stem cell as the cell of origin. However, in other cases, such as acute promyelocitic leukemia and chronic myelogenous leukemia, there is evidence for the transformation of progenitor cells. The transformation of progenitor cells might require mutations that allow them to undergo self-renewal, normally a process limited to stem cells.
Since specific pathways are involved in the embryonic and/or tissue stem cells self-renewal, it is not surprising that their altered maintenance in NSCs or their activation in non-stem cells, provide for a subpopulation with features indicative of stem cells. In several types of cancer, poorly differentiated tumors show expression of genes normally found in embryonic stem cells, including Nanog, Oct-4, Sox-2 and c-Myc (Ben-Porath et al. 2008).
Recently, it has been proposed that progenitors or differentiated cells gain features of stem cell through the induction of the epithelial-mesenchymal transition (EMT), a process that triggers the transition of epithelial cells to a less differentiated (mesenchimal-like) stage. Studies have shown that EMT induction creates populations of cells that are highly enriched for CSCs properties in transformed mammary epithelial cells (Mani et al. 2008; Morel et al. 2008) and that CSCs stem isolated from mammary epithelial cells cultures express markers similar to those of HMLEs that have undergone an EMT.
Poor tumor differentiation due to extensive EMT may therefore predispose cells to becoming CSCs.

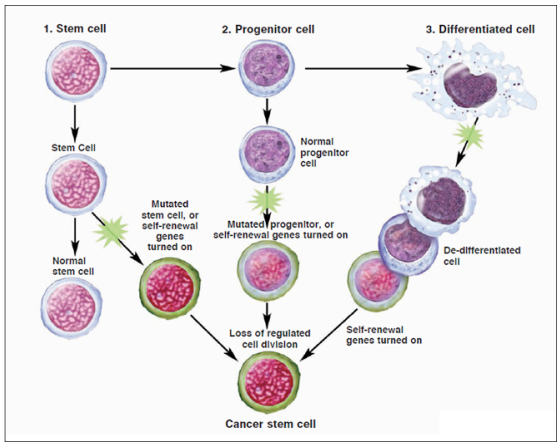

Figure 5. **Three are the hypotheses of how a cancer stem cell may arise:** (1) A stem cell undergoes a mutation (2) A progenitor cell undergoes two or more mutations or (3) A fully differentiated cell undergoes several mutations that drive it back to a stem-like state. In all three scenarios, the resultant cancer stem cell has lost the ability to regulate its own cell division.

1.2. The human mammary gland and stem cells in normal human breast

In humans, the mammary gland is a hormone sensitive, branching, and bilayered epithelial organ and is embedded within a fibrous and fatty connective tissue called stroma. The functional units of the mammary gland are comprised of terminal ducts and alveoli (lobules), which together form the terminal duct lobular units (TDLUs) and are responsible for the milk production during pregnancy. TDLUs form the branches of a greater ductal-lobular system composed of an inner layer of polarized luminal cells and an outer layer of myoepithelial cells. Myoepithelial cells are contractile cells that form a sheath around the ductal network of the breast and are characterized by expression of common Acute Lymphoblastic Leukaemia Antigen (CALLA) (Gusterson et al., 1986), Thymocyte Differentiation Antigen (Thy-1) (Gudjonsson et al., 2002), Alpha-Smooth Muscle actin (α-SMA) (Gugliotta et al., 1988), Vimentin (Guelstein et al., 1988), and Cytokeratin (CK) 5 and CK14 (Taylor-Papadimitriou et al., 1989). Luminal epithelial cells are cuboidal/columnar cells that line the ducts and alveoli. They are characterized by expression of Mucin-1 (MUC-1) (Petersen et al. 1986), epithelial cell adhesion molecule (EpCAM) (Latza et al. 1990), and CK7, CK8, CK18, and CK19 as well as estrogen receptor (ER) and progesterone receptor (PR).

The human breast is a dynamic gland and, unlike other organs that develop during embryogenesis and preserve their architecture throughout adult life, its morphology changes during embryonic development, pubertal, pregnancy/lactation, within menstrual cycles, and involution during menopause. These changes are under control of estrogen and progesterone hormones. During pregnancy and lactation, the breast goes through further rounds of development with an increase in cell growth and differentiation of the luminal epithelial lineage into functional milk-secreting alveolar cells. Cessation of lactation following weaning is accompanied by massive apoptosis and tissue remodeling, and the gland reverts to a structure resembling that before pregnancy.

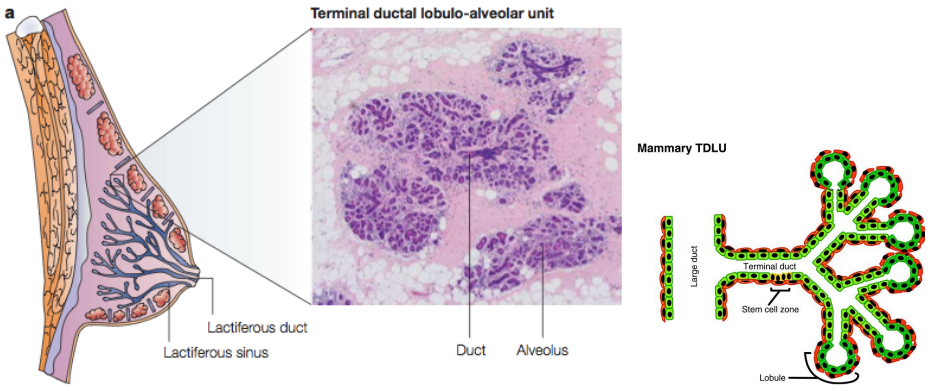

Figure 6. **Schematic representation of the human breast structure.**

Development and remodeling of mammary epithelium is hypothesized to be via the mammary stem cells, which are defined as those cells with high proliferative potential and differentiation ability in order to generate both the ductal and alveolar structures of the mammary gland.

The first who demonstrated, in the late 1950s, the existence of adult stem cells in mammary was DeOme and colleagues. Using a vivo serial transplantation assays, DeOme demonstrated that a small tissue fragment, transplanted into pre-cleared fat pads, could generate a mature functional mammary tree containing ductal, alveolar and myopethelial cells (Daniel et al. 1968). More recently, Dontu and colleagues developed a culture system to isolate and propagate in vitro human breast stem/progenitor cells in an undifferentiated state as non-adherent spherical

clusters, termed ‘mammospheres’. Mammospheres have been demonstrated to be clonally derived from single self-renewing cells. Moreover, they encompass undifferentiated precursors capable of differentiating along the three cell lineages of the mature mammary gland epithelium (luminal, myoepithelial and alveolar cells) in reconstituted three-dimensional culture systems (Dontu et al. 2003).
The isolation and characterization of mammary stem cells is fundamental to understanding mammary gland development and tissue homeostasis as well as breast oncogenesis. The most useful markers for isolating human mammary stem cells are the epithelial cell adhesion molecule EpCAM, the CD49f and, to a lesser degree, the luminal cell-specific glycoprotein MUC1, the common acute lymphoblastic leukemia antigen CALLA and the GPI-linked glycoprotein Thy-1 (Stingl et al., 1998; Stingl et al., 2001; Eirew et al., 2008; Villadsen et al., 2007; Raouf et al., 2008). EpCAM is an epithelial-specific molecule that is expressed at high level by luminal cells; CD49f is an integrin known to participate in cell adhesion as well as cell surface mediating signaling and displays a pattern of expression inverse to that of EpCAM (Stingl et al. 1998; Carter et al. 1990). Although both EpCAM and CD49f are not particularly useful for identifying different subsets of HMECs when separately used, when combined they permit a number of distinct cell types to be resolved by flow cytometry. MUC1 is a cell surface glycoprotein expressed by most epithelial cells and involved in cell-cell and cell-substrate adhesion. CALLA is a membrane-associated endopeptidase overexpressed in many neoplastic cell types and Thy1 is a membrane-associated glycoprotein involved in cell adhesion, migration and proliferation. In vitro analyses of human mammary epithelial colony-forming cells (Ma-CFCs) have demonstrated the existence of three distinct progenitors within the human mammary epithelium. 1) The luminal-restricted progenitors which generate colonies that are composed of cells with a luminal cell phenotype (expressing CK8, 18, 19 and 9). Replating of these colonies in secondary CFC assays reveals that cells of these colonies are indeed restricted to a luminal cell fate, since they only form pure luminal cell colonies. These luminal restricted progenitors display the phenotype EpCAM high $CD49f^{+}$ $MUC1^{+}$ $CALLA^{-}$ $Thy1^{-}$ (Stingl et al. 2001; Villadsen et al. 2007). 2) The bipotent progenitors display EpCAM low CD49f high $MUC1^{-}$ $CALLA^{+}$ $Thy1^{+}$ antigenic phenotype. These progenitors generate colonies that are characterized by a central core of $CK14^{-}CK18^{+}CK19^{+}MUC1^{+}$ luminal cells and peripheral zone of $CK14^{+}$ basal cells. Single-cell cloning of these progenitors confirms that these mixed colonies are clonal in origin (Stingl et al. 2001) 3) The myoepithelial restricted progenitors generate colonies that are composed solely of $CK14^{+}CK18^{-}CK19^{-}MUC1^{-}$ basal-like cells. Although recent findings have clarifying some aspects of mammary hierarchy, the precise number and nature of the intermediates remain still elusive. A simple model that accommodates much of the mounting data was proposed by Visvader and

colleagues and show that the stem cell gives rise to a common progenitor that produces committed progenitor cells for either the myoepithelial or luminal epithelial lineages (ductal and alveolar sublineages). Furthermore, the luminal progenitor subpopulation can commit to either a ductal or alveolar cell fate, dependent on the developmental stage (puberty or pregnancy) (Visvader et al. 2009).

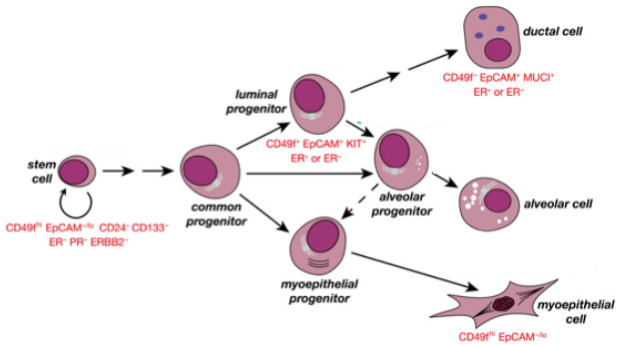

Figure 7. Model of the differentiation hierarchy within mammary epithelium. A stem cells give rise to a common progenitor, also referred as a bipotent progenitor cell. The common progenitor produces committed progenitor cells for either the myoepithelial or luminal epithelial lineages. During pregnancy, the alveolar progenitor may exhibit bipotential capacity.

1.3 Breast cancer

Breast cancer is a heterogeneous group of tumors originating from the epithelial mammary tissue. It is one of the most common causes of cancer-related mortality in women worldwide with more than one million women diagnosed every year and half a million dying for this disease. Worldwide, breast cancer accounts for 22.9% of all cancers (excluding non-melanoma skin cancers). Thanks to the widespread use of screening mammograms, the majority of breast cancers is diagnosed before clear symptomatology appears (Akhigbe et al. 2010). In other cases, the most common symptoms are the identification of a new lump or mass, a change in the aspect of the skin (redness, swelling), the presence of a secretion (in particularly if blood) or the retraction of the nipple. Sometimes a breast cancer can spread to

lymph nodes under the arm or around the collarbone and cause a lump or swelling there, even before the original tumor in the breast tissue is large enough to be felt. Multiple factors, known and unknown, contribute to human breast cancer. Environment, hereditary, hormonal, and reproductive factors are the most important associated risk factors associated with breast cancer (Anderson et al. 2014). The majority of breast cancer cases are not hereditary and over 70% of breast cancers have been attributed to environmental agents (chemical carcinogens exposition, tobacco smoke, alcohol, and diet). However, about 5% to 10% of breast cancer cases are thought to be hereditary and the most common cause of hereditary breast cancer is an inherited mutation in the BRCA1 and BRCA2 genes (Teng et al. 2008). Estrogen hormone exposition and reproductive factors also play a crucial role in breast cancer onset. After an early embryonic/fetal growth of breast, the major development processes takes place during puberty and pregnancy. From puberty and onward, systemic steroid hormones inflict changes in the epithelium as well as the adjacent stroma through each estrous cycle, and at menopause when hormonal levels change, the gland involutes. Developmental changes associated with cycling and pregnancy are critical parameters of cancer susceptibility and for this reason the risk factors for the breast cancer include early menarche, late menopause and late first pregnancy.

1.3.1 Breast cancer: a heterogeneous disease

Breast cancer, rather than constituting a unique entity, comprises heterogeneous tumors with different clinical characteristics, biologic behavior, and responses to specific treatments (Bertos et al. 2011). Over the years, many breast cancer classification systems have been developed and have tried to be used as toll for prognosis and treatments dosing. The different breast cancer classification systems are based on several tumor-intrinsic features that categorized breast tumors into multiple subtypes, including histological, immunopathological and molecular subtypes.

1.3.2 Histopathology and immunological classification

Classical pathology has segregated breast tumors into multiple categories, based on their overall morphology and structural organization. Breast cancers can be categorized into in situ carcinoma and invasive or infiltrating carcinoma (Figure 1.4 A) In situ breast carcinoma is further sub-classified as ductal or lobular, if they originate from ducts or lobules respectively. In Situ Ductal Carcinoma (DCIS) is considerably more common than in situ Lobular Carcinoma (LCIS) and includes a heterogeneous group of tumors. Particularly, DCIS has traditionally been further sub-classified on the basis of the architectural features of the tumor which has given rise to five well recognized subtypes: Comedo, Cribiform, Micropapillary, Papillary and Solid (Gautam et al. 2010; Figure 1.4a). Similar to in situ carcinomas, invasive carcinomas are a heterogeneous group of tumors differentiated into histological subtypes. The major invasive tumor types include infiltrating ductal carcinoma (IDC) (Figure 1.4 B), invasive lobular (Figure 1.4 C), ductal/lobular, mucinous (colloid), tubular, medullary and papillary carcinomas. Among these tumor types, IDC is the most common subtype accounting for 70–80% of all invasive lesions (Li et al. 2005). IDC is further sub-classified as either well-differentiated (grade 1), moderately differentiated (grade 2) or poorly differentiated (grade 3) based on the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Figure 1.4 A). Although this classification has been a valuable tool for several decades and has been useful as prognostic value, the outcome and the variable clinical response of patients to therapy reveals that a considerable heterogeneity linking to therapy still exists. For this motif, it has become necessary to more accurately stratify patients based on relative risk of recurrence or progression. These demands have led to the generation of several newer classification systems that uses the presence of specific markers to both define subtypes with differential prognosis and provide tumors responds to treatments. The main markers assessed are estrogen receptor (ER), progesterone receptor (PR), and human epidermal receptor 2 (HER2). Combinations of these markers allow for the assignment of individual cases to specific categories, namely ER+ ($ER^+/HER2^-$), HER2+ ($ER^-/HER2^+$), triple negative (TN; $ER^-/PR^-/HER2^-$), and triple positive ($ER^+/PR^+/HER2^+$).

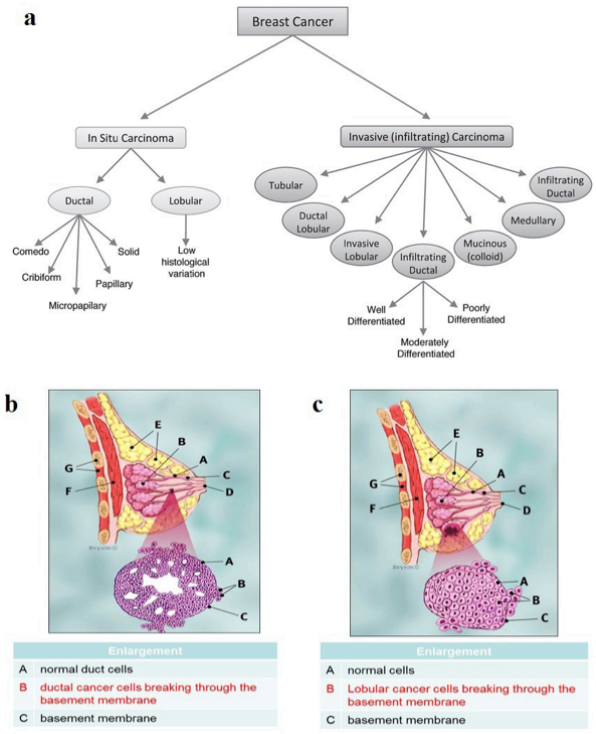

Figure 8. **Hystological subtypes of breast cancer.** (A) Hystological classification of breast cancer. (B, C) Schematic representation of invasive Ductal Carcinoma (B) and Invasive Lobular Carcinoma (C).

1.3.3 Molecular breast cancer classification

Over the past decade, the advent of high-throughput microarray-gene expression profiling has facilitated relatively large-scale studies leading to the identification of multiple molecular subtypes. These molecular subtypes has been described and defined by distinct transcriptional signatures that partially recapitulate the original immunopathological classes, and adding an additional level of detail (Sorlie et al. 2003; Perou et al. 2000; Sorlie et al. 2001). Two luminal subtypes (A and B) contain principally ER+ cases and are distinguished by the presence of genes regulated by the ER signaling pathway. The luminal A subtype is associated with higher levels of ER and ER-regulated genes, low proliferation, and improved overall outcome (Sorlie et al. 2001; Perou et al. 1999; Perreard et al. 2006).

Luminal B tumors are ER^+ and/or PR^+ and appear to have an increased proliferation and relatively worse prognosis. The molecular ERBB2+ subtype generally (but imperfectly) overlaps with IHC-defined HER2+ tumors (Perou et al., 2000), and previously was associated with poor outcome, now exhibit an improved overall outcome when treated with therapies anti-ERBB2. The basal or basal-like subtype broadly corresponds to the TN ($ER^-/PR^-/HER2^-$) cohort and is linked to the worst prognosis (Nielsen et al. 2004). A normal-like molecular subtype resembles normal epithelial tissue and may comprise cases in which samples contain large amounts of non-tumor tissue (Parvin et al. 2012; Huang et al. 2012; Parker et al. 2009)

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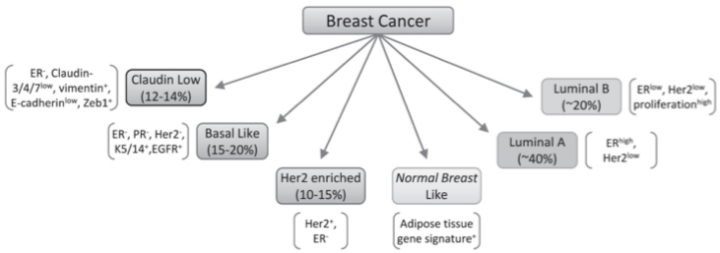


Figure 9. Molecular classification of breast cancers.

1.4 Breast cancer stem cells

Al Hajj and colleagues were the first that identify a subpopulation of human breast cancer stem cells, which is able to initiated tumors in immune-deficient NOD/SCID mice (Al Hajj et al., 2003). They used a set of cell surface markers to sort cells with an increased tumorigenic capacity. In particular, cells which were $CD44^+$ CD24 low $EpCAM^+$ and lineage- (it refers to non-CD45 and CD31 expressing cell to rule out blood or endothelial cell contamination) isolated from one primary breast cancer and eight metastases, were able to form heterogeneous tumors eight out of nine times. As few as 200 $CD44^+$ CD24 low $EpCAM^+$ lin^- cells transplanted into NOD/SCID mice could form tumors with 100% efficiency, while $CD44^-$ $CD24^+$ $EpCAM^-$ cells formed no tumors. CD24, also known as heat stable antigen (HAS), is an adhesion molecule specifically expressed in luminal

epithelial cells in human mammary gland. The tumors generating by this subset of cells contained not only the $CD44^+$ CD24 low $EpCAM^+$ lin^- population but also the phenotypically diverse non-tumorigenic cells that comprise the bulk of tumors. Subsequently, Ponti and colleagues carried out a study on sixteen breast lesions (thirteen primary invasive carcinomas, one recurrent carcinoma and two fibroadenomas) and using the sphere culture technique, were able to obtain three long-term primary cultures, which had self-renewing capacity and could differentiate into the different breast lineages (Ponti et al. 2005).

1.4.1 Pathways involved in breast cancer self renewal

The understanding of the pathways that govern the self- renewal of normal stem cells, and the ways in which these are deregulated during carcinogenesis, results fundamental for understanding the cancer stem cells biology. The pathways of Hedgehog, Notch, Wnt, RTKs and a transcription factor Bmi-1 have been found to play an important roles in mammary gland development and to be involved in the regulation of stem cell self-renewal and differentiation. Recent evidence has shown that the deregulation of these pathways has a pivotal role in mammary carcinogenesis.

The Hedgehog signaling. The Hedgehog ligands (Shh, Dhh, and Ihh) are secreted glycoproteins. After secretion, these ligands bind to the hedgehog-interacting protein 1 (Hip1) and Patched (Ptch), which are transmembrane receptors for these ligands (Ingham et al. 1991). Two transmembrane proteins, Ptch and Smoothened (Smo), form the receptor complex in the absence of ligands. Ptch binds to Smo and blocks its function (Taipale et al. 2002). This inhibition is relieved in the presence of ligands, and Smo interacts in a signalling cascade that results in activation of the transcription factors Gli1, Gli2, and Gli3 (Sasaki et al. 1999). Gli proteins in turn translocate into the nucleus and control target gene transcription. Gli regulates the transcription of several genes, including those controlling cell proliferation such as cyclin D, cyclin E, Myc, components of the epidermal growth factor pathway, and angiogenesis components including PDGF (platelet derived-growth factor) and VEGF (vascular endothelial growth factor) (Lewis et al. 2004). GLI family also directly regulate Nanog gene, a critical mediator of self-renewal (Po et al. 2010). Using the mammosphere-based culture system to examine the role of hedgehog signalling in mammary cell fate determination, Dontu showed that the addition of recombinant Shh can stimulate the formation of primary and secondary

mammospheres suggesting that hedgehog signalling is involved in mammary stem cell self-renewal (Liu et al. 2006). The importance of hedgehog signalling in carcinogenesis has been demonstrated by the fact that many of the genes involving hedgehog signalling are known oncogenes, including Smo, Shh, Gli-1, and Gli-2, or that Ptch1 can function as a tumor suppressor (Lewis et al. 2004)

Notch signalling. Activation of the Notch pathway depends on binding of a Delta-type or Jagged-type ligands expressed on the cell surface to a Notch-like receptor on the surface of a neighboring cell (Bettenhaussen et al. 2005, Rebay et al. 1991). The interaction between ligand and receptor causes cleavage of NOTCH, releasing the soluble intracellular domain (Schroeter et al. 1999). This intracellular NOTCH fragment translocates to the nucleus, where it associates with CBF1/Su(H)/LAG1 (CSL) family proteins (Jarriault et al. 1995). Notch binding switches the activity of CSL DNA binding proteins from transcription repressors to transcription activators to mediate the expression of target genes (Jarriault et al 1995).
Notch signaling plays an important role in regulation of embryonic development (Poulson et al. 2012, Swiatek et al. 1994), by regulating both restriction and specification of cell fate (Parks et al. 1997, Kim et al. 1996).
Dontu and colleagues found that Notch signalling acts as a regulator of asymmetric cell fate decisions during development and that Notch activation promoted the self-renewal of stem cells, whereas in later stages of development it biased cell fate decisions in mammary progenitor cells toward the adoption of a myoepithelial cell fate versus an epithelial cell fate (Dontu et al. 2004).
In cancer, increased expression of Notch pathway components has been associated with EMT process, and enhanced migration and invasion of mammary cancer cells, whereas inhibition of Notch signaling reverted the EMT phenotype and decreased expression of vimentin, Snail, Slug, and ZEB1 in human breast cancer cell lines (Wang et al. 2009, Bao 2011). These proteins are positive marker and/or regulator of the EMT allowing a change in the phenotype toward and undifferentiated status. Besides induction of EMT, Notch overexpression mediated upregulation of CSC markers CD44 and ESA (Bao et al. 2011). In vitro, overexpression of the constitutively active form of Notch4 inhibits the differentiation of normal breast epithelial cells. Uyttendaele and colleagues demonstrated that Notch4 has also an important role in carcinogenesis (Uyttendaele et al. 1998). Transgenic mice harbouring a constitutively active Notch4 exhibited arrested mammary gland development, and eventually developed poorly differentiated adenocarcinomas.

Wnt signalling. The canonical WNT pathway describes binding of extracellular Wnt to Frizzled family cell surface receptors and LRP co-receptors, leading to activation of Dishevelled (Bhanot et al. 1996,Cong et al. 2004). Dishevelled inhibits activity of the axin/GSK-3/APC complex which mediates proteolytic degradation of β-catenin, resulting in an increase of β-catenin levels (Kishia et al. 1999, Rubinfield et al. 1996). Interaction of β-catenin with LEF/TCF transcription factor family members promotes the expression of target genes (Behrens et al. 1996,Molenaar et al. 1996). Wnt/β-catenin signaling is important for maintenance of the self-renewal capacity in embryonic stem cells and induces Oct-3/4 dependent upregulation of the stem cell factor Nanog (Takao et al. 2007). Conversely, overexpression of Oct3/4 increased β-catenin activity and inhibited cell differentiation (Hochedlinger et al. 2005). Besides the canonical Wnt/β-catenin pathway, there are at least two non-canonical pathways downstream of WNT. The WNT/calcium pathway signals via heterotrimeric G-protein dependent activation of the phosphatidylinositol pathway, which leads to release of intracellular Ca^{2+} (Slusarski et al. 1997). As a consequence, Ca^{2+} sensitive enzymes including PKC and Ca^{2+}/calmodium-dependent kinase (CaMK)II are activated (Sheldahl et al. 1999, Küh et al. 2000). Wnt/calcium mediated upregulation of PKC leads to increased cell migration (Dissanayake et al. 2007). Moreover, acquisition of mesenchymal morphology, accompanied by upregulation of Snail and loss of E-cadherin, a key molecule at the tight junction that establish and maintain cell-cell adhesion and epithelial phonotype, indicates that increased expression of PKC also contributes to EMT.

Bmi-1. Bmi-1 is a transcriptional repressor belonging to the polycomb (PCG) group of transcription factors and is essential in maintaining chromatin silencing (Valk-Lingbeek et al. 2004). Bmi1 was first identified as an oncogene in lymphoma where it collaborates with c-Myc to promote lymphomagenesis and regulated cell proliferation and senescence through inhibiting the INK4A (Jacobs et al. 1999, Jacobs et al. 1999). Bmi1 was shown to be required for maintaining of both normal and leukaemia stem cells (Lessar et al. 2003, Park et al. 2003). It was later shown to be involved in the self-renewal of neuronal, mammary epithelium, pancreatic (including β-cell). Several recent studies have suggested a link between Bmi-1 and mammary carcinogenesis and that it resulted to be overexpressed in several human breast cancer cell lines (Liu et al. 2006). Furthermore, it was found that Bmi-1 regulates telomerase expression in mammary epithelial cells, avoiding the senescent process (Dimri et al. 2002).

RTK-Akt signalling. Receptor Tyrosine Kinases (RTKs) mediate signal transduction of multiple oncogenic cytokines or growth factors, including the epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) that are

used in culturing cancer stem cells in vitro (Dontu et al., 2003). Among these RTK pathways, the EGFR-mediated growth signalling through phosphoinositide 3-kinase (PI3K)/Akt is one of the most critical and best characterized pathways in malignant cancers. Frequently, these receptors are upregulated or mutated, leading to increase EGFR-Akt signalling in cancer cells (Moscatello et al., 1998; Choe et al., 2003). Isteed, Chan and colleague demonstrated that EGFR kinase inhibitor reduces epithelial proliferation in normal and premalignant breast cancer cells (Chan et al. 2002). In breast cancer, HER2, a member of EGFR family, plays an important role in breast development and mammary carcinogenesis (Palafox, 2012). Although HER2 itself has no known ligand, it forms heterodimers with ligand-activated EGFR, HER3, and HER4. Several lines of evidence indicate that HER2 is an important regulator of the CSC population in HER2 breast cancers. Korkaya and colleagues showed that HER2 overexpression increases CSCs and HER2 blockade decreases the CSC population in breast cancer cell lines and mouse xenografts. In addiction, HER2 overexpression induces the expression of stem cell-related genes and activates the PI3-K/Akt pathway (Korkaya et al. 2008).

Specific transcriptional factors and maintenance of breast cancer stem cells. Sox2, Oct3/4 and Nanog are core components of stem cell transcription factor network and play crucial roles in maintaining embryonic stem cells and somatic stem cells (Ellis et al., 2004; Loh et al., 2006; Tay et al., 2008; Fong et al., 2008). They are also critical factors for cell reprogram and the generation of inducible pluripotent stem cells (iPS) (Takahashi and Yamanaka 2006). These transcriptional factors are differentially expressed in breast cancer cell line and are important for BCSCs maintenance (Ben-Porath et al. 2008). Overexpression of Sox2, Oct3/4 or Nanog enhanced tumorigenicity of breast cancer cells and promoted the self-renewal expansion of $CD24^{-}$ $CD44^{+}$ CSC subpopulation (Luet al. 2013, Leis et al. 2012, Kim et al. 2011). In contrast, knockdown of these genes significantly affected the growth of breast CSCs.

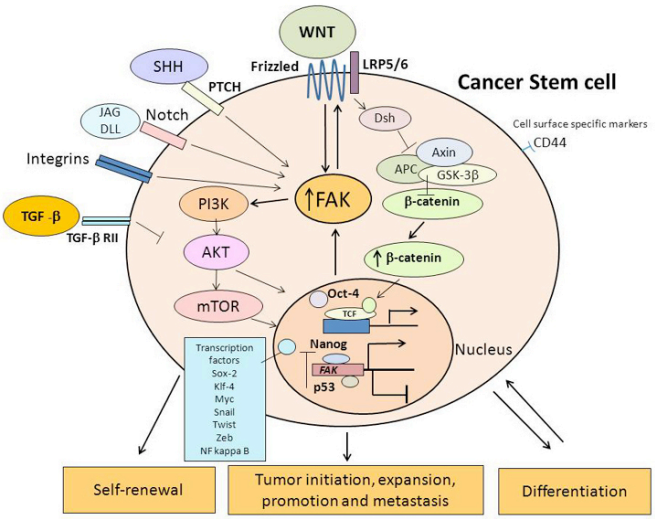


Figure 10. Pathways involved in breast cancer stem cells regulation.

1.5 microRNAs

In the last decade, many non coding-RNAs were found to regulate a wide variety of biological process. Among these, microRNAs (miRs) are the best characterized. miRs are a class of endogenous non coding-RNA with a length of 21-25 nucleotide that play an important role in the negative regulation of gene expression blocking the translation or directly cleaving the target mRNA. The biogenesis of miRs is a complex and coordinate process in which are involved different enzyme and protein (Bartel et al. 2004).
MiRs gene is first transcribed by RNA polymerase II into a long primary transcript (pri-miR) in the nucleus. Generally, pri-miRs show a 33 bp stem and a terminal loop structure with flanking segment. Primary miR processing starts in the nucleus where Drosha, a RNAseIII enzyme, removes the flanking segment and 11 bp of the stem region and convert the pri-miR into precursor miR (pre-miR). Pre-miRs are 60-70 nt long hairpin RNAs with 2-nt overhangs at 3' end. Pre-miRs are transported into the cytoplasm for further processing to become mature miRs. The transport occurs through a nuclear pore complex and is mediated by the RanGTP-dependent nuclear transport receptor exportin-5 (EXP5). EXP5 exports the pre-miR out of the nucleus where hydrolysis of the GTP results in the realise of pre-miR. In the cytoplasm the pre-miR is subsequentially processed by Dicer, an endonuclease cytoplasmatic RNAse II enzyme, to create a mature miR. Dicer is a highly specific enzyme that cleaves pre-miRs in 21-25 nt long miR duplex, of

which each strand bears 5' monophoshate, 3' hydroxyl group and 3' 2-nt overhang. Of a miR duplex, only one strand, designed the miR strand, is selected as the guide of the effector RNA-induced silencing complex (RISK). The core component of RISK is a member of Argonaute (Ago) subfamily proteins. During RISK loading, the miR duplex are incorporated into Argo proteins. Risk loading is not a simple process but also an ATP dependent active process. After risk loading, the duplex is unwound and the complex maintains only the miRstrand (Bartel et al. 2004, Lee et al. 2002, Gregory and Shiekhattar 2005).
miRs target sites lie in the 3'untranslating region (UTR) because the movement of ribosome (the translation) counteracts RISK binding. Typically, a target mRNA bears multiple binding site of the same miR and/or several different miRs. Not all nucleotidies of a miR contribute equally to RISK target recognition. The recognition of the target is largely determined by base paring of nucleotides in the seed region and is enhanced by additional base-pairing in the middle of the 3'UTR region. The binding of RISK to 3'UTR of mRNA, through the action of AGO protein, is capable of RNA cleavage, but this reaction requires extensive base pairing between the miR strand and mRNA target. This is the same mechanism used by siRNA.

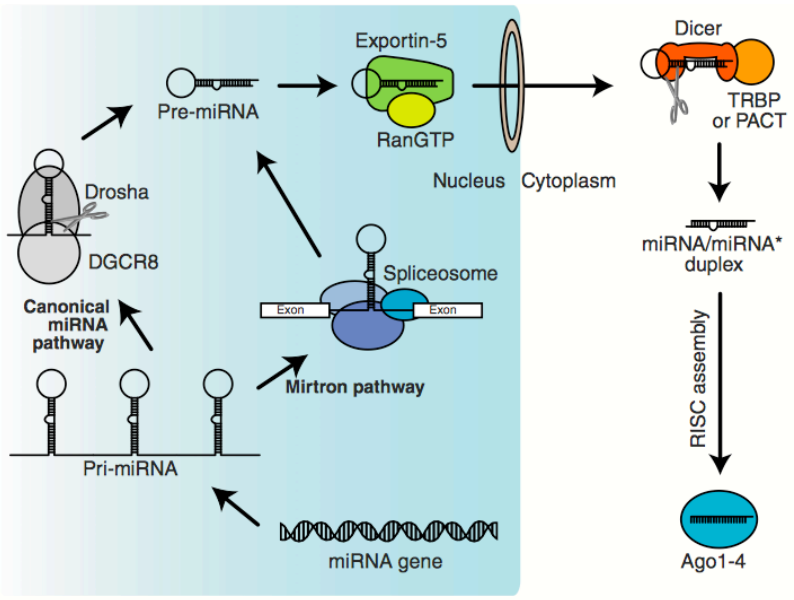


Figure 11. Biogenesis of miRs

If the complementary between the miR strand and the mRNA is limited, RISK is incapable to cleave target. In such case, Ago protein can recruit other factors required for translation repression and subsequentially mRNA deadenylation/degradation (Lewis et al. 2003). To date, the exact mechanism used by RISK to repress translation is subjected of debate. Between the mechanisms proposed at least six seems to be possible: RISK could induce deadenylation of mRNA which decrease the efficiency of translation by blocking mRNA circularization, RISK could block the cap at 5' or the recruitment of ribosomal subunit 60S; RISK could block the initial step of elongation or could induce proteolysis of nascent peptides; RISK could initial recruits mRNA to processing bodies, in which mRNA is degraded or temporary stored in an inactive form. These models do not necessarily exclude each other.

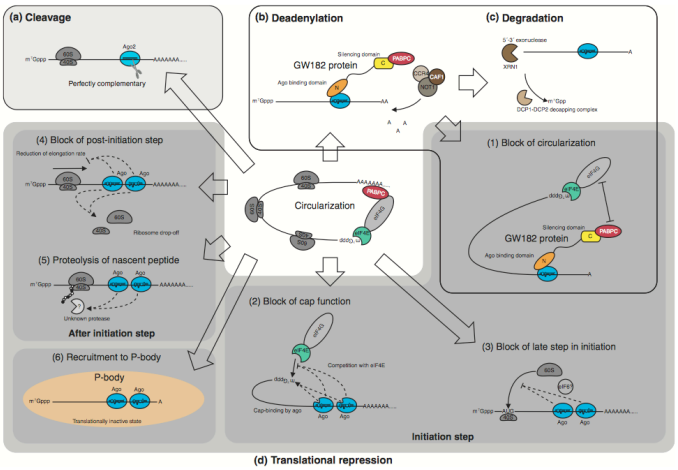


Figure 12. Various mechanism of regulation of gene expression by RNA-induced silencing complex (RISK).

1.5.1 Function of miRs in animals

MiRs have key roles in the regulation of distinct process in mammals. MiRs play an evolutionally conserved role in the development and in physiological function in animal. Knockout gene strategies have been used in different mammals to study the role of miRs in development processes. A dicer knockout was made in Zebrafish and this revealed a role of the family of miR-140 in Zebrafish

neurogenesis. MiRs can also control late stage mouse development by miR-196 which act upstream Hox B8 and Sonic hedgehog in limb formation. miR-182 is preferentially expressed in B-lymphocytes and regulates mouse hematopoietic lineage differentiation (Chen et al. 2004). miR-133 and miR-1 are essential for the differentiation of ESCs into cardiomyocytes. MiR-122a is highly express in adult livers and its expression is upregulated during mammalian liver development (Chang et al. 2004). In mouse ESCs several miRs have been shown to promote differentiation by targeting genes encoding transcription factors involved in the maintenance of stem cell identity. For instance, miR-200c, miR-203 and miR-183 cooperate to repress Sox2 and Klf4 (Wellner et al. 2009). Upon retinoic-acid-induced differentiation of mESC, miR-134, miR-296 and miR-470 are up-regulated and target the coding regions of Nanog, Oct3/4, and Sox2 (Tay et al. 2008). Recent studies have also reveal that miR can act as important molecular mediator of the stemness process in adult stem cells. For example, miR-125a controls HSC population size by inhibiting their apoptosis via translational repression of the pro-apoptotic protein Bak1 (Guo et al. 2010). miR-128 and miR-181 are expressed only in early progenitor cells and prevent the differentiation of all hematopoietic lineages. Let-7b overexpression inhibits NSC proliferation and accelerates neural differentiation by targeting TLX and cyclinD1 involved in the regulation of cell cycle progression.
Some miRs regulate diverse physiological process, for example miR-375 is express in pancreatic islet and inhibits glucose-induced insulin secretion.
Several studies have establish a role of MiRs in also in other cellular processes including apoptosis, proliferation, stress response, metabolism etc.

1.5.2 miRs in cancer

Recent studies have demonstrated a link between deregulated expression of miRs and carcinogenesis. A number of miRs have been shown to function as oncogenes or tumour suppressor during tumor development. The first evidence for miR involved in human cancer came form a study by Calin (Calin et al. 2002). Examining a recurring deletion at chromosome 13q14 in the search of tumour suppressor gene involved in Chronic Lymhocitic Leukaemia (CLL), it was found that the region of deletion encodes two miRs, miR-15a and miR-16-1. Subsequent investigations have confirmed the involvement of these two miRs on the pathogenesis of CLL (Calin et al. 2005, Cimmino et al. 2005).
To date, a lot of miRs have been characterized for their function in cancer.

Constinean and colleagues reported, for the first time, that a miR by itself could induce a neoplastic disease (Constinean et al. 2005). In fact, by using a transgenic mouse model, they demonstrated that overexpression of miR-155 in B-cell induce a lymphoma pre-B leukaemia. Then, Petrocca and colleagues (Petrocca et al. 2008) have demonstrated that miR106b-25 cluster plays a key role in gastric cancer interfering with proteins involved both in cell cycle and apoptosis, whereas Johnson have showed that Let-7 family contains miRs that are able to regulate the RAS family of oncogenes (Johnson et al. 2005).

Deregulation of miR expression levels emerges as the main mechanism that triggers their loose or gain of function in cancer cells. The activation of the oncogenic transcription factor such as Myc, represents an important mechanism for altering miRs expression. Genomic aberration such as amplification, chromosomal deletions, point mutation or aberrant promoter methylation might alter miRs expression. Chromosomal abnormalities can trigger oncogenic action of miRs by modulating miR expression in the wrong cell type or at wrong time.

Several examples of miRs whose expression is deregulated in human cancer have been reported. miR-155 is overexpressed in Hodgkin lymphoma, in pediatric Burkitt lymphoma and in diffuse large B-cell Lymophoma (Eis et al. 2005, Kluiver et al. 2005). MiR-21 is upregulated in breast cancer and in glioblastoma, while miR-143 and miR-145 genes are significantly downregulated in colon cancer tissue compared with colon mucosa (Micheal et al. 2003). Evidence now indicates that the involvement of miRs in cancer is much more extensive that initially expected. Studied that investigated the expression of the entire microRNAome in various human solid tumours and hematologic malignancies have revealed difference in miR expression between neoplastic and normal tissue (Calin et al. 2005, Pallante et al. 2006; Weber et al. 2006). These studies show that each neoplasia has a distinct miR signature that differs from that of other neoplasm and that of the normal tissue counterpart. Moreover, it has become clear that some miRs are recurrently deregulated in human cancer. In the most case, miRs are upregulated or downregulated in all tumours suggesting a crucial role of these miRs in tumorigenesis. However, there are some unusual situation: for example member of miR-181 family are up-regulated in some cancer, such as thyroid (Pallante et al. 2006), pancreatic (He et al. 2005), and prostate carcinoma (Volina et al. 2006) but downregulated in others such as glioblastomas and pituitary adenomas (Cianfrè et al. 2005, Bottoni et al. 2007).

In this context we showed that, in glioma cell lines, high expression levels of miR-21 and -30b/c are needed to maintain the TRAIL-resistant phenotype (Quintavalle et al. 2013). Furthermore, in the same cell lines we found that miR-221-222 overexpression induced an increase in cell migration and invasiveness by downregulating the tumor suppressor protein PTPμ (Quintavalle et al. 2012). On the contrary in Non Small Cell Lung Cancer (NSCLC) cell lines we demonstrated

that miR-212 behave as a tumor suppressor because it negatively regulates the antiapoptotic protein PED (Incoronato et al. 2010).

1.5.3 MiRs in CSCs

Since MiRs are critical for both cancer and stem cell properties, their role in CSC self-renewal is under intense investigations. Recent studies showed that MiRs are the major components of acquisition and maintenance of "stemness" of CSCs (Zimmerman et al. 2011). It is interesting that several of the MiRs found to control CSC properties are MiRs involved in the regulation of Embryonal Stem Cells (ESC) self-renewal and differentiation. For example, MiR-371–373 cluster is specifically expressed in human ESCs and is involved in stem cell maintenance. Recently, this cluster is found upregulated in undifferentiated aggressive hepatocellular cancer cells (Cairo et al. 2010), whereas in breast cancer cells, it is linked to high aggressiveness and promotes tumor invasion and metastasis by targeting CD44 (Hang et al. 2008).
Usually, MiRs that positively regulated the stemness state are upregulated. For instance, MiR-17–92 polycistron is more abundant in leukemic stem cells than in non-stem leukemic cells or than in their normal counterpart precursors and regulate stem cells by targeting p21, resulting in more proliferative cells (Wong et al. 2010). MiR-130b is upregulated in $CD133^{+}$ liver cancer stem cells compared to $CD133^{-}$ cells and promotes liver CSC growth and self-renewal via targeting of TP53 (Ma et al. 2010). While ESC-enriched miRs seem to be important in CSC functions, miRs able to repress pluripotency of ESC are usually inhibited in CSC. For instance, members of the Let-7 family have a role of tumor suppressor by targeting K-Ras and cMyc and their expression is repressed in lung, breast, liver and head and neck CSCs (Lo et al. 2011, Yuet al. 2011, Kong et al. 2010, yang et al 2010). In particular, Let-7 overexpression reduces proliferation, mammosphere formation, and the proportion of undifferentiated cells in vitro, as well as tumor formation and metastasis in vivo (Yu F et all. 2007). Members of the miR-200 family are downregulated in CSCs isolated from lung, ovarian, head and neck, liver, pancreatic and breast cancer compared to their non-stem cancer counterparts (Lo et al. 2011, Wu et al. 2011). As discussed earlier, expression of miR-200 represses EMT and this process of EMT an important role in the progression of cancer by promoting invasion, metastasis (Cannito et al. 2010) and stemness phenotype in differentiated cells. (Floor et al. 2011, Kong et al. 2011, Scheel et al. 2011). In breast cancer, miR-200c targets proteins involved in invasiveness,

resistance to apoptosis and induction of breast CSC characteristics (Chang et al. 2011).
In addition to the alteration of miR related to embryonal stem-regulation, studies demonstrated that miRs involved in the regulation of various adult stem cell functions also play important roles in regulation. For instance, miR-125b suppresses glioma SC proliferation by targeting CDK6 and CDC25A, thus inducing cell cycle arrest in G1 (Shi et al. 2010).

2. AIM OF THE STUDY

Currently, breast cancer represents a worldwide emergence. Although advances in classification and standard treatments have improved the life prospective of patients, the incidence of death is still high due to recurrence and metastasis. Recently, the discovery of a putative CSCs subpopulation has shed new light on the conception of tumor. This population is responsible for sustaining the tumor growth and, under determinate conditions, can disseminate and migrate to give rise to a secondary tumor or metastasis to distant organ. Furthermore CSCs have shown to be more resistant to anti-tumor treatments than the non stem cells, suggesting that surviving CSCs could be responsible for tumor relapse after therapy. These important properties have raised the interest in understanding the mechanisms that govern the generation and maintenance of this special population of cells. In the last decade it has became clear that miRs are critical players of most cellular events, including stem cell and cancer stem biology, and that they contribute actively to the regulation of cancer stem cell features such as metastasis and response to chemotherapy.

The present work aims to identify new miRs differentially expressed in cancer stem cell compared to differentiated cells and to investigate their role in the regulation of key proteins involved in the stemness homeostasis. MiRs act through the repression of target genes and the identification of "disregulated miRs" led to a deeply comprehension of the aberrant signals that operate in cancer stemness fate. The characterization of miRs able to regulate CSC self-renewal should have important implications in understanding breast cancer malignancy and behaviors as metastasis spread and drug resistance.

3. MATERIALS AND METHODS

3.1 Cells and mammospheres culture

Breast tumor differentiated cells from three patients (#1, #2,#3) and BTSCs (Breast tumor stem cells) have been obtained as previously described by Dontu and colleague(Dontu et al. 2003) and have been used for miR array. T47D cells were grown in RPMI 1640 Media supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. For mammospheres culture, single cells were plated at a density of 1,000 cells/ml. Cells were grown in a serum-free DMEM-F12 (Sigma, Milan Italy), supplemented with B27 (Life technologies Milan Italy), 10 ng/ml EGF (Sigma, Milan Italy) and 20 ng/ml bFGF (BD Biosciences, Milan Italy) and 1X antibiotic-antimycotics (Life technologies, Milan Italy). After 5-7 days, mammospheres, appearing as spheres of floating viable cells, were collected by gentle centrifugation (800 rpm) and dissociated with 0.25% trypsin for 5 min. HEK-293 were grown in DMEM Media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin.

3.2 Cells transfection

For miRs transient transfection, cells at 50% confluency were transfected using Oligofectamine (Invitrogen, Life technologies Milan Italy) with 100nM of pre-miR-221, scrambled or anti miR-221 (Ambion, Life technologies Milan Italy). In order to overexpress DNMT3b, cells were transfected using Lipofectamine 2000 with 3 μg of DNMT3b cDNA, a kind gift of Anna Portela (IDIBELL, Barcellona). To knock down DNMT3b gene expression, specific DNMT3b siRNA was transfected using Lipopfectamin 2000 at a final concentration of 100 nm (Santa Cruz Biotechnology, MA).

3.3 Virus infection

Breast primary cell line obtained from patient (patient #5) was infected using TWEEN miR-221 or TWEEN control vector as already described by Quintavalle (Quintavalle et al. 2012). Briefly, on the day of infection, the medium was removed and replaced with viral supernatant with the addition of 4 mg/ml of Polybrene. Cells were then centrifuged in their plate for 45 min in a Beckman GS-6KR centrifuge, at 1800 rpm and 32°C. After centrifugation, cells were kept for O/N in a 5% CO2 incubator at 37°C. After exposure, cells were washed twice with cold PBS and fresh medium was added. At 48 h after the infection, cells were washed with PBS, harvested with trypsin/EDTA and analyzed by FACS for GFP expression. T47D cells were infected with a lentiviral construct overexpressing a short hairpin for DNMT3b (Santa Cruz Biotechnologies, MA).

3.4 Protein isolation and Western blotting

Cells were washed twice in ice-cold PBS, and lysed in JS buffer (50 mM HEPES pH 7.5 containing 150 mMNaCl, 1% Glycerol, 1% Triton X100, 1.5mM $MgCl_2$, 5mM EGTA, 1 mM Na_3VO_4, and 1X protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (BioRad) using bovine serum albumin as the standard, and equal amounts of proteins were analyzed by SDS-PAGE (12.5% acrylamide). Gels were electroblotted into nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hr with 5% non-fat dry milk in Tris Buffered Saline (TBS) containing 0.1% Tween-20, and incubated at 4°C over night with the primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (Thermo, Euroclone Milan Italy). Primary antibodies used were: anti-Zeb-1, anti-Oct-3/4, anti-Nanog, cytokeratin 18, cytokeratin 8 (Santa Cruz Biotechnologies, MA), anti DNMT3b (Abcam) and anti-bActin (Sigma, Milan Italy).

3.5 MiR Microarray

5 μg of total RNA from each sample was reverse transcribed using biotin end labeled random-octamer oligonucleotide primer. Hybridization of biotinlabeled complementary DNA was performed on Ohio State University custom miR microarray chip (OSU_CCC version 3.0), which contains 1150 miR probes, including 326 human and 249 mouse miR genes, spotted in duplicates. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments, Sunnyvale, Calif).
Raw data were normalized and analyzed with GENESPRING 7,2 software (zcomSilicon Genetics, Redwood City, CA). Expression data were median-centered by using both the GENESPRING normalization option and the global median normalization of the BIOCONDUCTOR package (www.bioconductor.org) with similar results. Statistical comparisons were done by using the GENESPRING ANOVA tool, predictive analysis of microarray (PAM) and the significance analysis of microarray (SAM) software(http://www-stat.stanford.edu/~tibs/SAM/index.html).

3.6 Breast Primary cell cultures

Breast ductal carcinoma specimens were collected at surgical Unit of ClinicaMediterranea SPA (Naples). All samples were collected according to a prior consent of the donor before the collection, acquisition or use of human tissue. To obtain the cells, samples were mechanically and enzymatically disaggregated and then the lysates were grown in DMEM-F12 medium supplemented with 10% FBS 1% penicillin streptomycin and 20 ng/ml EGF (Sigma-Aldrich, Milan Italy). To exclude a fibroblast contamination cells were fixed and then stained for Pan Keratin and analyzed by Immunohistochemistry.

3.7 Mammospheres forming assay

Mammospheres were resuspended in 0.5% agar (Bacto-Agar, Difco Laboratories) and layered on preformed 0.8% agar layer using a 60 mm Petri dishes (BD). Colonies were counted under an inverted microscope (Nikon) and then photographed.

3.8 RNA extraction and Real-time PCR

Total RNAs (miR and mRNA) were extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription of total miR was performed using miScript reverse Transcription Kit (Qiagen, Milan Italy), for mRNA was used SuperScript® III Reverse Transcriptase (Life Technologies). Quantitative analysis of Nanog, Oct3/4 Sox2, Actin (as an internal reference), miR-221, and RNU6B (as an internal reference) were performed by Real time PCR using specific primers (Qiagen), miScript SYBR Green PCR Kit (Qiagen), and iQ™ SYBR Green Supermix (Biorad), respectively. Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (Bio-Rad).

3.9 Luciferase assay

The 3' UTR of the human DNMT3b gene was PCR amplified using the following primers: DNMT3b-Fw:5'GCTCTAGACAGCCAGGCCCCAAGCCC3'; DNMT3b-Rv: 5'GCTCTAGAACCTCAGGCTACCCCTGC3', and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega, Milan Italy). An inverted sequence of the miR-binding sites was used as negative control. HEK-293 cells were co-transfected with 1.2μg of plasmid and 400 μg of a Renilla luciferase expression construct, pRL-TK (Promega, Milan, Italy), with Lipofectamine 2000 (Invitrogen, Milan, Italy). Cells were harvested 24 hrs post-transfection and assayed with Dual Luciferase Assay (Promega, Milan, Italy) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

3.10 RNA extraction and Real time PCR

Total RNA (miR and mRNA) was extracted using Trizol (Invitrogen, Milan, Italy) according to the manufacturer's protocol. Reverse transcription of total miR was performed starting from equal amounts of total RNA/sample (1µg) using miScript reverse Transcription Kit (Qiagen, Milan, Italy), and with SuperScript® III Reverse Transcriptase (Invitrogen, Milan Italy) for mRNA. Quantitative analysis of Nanog, Oct3/4, DNMT3b, β-actin (as an internal reference), miR-221, miR-222, and RNU5A (as an internal reference) were performed by Real time PCR using specific primers (Qiagen, Milan, Italy), miScript SYBR Green PCR Kit (Qiagen, Milan, Italy), and iQ^{TM} SYBR Green Supermix (Bio-Rad, Milan, Italy), respectively. The reaction for detection of mRNAs was performed as follows: 95°C for 15', 40 cycles of 94°C for 15'', 58°C for 30'', and 72°C for 30''. The reaction for detection of miRs was performed as follows: 95°C for 15', 40 cycles of 94°C for 15'', 55°C for 30'', and 70°C for 30''. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For relative quantization, the $2^{(-\Delta CT)}$ method was used as previously described [26]. Experiments were carried out in triplicate for each data point, and data analysis was performed by using a Bio-Rad software (Bio-Rad, Milan, Italy).

3.11 FACS analysis

Cells were stained with PE anti-CD24- and anti- CD44-PerCPCy 5.5 or with negative control PE-conjugated and PerCPCy 5.5-conjugated antibody (BD). After enzymatic detachment from saturated cultures, cells were counted, resuspended in the analysis buffer at $2x10^4$ for 100µl and stained by incubation at 4°C for 20 minutes with the appropriate amount of above mentioned MoAbs. After staining, all samples were washed twice with PBS solution, centrifuged and resuspended in 0.1 ml of PBS for FACS analysis. The negative control was prepared to exclude the signal background caused by the cellular autofluorescence.

4. RESULTS

4.1 MiRs involved in stemness property

To identify miRs differentially expressed in BCSCs and involved in stemness maintenance, we performed a microarray analysis. To obtain a data that was more related to the "physiological/pathologial condition", we used primary cell line obtained by three patients' speciements (Fig 13). Briefly, biopsies were enzymatically disaggregated and epithelial cells were seeded in two different conditions. In the first, the cells were classically seed in adherence condition and grown in medium containing FBS. In the second, cells were grown using the culture system adopted by Dontu and colleagues (Dontu et al. 2003). Cells were grown in ultra-low attachment flasks, using a medium without serum but implemented with EGF and FGF growth factor. This system allows to isolate and propagate in vitro breast stem/progenitor cell as non-adherent spherical clusters, termed 'mammospheres'. Instead, in these conditions, only the cells able to be resistant to anoikis and responsible to EGF and FGF growth factors are able to survival and these characteristics are both exhibit by stem/progenitors cells. All this procedure was done in collaboration and under the supervision of MatildeTodaro and Girogio Stassi (Department of Surgical and Oncological Sciences, University of Palermo).

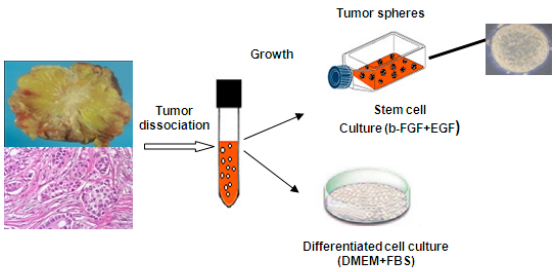


Figure 13: Biopsy disaggregation. Suspension culture is a strategy for the in vitro enrichment and propagation of human mammary stem /progenitor cells.

To confirm that these cells were really enriched for stem/progenitors cells, we analyzed the expression of some stem markers. These markers are normally express in embryonic cells, sustaining self-renewal, but their expressions have been observed also in several cancer stem populations. In particular, we analyzed the expression of Nanog, and Sox2 that usually are more expressed in cancer stem cells than differentiated counterpart. We confirmed, by Real time-PCR that the "putative stem culture" isolated from patients, expressed high levels of these genes respect to the differentiated cells in all three patients derived cell lines (Figure 14). Then, we moved on with the analysis of the global miR expression profile that characterizes breast stem cells population.

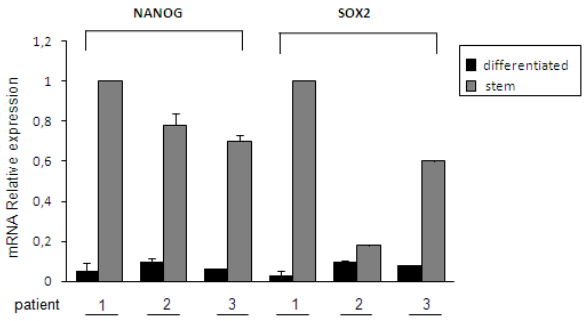


Figure 14: **Real time-PCR of Nanog and Sox2 in primary cell populations**. Nanog and Sox2 are over-expressed in cancer stem cells than differentiated cells. Representative of at least three independent experiments.

The array was performed analyzing the miR expression profile of primary BCSCs compared to the expression profile of the breast cancer cells growing in adherence (differentiated cells). The analysis was performed with a microarray chip containing 1150 miR probes, including 326 human and 249 mouse miRs, spotted in duplicates. The microarray results revealed a significant up-regulation of three miRs in cancer stem cells (miR-221, miR-24 and miR-29a) (Table 1) and a down-regulation of other miRs (miR-216a, miR-25, and let-7d). We focused our attention on miR-221 since its role in tumorigenesis was already reported in several tumor types (Quintavalle et al. 2011,Garofalo et al. 2008, Visone et al. 2007). Through a Real time-PCR on the same samples, we validated the microarray results and confirmed that miR-221 was upregulated in the stem cells populations respect to the differentiated cells. This finding led us to hypothesize

that the expression of those miR can be functional to the maintenance of stemness phenotype in BCSCs (Figure 15).

Unique id	Parametric p-value	Fold change (stem vs differentiated)
Hsa-miR-221	0,013	1,8
Hsa-miR-24	0,003	2,55
Hsa-miR-29a	0,023	2,47
Hsa-miR-25	0,001	-1,89
Hsa-miR-216a	0,003	-2,46
let-7d	0,004	-1,4

Table 1. All miRs differentially express have p value 0,05.

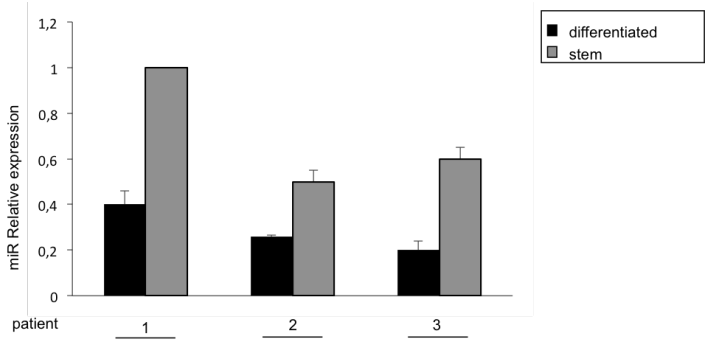


Figure 15. **MiR-221 expression levels is higher in breast cancer stem cells of patient than differentiated cells.** Relative expression of miR-221 was calculated using the comparative CT methods. Representative of at least three independent experiments.

4.2 T47D mammospheres culture is enriched of mammary stem progenitors and express high levels of miR-221

To uncover the effect of miR 221 on the stemness properties, we adopted a suspension culture of T47D breast cancer cell line. We chose a tumor cell line as an easy and reproducible model for the identification of those miRs involved in stemness functions. As well as for primary cells described before, T47D cells were seeded in non-adherent conditions and using a medium supplemented with EGF, b-FGF, B27. This system allows isolating CSCs present in to the T47D population as classical mammosphers clusters (Figure 16). We investigated the expression of stemness genes and EMT markers associated with the stem-like phenotype to verify the reliability of the method. We chose Sox2, Nanog, and Oct3/4 as stemness markers and E-cadherin and Zeb1 as EMT markers. As expected for a stemness phenotype, we found an upregulation of Zeb-1, Oct3/4 Nanog but a decrease of expression of E-cadherin, a repressor of EMT transition. Moreover, we evaluated also the levels of Cytokeratin 18 and 8, known structural proteins that regulate the epithelial structure of luminal cells. After 7 days of culture, by Western blot we observed that the levels of these two additional markers resulted downregulated in suspension-stem like cultures (Figure 17A and B).

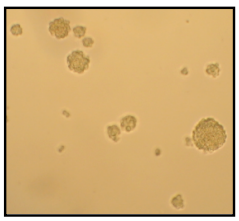
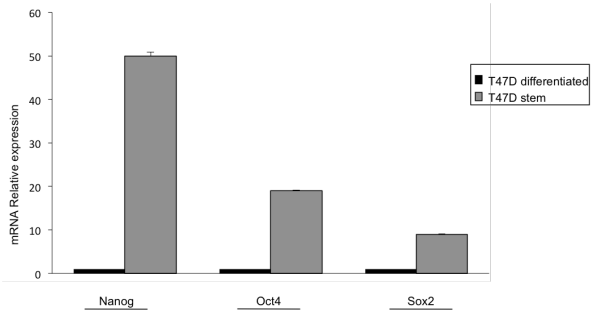


Figure 16. T47D suspension culture. The growth of T47D in non-adherent condition allow to isolate cluster of cells called “mammospheres”.

A



B

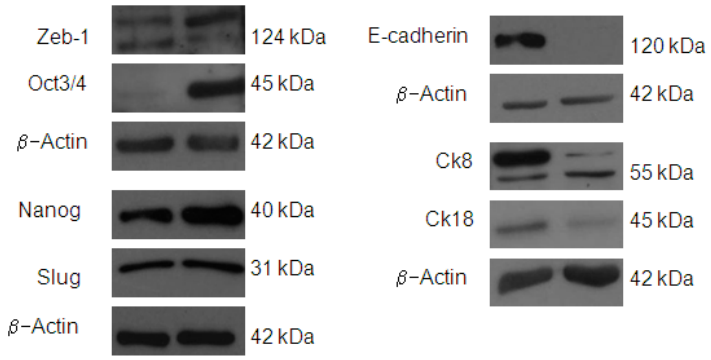


Figure 17. **T47D suspension culture is enriched for stem cells/progenitors stem.**
(A) Zeb1, Oct3/4, Slug, Nanog, Ck8, 18, E-cadherin levels were evaluated by immunoblotting. To confirm equal loading, the membrane was immnunoblotting with anti b-actin antibody. (B) Nanog and Oct3/4 for were also evaluate through Real time PCR together with Sox2. Representative of at least three independent experiments

These data confirm that the mammospheres culture of T47D can be used as a tool to obtain population enriched of stem/progenitors that express higher level of gene involved in the positive modulation of the stemness state and lower genes of those involved in epithelial phenotype.
Next, we found that the levels of miR-221 was increased also in T47D stem cells population in a similar manner to primary breast cancer cells line (Figure 18).

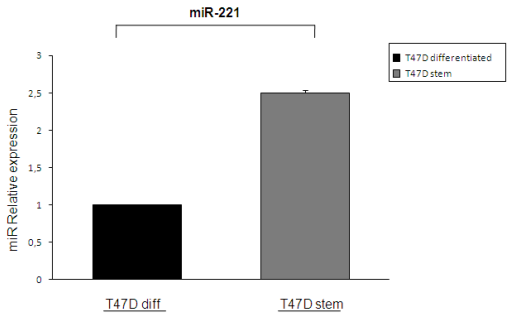


Figure 18: miR-221 levels are higher also in T47D stem cells.

4.3 MiR-221 and stemness

To address the biological role of miR-221 in BCSCs' behaviour, we modulated the levels of miR-221 through transfection and looked for T47D mammospheres formation in non-adherent conditions. After 3 or 6 days of culture in stem medium, we found that miR-221 overexpression increased the number of mammospheres compared to the scrambled oligonucleotide (control) (Figure 19A).

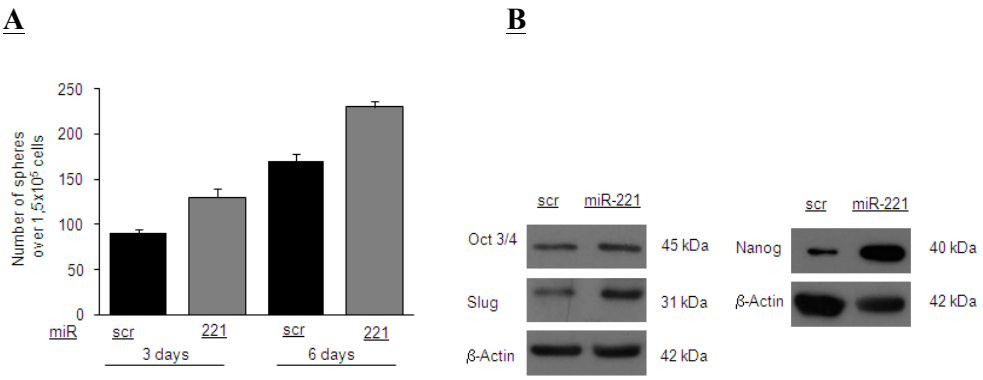


Figure 19. miR-221 expression induces the switch on pathway involved in stemness phenotype. (A) T47D transfected with miR-221 were grown in non-adherent conditions. Mammospheres are counting after 3 and 6 days. (B) Effect of miR transfect in T47D differentiated cells on Oct3/4, Slug and Nanog expressions

To evaluate whether miR-221 may directly affect the pathways involved in stemness maintenance, we transfected miR-221 in T47D differentiated cells and analysed by Western blot, the levels of the stem cells markers. As shown in Figure 18B, Nanog, Oct3/4 and Slug were upregulated upon miR-221 expression. Therefore, this data indicated that miR-221 expression trigger the switch on pathway involved in stemness phenotype. To further confirm our observations, we infected a primary breast cell line obtained from patient's specimen (patient #5) with a lentiviral construct encoding for miR-221. As shown in Figure 20A the infection resulted in miR-221 over-expression as assessed by qRT-PCR (Figure 20B). Thank to the stable expression of miR-221 over the time, we performed a replating assay in which the formed "primary" mammospheres were dissociated into single cells and grown as "secondary" mammospheres. We observed that cells overexpressing miR-221 showed increased spheres formation capacity (Figure 20B) that was enhanced upon first stem spheres replating (Figure 20C), indicating an expansion of stem cells population respect to the control. Interestingly, this stably over-expressing miR-221 cells also showed an enrichment in CD44+/CD24- marker that is known to represent the signature of breast cancer stem cells (Figure 21).

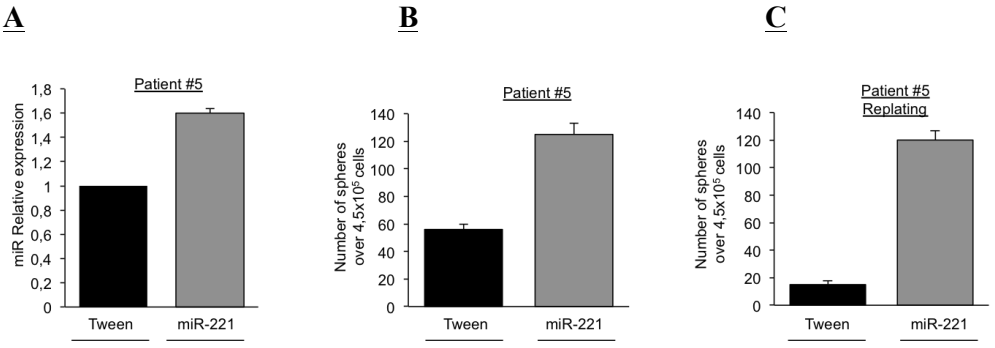


Figure 20. MiR-221 stably expression affect the capacity of primary breast cancer to form primary and secondary mammospheres respect to the tween control. (A) miR-221 constitutiveexpression was evaluated through Real time PCR. Primary cells infected were seed in non-adherence condition (B) and after a week were disaggregated and replated to obtain secondary mammospheres (C).

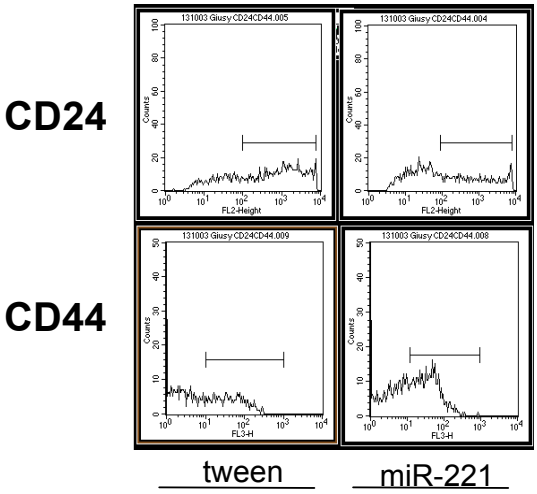


Figure 21. Primary cells stably infected with a lentivirus encoding for miR-221. Overexpression of mir-221 in primary cells induces an upregulation of CD44 marker levels and a downregulation of CD24 respect to the Tween control.

4.4 Identification of DNMT3b as a new target of miR-221

We then investigated miR-221 targets possibly involved in the stemness phenotype. Among the potential targets predicted by bioinformatics analysis programs, we found that the protein DNMT3b, a DNA methyltrasferase involved in de novo DNA methylation, was predicted as a target of miR-221. The most widely used approach for experimentally validating miR target is to clone the predicted miR-binding sequence downstream of a luciferase report construct, and to cotrasfected it with a miR of interest in a luciferase assay. To this end we cloned the 3'UTR sequence of human DNMT3b into the luciferase expressing vector pGL3-control downstream of the luciferase stop codon; HEK-293 cells were transiently transfected with this construct in the presence of pre miR-221 or in the presence of a scrambled oligonucleotide acting as negative control. As reported in Figure 22, miR-221 significantly reduced luciferase activity compared to the scrambled oligonucleotide. This indicated that miR-221 binds the 3'UTR of DNMT3b and impair its mRNA translation. In order to determine that the region was specific for the binding with miR-221, we generated a mutant, in which the seed sequence was cloned with an inverted orientation, lacking in this manner of the binding site. As shown in figure 22, miR-221 did not significantly reduce

luciferase activity in the presence of the 3' UTR-DNMT3b mutated sequence. This result indicates that miR-221 targets DNMT3b mRNA.

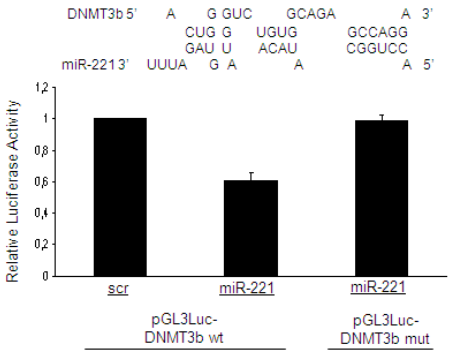


Figure 22: Identification of target site in 3'UTR of DNMT3b. Complementary site for miR-221 on wild type DNMT3b 3'UTR. For luciferase activity HEK293 cells were transiently cotransfected with the luciferase reporter containing wild type DNMT3b-3'UTR or DNMT3b mutant in the presence of miR-221 or a scrambled oligonucleotide. Luciferase activity was evaluated 24h after transfection as described in Material and Methods. Representative of at least three independent experiments.

4.5 MiR-221 specifically represses DNMT3b expression

In order to find a causative effect between miR-221 and DNMT3b expression, we transfected T47D with a pre-miR-221 for 48 hrs and then analyzed DNMT3b levels by Western blot and qRT-PCR. After transfection, we found that both DNMT3b protein and mRNA levels were downregulated (Figure 23 A-B) confirming that DNMT3b mRNA is targeted by miR-221. In contrast, T47D transfected with an antimiR-221, showed an increase of DNMT3b protein and mRNA (Figure 23 C-D).

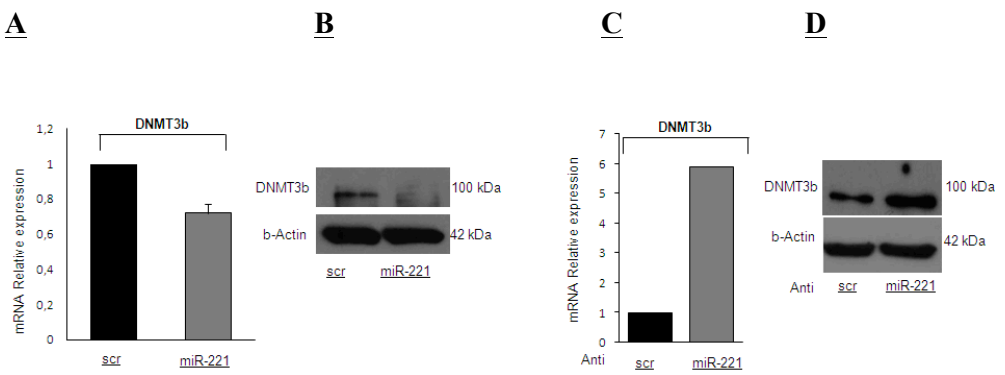


Figure 23. DNMT3b and miR-221 expression levels are inversely correlated in T47D stem cells. Effect of miR-221 or Anti-miR-221 transfection on DNMT3b expression: miR-221induced a decrease of DNMT3b mRNA (A) and protein (B) if compared to the scrambled sequences. On the contrary, anti miR-221 was able to increase DNMT3b expression levels (C-D).

To assays whether the expression of miR-221 was inversely correlated with DNMT3b also in T47D mammospheres, we analyzed the levels of the protein in stem and differentiated T47D cells. As shown in Figure 24 A and B, we found a reduced DNMT3b expression in T47D stem cells. Furthermore, we observed the same decrease of DNMT3b mRNA levels also in stably expressing miR-221 primary cells (Figure 24C).

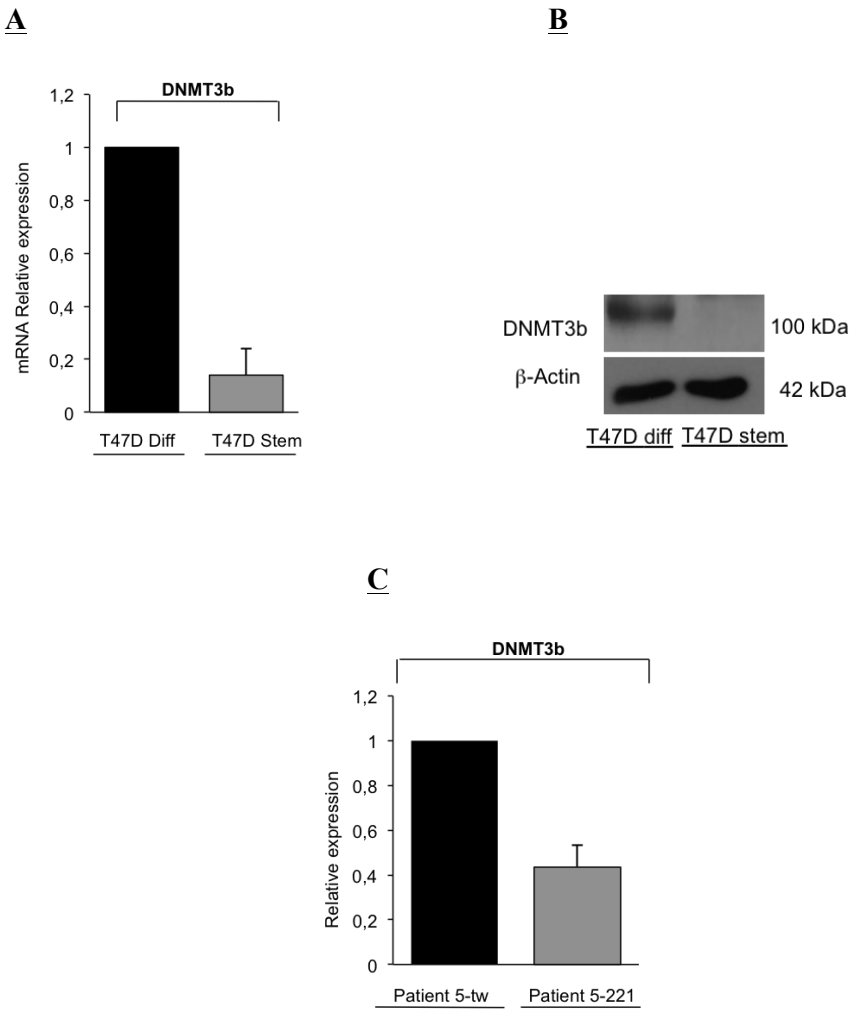


Figure 24. DNMT3b is inversely correlated with miR-221. DNMT3b is expressed at lower levels in T47D stem cells (A-B) and primary cells that overexpressing miR-221 rispect to the controls (C).

4.6 MiR-221 control stem proprieties through DNMT3b

DNMT3b is a master regulator of Nanog and Oct3/4 expression and, through its methylation activity, is able to repress their expression during embryogenesis. Therefore we wondered whether the up-regulation of Nanog and Oct3/4 observed upon miR-221 expression was related to the down regulation of DNMT3b levels and consequently to its reduced methylation activity. We transfected T47D with a DNMT3b cDNA and investigated the effect on stem markers expression and mammospheres formation. As shown in Figure 5, DNMT3b was able to inhibit the expression of Nanog and Oct3/4 as well as mammospheres formation (Figure 25A-B). To confirm the direct implication of DNMT3b in stemness maintenance, we transfected T47D with a siRNA to obtain a transient silencing of DNMT3b (Figure 25C-D) and observed an increase of mammosphere formation in cell silenced for DNMT3b, mimicking the effect of miR-221 on mamospheres numbers. These data confirmed our hypothesis of an involvement of DNMT3b in the stemness properties.

MiRs may target different proteins. In order to demonstrate that stemness features observed by miR-221 overexpression were carried out by miR induced DNMT3b downregulation, we performed a rescue experiment transfecting simultaneously pre miR-221 and a DNMT3b cDNA lacking of 3'UTR in T47D cells. As shown in Figure 25 A-B, the effect of the miR-221 on Nanog and Oct3/4 expression was abolished by the overexpression of DNMT3b cDNA. The simultaneous expression of DNMT3b protein was also able to partially revert the miR-221 effect on mammaspheres formation. This rescue experiment provides the causative connection between miR-221 expression, DNMT3b down regulation and stemness phenotype.

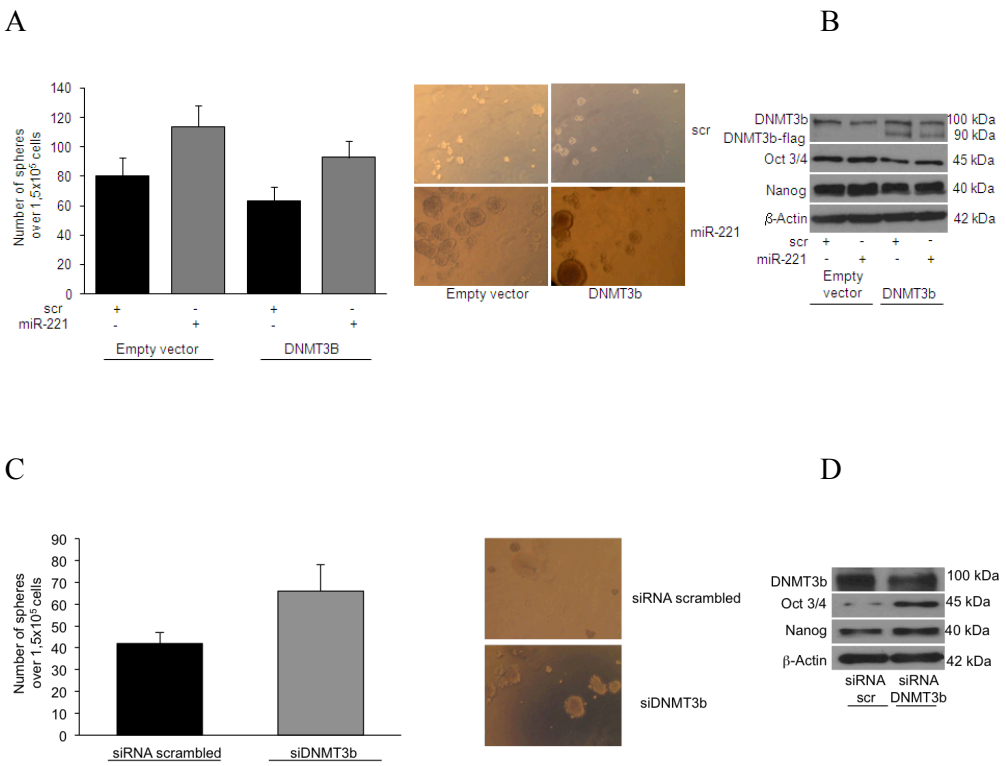


Figure 25. MiR-221 regulates stemness properties by targeting DNMT3b. (A) DNMT3b is able to repress stem marker and mammospheres formation as assessed by mammospheres counting assay (A) and Western blot. (B) DNMT3 silencing mimics the effect of miR-221 on stemness properties (C-D).

Furthermore, we stably infected T47D with a lentiviral construct overexpressing a short hairpin for DNMT3b and observe an increasing ability of mammospheres formation in DNMT3B silenced T47D cell lines, and that this effect is enhances after the first replating. This experiment testify that DNMT3b silencing determine a progressive expansion of the stem cells compartment, in a similar way to the primary cell line stably expressing the miR-221 (Figure 26).

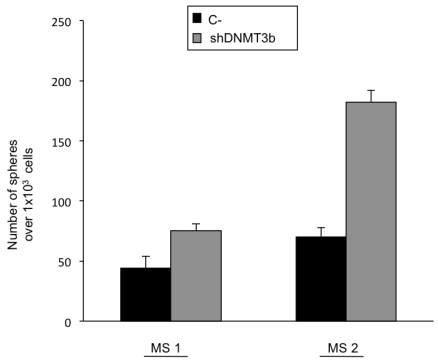


Figure 26. Analysis of serial mammospheres formation. T47D infected with a lentivirus encoding for a short hairpin for DNMT3b formed more primary (MS1) and secondary (MS2) mammospheres respect to the control.

5. DISCUSSION

The CSC model suggests that cancers arise from cells that can be defined, by analogy with totipotent stem cells, as cancer stem cells (CSCs) displaying self-renewal and differentiation potentials, to give rise to the phenotypically diverse cancer cell populations. However, little is known about what regulates their critical ability to self renewal and initiate tumors.

In less than 10 years, miRs have experienced a radical shift in people's mind, once considered as junk RNA not useful for the individual, they now appear as critical regulators of most cellular events. By their ability to target hundreds of mRNAs, they can induce a rapid switch in cell fate and fine tune genome expression and are now accepted as major post-transcriptional regulators. In the last years researchers showed that miRs regulate target genes involved in key cellular processes as well as stem cell biology. Interestingly, some miRs regulating ESCs are found important in regulating the CSC phenotype, suggesting that their alteration can contribute to maintain or trigger a stem-phenotype in cancer cells. Indeed ESC-enriched miRs such as miR-17–92 cluster, miR-371–373 cluster or miR-130 induce "stemness" and aggressiveness in cancer cells, while let-7 and miR-200 inhibit CSC properties (Wong et al. 2010, Wu et al. 2011).

Breast cancer is the leading cause of death in woman and is characterized by an elevated heterogeneity, different responses to therapies and metastatic variability among patients. Advances in standard treatment of this disease, such as surgery, radiotherapy and chemotherapy have increase patients survival, but the incidence and the death related to this cancer are still high. Breast cancer is the first human carcinoma for which a putative cancer stem cell subpopulation has been isolated at the basis of its CD44+/CD24-/low antigenic phenotype (Al-Hajj et al. 2003). Actually, breast cancer stem cells are objected of several studies because they are considered as the driving force of tumor and the understanding the molecular characteristics that govern their stemness could represent a therapeutic promise to erase the cancer.

In this study, we identify miR-221 as important player in the regulation of the cancer stem cells homeostasis and a new mechanism by which miR-221 is able to hold on the stemness state of cancer cells.

In order to identify new miRs that could act as molecular switchers in the maintenance of stemness properties, we investigate miR expression profile in breast cancer stem cells compared to their differentiated counterparts. We identified three miRs upregulated in breast cancer stem cells and three downregulated. Among the different miRs, we focus our attention on miR-221.

We found that miR-221 is expressed at higher levels in stem population of both primary and T47D cell lines compared to differentiated cells.
We focused on miR-221 because its expression in human cancer has been extensively investigated (Garofalo et al 2009, Visone et al 2007). MiR-221 has been frequently found overexpressed in a number of human tumors (Pallante et al 2006, Conti et al 2009, Pineau et al 2010) and is commonly identify as an oncomiR thanks to its ability to regulate cancer development and progression. For instance, we found that miR-221 increase cell migration and invasiveness in glioma cells (Quintavalle et al. 2012) and that is able to induce DNA demage, conferring oncogenic traits. (Quintavalle et al. 2013). Interestingly, the upregulation of miR-221 was reveled in breast cancer cells resistant to the tamoxifen and fulvestrant (Rao X et al. 2010) and other authors demonstrated that it was involved in the promotion of an aggressive basal-like phenotype triggering EMT process (Maitri Y Shah 2011). Since EMT is a process that is usually associated with a cancers stemness phenotype, we hypothesize that miR-221 can be part of a more complex signaling that governs the stemness properties. In agreement with this hypothesis, we found that miR-221 is able to induce the expression of Slug, Nanog, Oct3/4 genes, which enforce the stemenss state. Interestingly, the re-expression of pluripotency associated genes and adult stem cell gene expression signature in cancer cells, as well as the upregulation of EMT correlates with poor prognosis and with resistance to chemotherapy. In addition, miR-221 has an impact on the expansion of the cancer stem cells compartment in T47D cells, justified by an increasing of mammospheres number. MiRs have an important role in regulating expression profile by repressing the translation of selected mRNAs. In this manner, they can act on multiple pathways and influence several biological processes. Therefore, we wonder if the up-regultion of miR-221 could be functional in regulating self-renewal and differentiation signaling of cancer stem cells. We looked for possible target involved in the regulation of self-renewal and differentiation processes and focuses our attention on DNMT3b as putative target gene. DNMT3b is a de novo DNA methyl transferase and its involvement in stemness regulation derived our hypothesis. In fact it is known that epigenetic mechanisms are involved in the regulation of the embryonic and adult stem cell transcriptional program, controlling self-renewal and differentiation. Multiple observations indicate that the establishment and maintenance of CSC features can be orchestrated by a similar way. DNMT3b has been described to be able to methylate and consequently repress Nanog and Oct3/4 gene during embryogenesis. In embrionic stem cells, Nanog is involved in a complicated stemness regulatory network in cooperation with other key transcriptional factors, such as Oct3/4, Sox2, and Lin 28, to precisely balance between pluripotency and differentiation tendency (Kalmaret al. 2009). During tumorigenesis, some embryonic genes may be re-expressed or activated and influence the tumor

features and contribute to generate new cancer stem cells. For instance, the upregulation of Nanog and Oct3/4 regulates several aspects of cancer development such as tumor cell proliferation, self-renewal, motility, epithelial-mesenchymal transition, immune evasion, and drug-resistance, which are all defined features for cancer stem cells (Ibrahim et al. 2012, Chiou et al. 2010, Lin et al. 2012, Noh et al. 2012). Functional studies have demonstrated that Nanog and Oct3/4 are not only a CSC marker, but also promote CSC-like characteristics in several cancers. For example, ectopic overexpression of the Nanog gene in prostate cancer cells enhanced clonal growth and tumor regenerative capacity (Jeter et al. 2011), and the activation of embryonic Nanog gene drives a subpopulation of colorectal cancer cells to adopt a stem-like phenotype (Ibrahim et al. 2012). In addiction, overexpression of Nanog, along with Oct3/4, increased clonogenic growth and spheroid body formation of lung adenocarcinoma cells (Chiou et al. 2010). On the basis of these data, we hypothesized that miR-221 promote stemness features by downregulating DNMT3b and consequently Nanog and Oct3/4 gene methylation status (Li et al. 2007, Wongtrakoongate et al. 2014). We provided for the first time evidence that miR-221 is able to down-regulate DNMT3b expression levels and that its down regulation affects the breast cancer stem cell behaviour. In fact, DNMT3b siRNA is able to enhance the capability of T47D cells to form mammospheres and to up-regulate Nanog and Oct3/4 respect to the control. We also demonstrated that the endogenous levels of DNMT3b are lower in T47D mammospheres than adherent cells. These findings confirmed the inverse relation between miR-221 and DNMT3b in breast cancer stem cells. Because miRs can affect many different proteins, we validated the effect of dnmt3 by costrasfecting of miR-221 and ectopic DNMT3b lacking the miR binding sites in its 3'UTR. This rescue experiment provided the causative link between miR-221, DNMT3b and stemness. The idea that DNMT3 can act repressing the stemness properties is in agreement with Challen's work. Challen and colleagues demonstrated that the deletion of Dnmt3a (member of the family) in hematopoietic stem cells show a dramatic expansion of the stem cell compartment, in secondary but not primary transplants (Challen et al. 2011). Moreover, despite a marked expansion of stem/progenitor cells, Dnmt3a deficient cells did not show a parallel increase in contribution to differentiated hematopoietic lineages, suggesting a differentiation defect. Since DNMT3b and DNMT3a have similar sequence and function, it possible postulate that dnmt3b downregulation mediated by miR-221 could affect the expansion of breast cancer stem cells compartment upregulating Nanog and Oct3/4 and consequently altering the differentiated phonotype. On the basis of our results, we can hypothesize that DNMT3b plays a negative role on breast cancer stemness and can be considered as a tumor suppressor gene. However, the role of DNMT3b in cancer development is not really clear. Early works demonstrated that DNMT3b was overexpressed in several tumors (Esteller et al. 2007, Robertson et

al. 1999) and that its overexpression correlated with tumorigenesis and with the methylation of specific groups of genes, usually belong to tumor suppressor genes (Esteller et al. 2007, Issa et al. 2004, Roll et al. 2008). In particular, DNMT3b is required for the survival and proliferation of human lung, breast, colon, and bladder cancer cells and in lung cancer contributes to the malignant transformation induced by SV40T antigen (Soejima et al. 2003). Although these studies point to an oncogenic role of DNMT3b in cancer, the discovery of genetic alteration of this gene suggests that its role in cancer is more complex than it was previously believed. In fact in samples from patient with immunodeficiency, centromere instability and facial anomalies (ICF), whole genome bisulfite sequencing identified global loss of DNA methylation associated with genetic defect in DNMT3b (Heyn et al. 2012). Usually, a global loss of DNA methylation is observed is several tumors and the alterations identified in DNMT3b gene could explain the open chromatin that charachterized the cancer stem cells. Nevertheless, recent publications have shown that this enzyme also suppresses tumorigenesis. Using a conditional DNMT3b knockout in APC^{MIN-} mice, Lin and collegues demonstrated that DNMT3b is involved in the transition stage between microadenoma formation and macroscopic colon tumor (Lin et al. 2006). Another study demonstrated that the conditional knockout of DNMT3b in a mouse model of myc-induced lymphomagenesis, increases cellular proliferation. The antitumor action of DNMT3b in this model suggested to be mediated by its role in maintenance DNA methylation of the putative oncogene MET (Hlady et al. 2012).
On the basis of these considerations, although DNMT3b was classically considered as oncogene due to its role in the hypermethylation of tumor suppressor genes, DNMT3b is now considered behave also as tumor suppressor in advanced tumor stage. So, DNMT3b can act as tumor suppressor or promoter depending on the stage, but it is not excluded that it can have a bivalent role also depending on the tumoral cells populations. In stem context, we postulate that DNMT3b activity could be not helpful because it can methylate and repress master gene involved in the control of stemness state. On the other hand, its expression in differentiated compartment can be an advantage and responsible for the methylation of tumor suppressor genes that inhibit the growth and aggressiveness of the cancer. Therefore, we can hypothesize that DNMT3b perform a double function and that its tumor suppressor function could occur in an advance step of tumorigenesis. In fact at this stages, its silencing could be an advantage for the cancer because could determine an expansion of stem cells compartment and consequently promote a greater aggressiveness of the tumor. According with this supposition, it was demonstrated that DNMT3a deletion in a lung cancer model promoted tumor progression but not initiation, and resulted in anchorage-independent growth and expression of metastasis-associated genes in breast cancer cell lines (Quing et al. 2011).

In conclusion, we have shown a potential involvement of miR-221 in the regulation of stemness features targeting DNMT3b. We identified a new mechanism by which miR-221 can influence the tumor characteristic and aggressiveness, adding a new piece in understanding the oncogenic mechanism of action of miR-221.

Future therapeutic interventions that target miRs acting as stemness inducer or suppressor, should be useful to target key pathways that regulate the stemness state of breast cancer cells. This is a rapidly emerging field in oncology and might represent a promising strategy for cancer therapy in the future. Therefore, targeting the stemness-like properties of cancer cells with agents that modify the expression of miR-221 can contribute to reduce the number and to the sensitization of CSCs to chemotherapy, impeding tumor relapse.

6. CONCLUSIONS

In this work, we described a new circuit of both genetic and epigenetic mechanisms that contributes to the acquisition and maintenance of self-renewal and stemness features by breast cancer stem cells. In this study, we show a potential involvement of miR-221 in the regulation of stemness features. We demonstrated that miR-221 act as a stemness promoter inducing indirectly the expression of key genes, Nanog and Oct3/4 and promoting the expansion of stem cells numbers. Our data showed that this mechanism is, in part, mediated by targeting the DNA methytransferase DNMT3b. The down regulation of this enzyme led to the upregulation of Nanog and Oct3/4 genes and to a positive effect on the expansion of the stem cells compartment.
In this manner, we identify a new loop by which miR-221 can influence the stemness phenotype of breast cancer stem cells, adding a new information in understanding the oncogenic role of miR-221 in breast cancer.

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ORIGINAL ARTICLE

Effect of miR-21 and miR-30b/c on TRAIL-induced apoptosis in glioma cells

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Glioblastoma is the most frequent brain tumor in adults and is the most lethal form of human cancer. Despite the improvements in treatments, survival of patients remains poor. To define novel pathways that regulate susceptibility to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in glioma, we have performed genome-wide expression profiling of microRNAs (miRs). We show that in TRAIL-resistant glioma cells, levels of different miRs are increased, and in particular, miR-30b/c and -21. We demonstrate that these miRs impair TRAIL-dependent apoptosis by inhibiting the expression of key functional proteins. T98G-sensitive cells treated with miR-21 or -30b/c become resistant to TRAIL. Furthermore, we demonstrate that miR-30b/c and miR-21 target respectively the 3′ untranslated region of caspase-3 and TAp63 mRNAs, and that those proteins mediate some of the effects of miR-30 and -21 on TRAIL resistance, even in human glioblastoma primary cells and in lung cancer cells. In conclusion, we show that high expression levels of miR-21 and -30b/c are needed to maintain the TRAIL-resistant phenotype, thus making these miRs as promising therapeutic targets for TRAIL resistance in glioma.

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Keywords: glioblastoma; TRAIL; therapy; microRNA; treatment; apoptosis

INTRODUCTION

Glioblastomas are the most common primary tumors of the brain and are divided into four clinical grades on the basis of their histology and prognosis.[1] These tumors are highly invasive, very aggressive and are one of the most incurable forms of cancer in humans.[2] The treatment strategies for this disease have not changed appreciably for many years, and failure of treatment occurs in the majority of patients owing to the strong resistant phenotype. Therefore, the development of new therapeutic strategies is necessary for this type of cancer.

A novel interesting therapeutic approach is the reactivation of apoptosis using member of TNF (tumor necrosis factor)-family, of which the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) holds the greatest interest. Apoptosis is a particularly desirable treatment outcome, as it eradicates cancer cells without causing a major inflammatory response, which could provide unwanted survival signals. However, many cancers develop functional defects in the drug-induced apoptosis pathway, which may lead to constitutive or acquired resistance. To this end, alternative pathways, such as the one activated by death receptors including Fas/Apo-1, or DR4 (TRAIL-R1) and DR5 (TRAIL-R2), are being explored for cancer treatment. TRAIL is a relatively new member of the TNF family, known to induce apoptosis in a variety of cancers.[3] Treatment with TRAIL induces programmed cell death in a wide range of transformed cells, both *in vivo* and *in vitro*, without producing significant effects in normal cells.[3,4] Therefore, recombinant TRAIL or monoclonal antibodies against its receptors (TRAIL-R1 and TRAIL-R2) are in phase II/III clinical trials for different kinds of tumors, either as a single agent or in combination with chemotherapy.[5,6]

However, a significant proportion of human cancer cells are resistant to TRAIL-induced apoptosis, and the mechanisms of sensitization seem to differ among cell types. Different studies relate resistance to TRAIL-induced cell death to downstream factors. It has been shown that downregulation of two anti-apoptotic proteins such as PED (Phosphoprotein enriched in diabetes) or cellular-FLICE such as inhibitory protein (c-FLIP) can sensitize cells to TRAIL-induced apoptosis.[7–9] However the mechanism of TRAIL resistance is still largely unknown.

miRs are a class of endogenous non-coding RNA of 19–24 nucleotides in length that has an important role in the negative regulation of gene expression blocking translation or directly cleaving the targeted mRNA.[10] miRs are involved in the pathogenesis of most cancers.[10] In the last few years, our understanding of the role of miRNA has expanded from the initially identified functions in the development of round worms to a highly expressed and ubiquitous regulators implicated in a wide array of critical processes, including proliferation, cell death and differentiation, metabolism and, importantly, tumorigenesis.[11] We have recently showed an important role of microRNAs in TRAIL sensitivity in non-small cell lung cancer (NSCLC).[12–14]

In this study, to identify novel mechanisms implicated in TRAIL resistance in human glioma, we performed a genome-wide expression profiling of miRs in different cell lines. We found that miR-30b/c and -21 are markedly upregulated in TRAIL-resistant, and downregulated in TRAIL-sensitive glioma cells.

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Our experiments indicate that miR-30b/c and -21 modulate TRAIL sensitivity in glioma cells mainly by modulating caspase-3 and TAp63 expression and TRAIL-induced caspase machinery.

RESULTS

Selection of TRAIL-sensitive vs TRAIL-resistant glioma cell lines

We analyzed TRAIL sensitivity of different human glioma cell lines. Cells were exposed to TRAIL at two different concentrations for 24 h and cell death was assessed using the MTT assay (Figure 1a) or propidium iodide staining (Figure 1b). As shown in Figure 1, we can distinguish two sets of cells: TB10, LN229, U251 and U87MG cells exhibited total or partial TRAIL resistance, whereas T98G and LN18 cells underwent TRAIL-induced cell death.

miRs expression screening in TRAIL-resistant vs TRAIL-sensitive glioma cell lines

To investigate the involvement of miRs in TRAIL resistance in glioblastoma cell lines, we analyzed the miRs expression profile in the most TRAIL-resistant glioma cells (TB10 and LN229) vs the TRAIL-sensitive cells (T98G and LN18). The analysis was performed with a microarray chip containing 1150 miR probes, including 326 human and 249 mouse miRs, spotted in duplicates. Data obtained indicated that seven miRs (miR-21, -30b, -30c, -181a, -181d, -146 and -125b) were significantly overexpressed in resistant glioma cells with at least >1.9-fold change (Table 1). Quantitative real-time-polymerase chain reaction (qRT–PCR) validated the microarray analysis (data not shown).

Role of miRs in TRAIL resistance in glioma

To test the role of these overexpressed miRs in TRAIL sensitivity in glioma, we transfected T98G TRAIL-sensitive cells with miR-21, -30b, -30c, -181a, -146 and -125b. TRAIL sensitivity was evaluated by MTT assay, propidium iodide staining and colony assay. We obtained significant results only for miR-30b/c and miR-21 that were then extensively investigated. In fact, data obtained with MTT assay and FACS analysis showed that the overexpression of miR-30b/c and -21 was able to revert TRAIL sensitivity in T98G (Figures 2a and b). Similar results were obtained in LN18 cells (Figures 2c and d). This effect was not restricted to glioma, as miR-30 and miR-21 were able to exert an anti-apoptotic action also in non small cell lung cancer (NSCLC) (Supplementary Figure 3B). We further evaluated TRAIL sensitivity by colony assay. T98G and LN18 cells were transfected with miR-scrambled, miR-30b/c and miR-21 for 48 h and then were treated with 50 or 100 ng/ml of superKiller TRAIL for 24 h. Cells were grown for 6 days and then coloured with crystal violet-methanol solution (Supplementary Figures 1A and B). The results indicated that both miRs induced an increase of TRAIL resistance.

To further explore the role of miR-21 and -30b/c on TRAIL sensitivity, we transfected U251 (Figure 3a) or LN229 (Figure 3b) TRAIL-resistant cells with anti-miR-21, -30b, 30c, or with a scrambled sequence. As shown in Figures 3a and b, transfection of the anti-miR sequences was able to sensitize U251 and LN229 cells to TRAIL. Anti-miR-21 and -30c were also able to sensitize to TRAIL the CALU-1-resistant non-small cell lung cancer (NSCLC) TRAIL-resistant cell lines (Supplementary Figure 3C), indicating that this effect was not restricted to glioma.

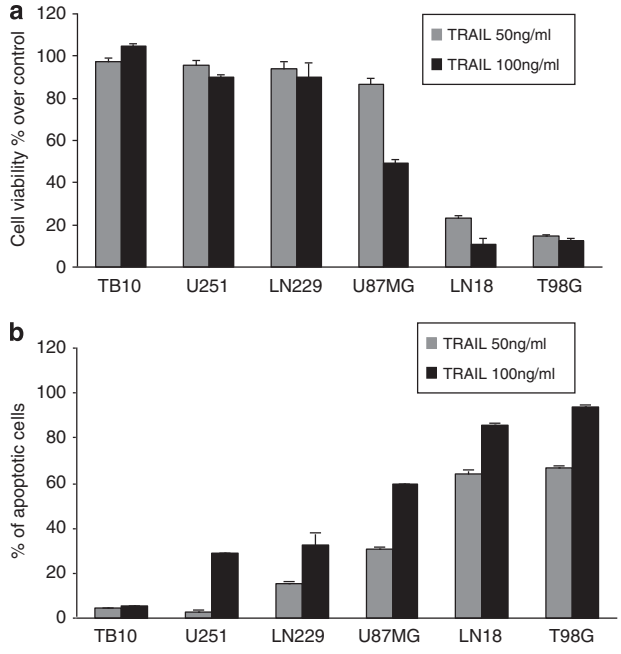

Figure 1. TRAIL sensitivity of glioblastoma cells. Glioblastoma cell lines (10^4 cell) were treated with superKiller TRAIL. After 24 h of treatment, the effect on cell death was assessed with MTT assay (**a**) or by propidium iodide staining and FACS analysis (**b**).

Identification of cellular targets of miR-30b/c and miR-21 in glioma cells

To identify cellular targets of miR-30b/c and -21, we used as first attempt a bioinformatic search, using programs available on the web including Pictar, TargetScan, miRanda and Microcosm target.

miR-21 targets different tumor suppressor genes and proteins potentially involved in TRAIL resistance in glioblastoma cells, such as PTEN (phosphatase and tensin homologue), PDCD4 (programmed cell death 4), TPM1 (Tropomyosin 1) and p53.[15–17] Computer-assisted analysis identified the presence of evolutionary-conserved binding sites for miR-21 in *TAp63* gene. We focused our attention on this p53 family member, as it regulates the expression of TRAIL receptors and molecules involved in TRAIL signaling.[18] We also searched for miR-30 targets and among them we focused on caspase-3.

TRAIL-resistant and TRAIL-sensitive glioma or NSCLC cells exhibited different levels of miR-21 and -30c assessed by either qRT–PCR (Figure 4a and Supplementary Figure 3A) or by northern blot analysis (Supplementary Figure 4). Interestingly, we observed a reduction of protein (Figure 4b and Supplementary Figure 3D) and mRNA (Figure 4c) levels of TAp63 and caspase-3 upon, respectively, miR-21 or miR-30c and miR-30b (data not shown) transfection in TRAIL-sensitive cell lines. We didn't observe a

Table 1. microRNA identified in TRAIL-resistant glioma (LN229 and TB10) compared with TRAIL-sensitive (T98G, LN18) cells

miR	P-*value*	*Fold difference*
hsa-miR-125b1-A	6.09e − 05	3.033
hsa-miR-30b-A	9.14e − 05	2.041
hsa-miR-30c-A	0.0001199	2.337
hsa-miR-146b-A	0.0001556	5.972
hsa-miR-181a-5p-A	0.0004698	2.66
hsa-miR-181d-A	0.0004817	3.035
hsa-miR-21-A	0.0032482	1.949

miRNA expression profiles in TRAIL-sensitive vs TRAIL-resistant cells. miRNA screening was performed in triplicate for TRAIL-sensitive and TRAIL-resistant cell lines by a microarray as described in Materials and methods. A two-tailed, two-sample *t*-test was used ($P<0.05$). Seven miRNAs were found to be significantly deregulated in TRAIL-resistant cells compared with the TRAIL sensitive.

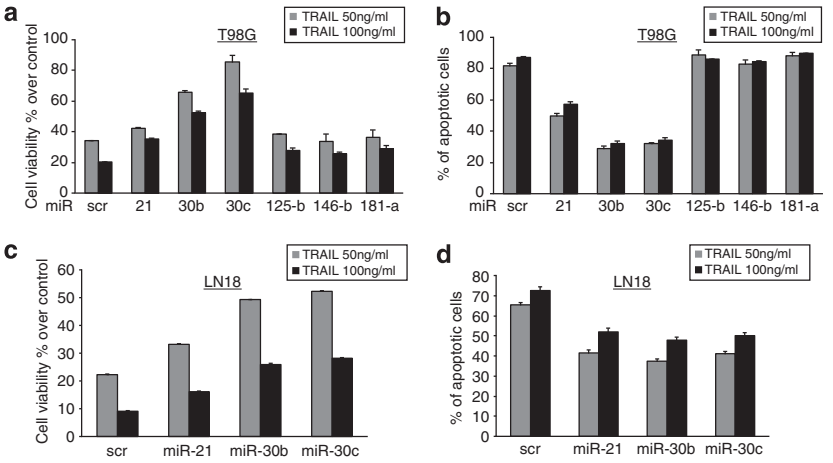

Figure 2. Effect of miR-21 and miR-30c overexpression on TRAIL-sensitive glioblastoma cells. T98G (**a**, **b**) cells were transfected with miR-21, miR-30b, miR-30c, miR-125b, miR-146b and miR-181a. LN18 (**c**, **d**) were transfected with miR-21, miR-30b and miR-30c. 10^4 cells were then treated with two different concentrations of superKiller TRAIL. After 24 h of treatment, cell viability was assessed with MTT assay (**a**, **c**) or with propidium iodide staining and FACS analysis (**b**, **d**). Both miR-21 and miR-30 induce TRAIL resistance in glioma cells.

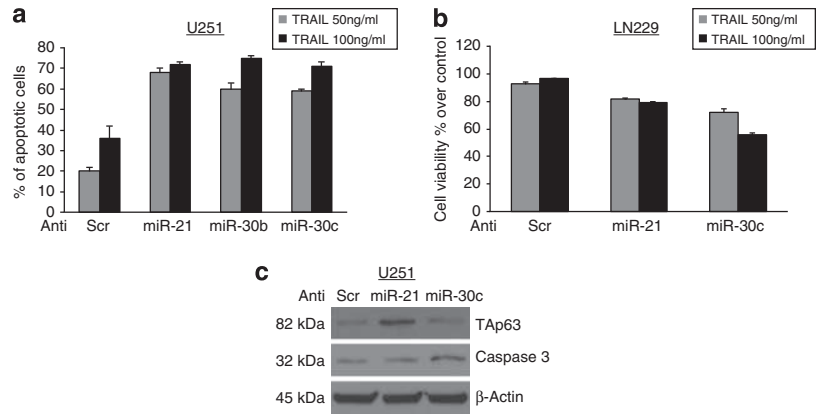

Figure 3. Effects of anti-miR-21 and anti-miR-30b/c on TRAIL sensitivity. Knock down of miR-21 and anti-miR-30b/c increases the percentage of apoptotic cells as assessed by propidium iodide staining in U251 cells (**a**) and decreases the cell viability of LN229 (**b**). (**c**) TAp63 and caspase-3 western blot analysis of U251 cells transfected with a scrambled sequence as negative control and with anti-miR-21 or anti-miR-30c, as indicated.

decrease in the levels of other caspases upon miR-30c transfection (Figure 4b). On the contrary, TAp63 and caspase-3 protein levels increased upon anti-miR-21 and anti-miR-30c transfection (Figure 3c and Supplementary Figure 3D) in TRAIL-resistant cell lines. To verify a direct link between the miR-21/TAp63 and miR-30b/c and caspase-3, we performed luciferase assay by co-transfecting pGL3-3′ untranslated region (UTR) vectors along with miR-21 or miR-30c. The results obtained indicated a direct interaction of miR-21 with TAp63 and miR-30c with caspase-3 (Figure 4d). As indicated in Figure 4d, miR-30b and -30c have the same seed sequence that recognizes caspase-3, differing only at the latest four nucleotides of the 5′. Therefore, miR-30b down-regulates caspase-3 at the same extent than miR-30c (data not shown). Deletions in seed complementary sites rescued the repression of miR-21 and miR-30c on their identified targets (Figure 4d).

Validation of miR-21 and miR-30b/c mechanisms of action

To demonstrate that miR-21 and miR-30b/c, by downregulating TAp63 and caspase-3, are responsible for the TRAIL resistance observed in T98G and LN18 cells, we transfected T98G with caspase-3 or TAp63 complementary DNAs lacking the miRNA-binding site in their 3′UTR or with a control vector and miR-30c (Figure 5a) or miR-21 (Figure 5b). Interestingly, transfection of TAp63 and caspase-3 was able to overcome the effects of miR-21 and miR-30c, decreasing cell viability and increasing apoptosis (Figures 5a and b). The data were confirmed by colony assay in T98G cells (Supplementary Figures 2A and B). Similar results were obtained when we analyzed miR-30b (data not shown). These rescue experiments proved the causative link between miR-21/TAp63 and caspase-3/miR-30b/c and TRAIL sensitivity.

Effect of miR-21 and miR-30c expression on TRAIL sensitivity in primary human glioma cell lines

MiR-21 and miR-30c expression levels were measured by qRT–PCR in nine different human primary cell lines (Figure 6a), eight derived from glioblastoma tumors (patient no. 1 to no. 8) and one from tissue surrounding the tumor (patient no. 9), and compared with TRAIL sensitivity. As shown in Figure 6b, TRAIL sensitivity correlated with miR-21 and miR-30c expression levels in all cases analyzed, with the exception of control sample that did not respond to TRAIL. Moreover, anti-miRs expression in TRAIL-

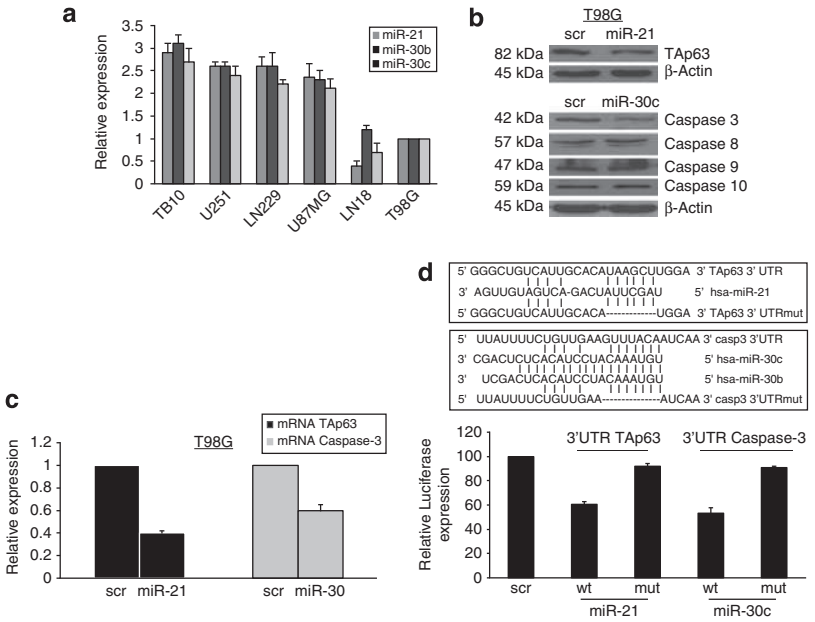

Figure 4. TAp63 and caspase-3 are targets of miR-21 and miR-30c. (**a**) qRT–PCR expression of miR-21, miR-30c and miR-30b in TB10, LN229, U251, U87MG, LN18 and T98G glioma cells. (**b**) TAp63 and caspase-3, caspase-8, caspase-9 and caspase-10 western blot analysis of T98G cells transfected with a scrambled sequence as negative control and with miR-21 and miR-30c, as indicated. (**c**) qRT–PCR of TAp63 and caspase-3 mRNA in T98G transfected with a negative control and with miR-21 and miR-30c, as indicated. (**d**) Alignment between miR-21 and 3′UTR TAp63 wild type or mutant and between miR-30c and 3′UTR caspase-3 wild type or mutant. Luciferase activity of PGL3-3′UTR TAp63 and of PGL3-3′UTR caspase-3 vector after HEK-293 transfection with scrambled miR, miR-21 and miR-30c wild type or mutated, as indicated.

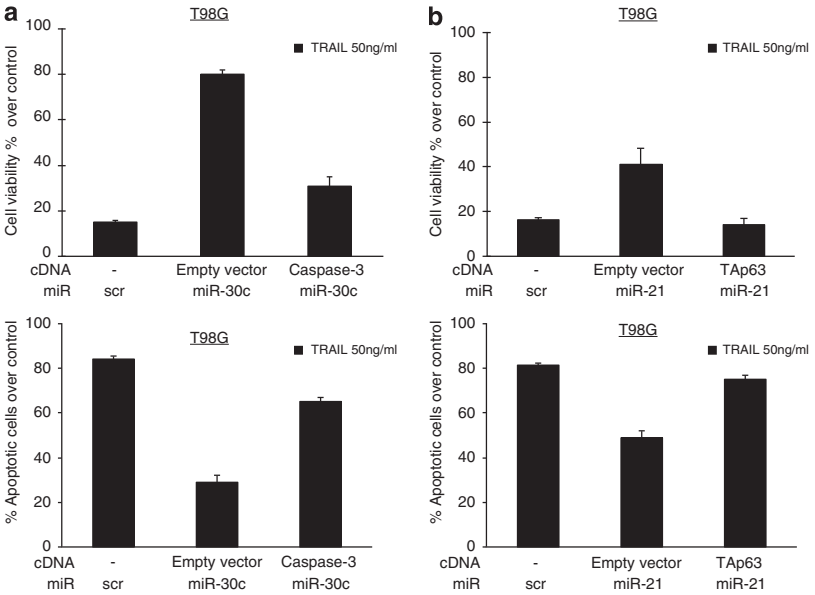

Figure 5. Validation of the involvement of caspase-3 and TAp63 in TRAIL sensitivity. Cell viability assay (upper panels) and propidium iodide staining (lower panels) of T98G cells transfected with miR-30c (**a**) and miR-21 (**b**) in the presence of cDNA for caspase-3 or TAp63.

resistant primary cultured cells (patient no. 1 and no. 2) was able to determine an increase of TRAIL sensitivity (Figure 6c) and concomitantly an increase of the levels of TAp63 and caspase-3 (Figure 6d).

DISCUSSION

Sensitization of cancer cells to apoptosis could be a valuable strategy to define new treatment options for cancer, in particular when using agents that aim to directly activate apoptotic pathways. A promising agent is the death receptor ligand TRAIL,[19] as it induces apoptosis in most cancer cells, but not in normal cells.[20,21] Moreover, TRAIL exhibits potent tumoricidal activity *in vivo* in several xenograft models, including malignant glioma.[22,23] Indeed, agonistic anti-TRAIL receptor monoclonal antibodies (mAbs), including mapatumumab (HGS-ETR1, anti-human DR4 mAb),[24] lexatumumab (HGS-ETR2, anti-human DR5 mAb)[25] and MD5-1 (anti-mouse DR5 mAb) are currently under

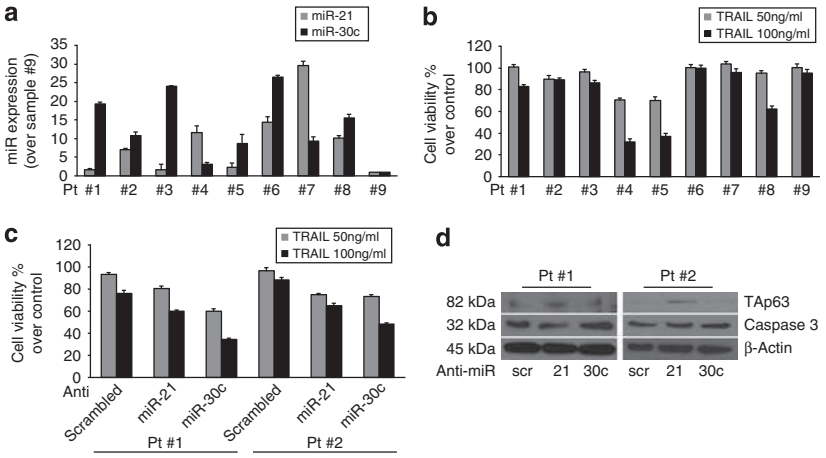

Figure 6. Effect of miR-21 and 30c on primary glioblastoma cell lines. (**a**) qRT–PCR analysis of miR-21 and miR-30c levels in eight primary glioblastoma cancer cell lines and one primary cell line derived from the surrounding tumor tissue used as control. (**b**) TRAIL sensitivity of primary cell lines (10^4 cells) treated with two different doses of SuperKiller TRAIL for 24 h, as indicated. (**c**) Cell viability assay of glioblastoma cells from two patients (#1 and #2) transfected with anti-miR-21 and anti-miR-30c and then treated with 50 ng/ml and 100 ng/ml of TRAIL for 24 h. Anti-miRs treatment sensitized glioblastoma cells to TRAIL. (**d**) Western blot analysis of TAp63 and caspase-3 after anti-miR-21 and anti-miR-30c transfection in patient #1 and #2.

intensive investigation. The former two mAbs have been tested in phase 1 clinical trials in patients with systemic malignancy, exhibiting excellent safety profiles. Anti-mouse DR5 mAb MD5-1 could also be administered safely without inducing hepatotoxicity either alone or in combination with histone deacetylase inhibitors in mice.[26] The induction of apoptosis by TRAIL is essentially dependent on the expression of specific TRAIL receptors and on the activation of caspases,[20] thus the regulation of the expression levels of those molecules is of fundamental importance.

MicroRNAs are emerging as key regulators of multiple pathways involved in cancer development and progression,[27–29] and may become the next targeted therapy in glioma. The present study shows that microRNA expression may modulate TRAIL-induced apoptosis in glioma cells, by the regulation of caspase-3 and TAp63 levels. We analyzed the miRs profile of TRAIL-resistant compared with TRAIL-sensitive glioma cells. We then focused our attention on miR-30b/c and miR-21, as only these miRs among those identified by the array, demonstrated the ability to revert the TRAIL-sensitive phenotype. We also provided evidences that this regulation is not restricted to glioma, but it is present also in a different type of cancer such as NSCLC.

MiR-21 has been found overexpressed in high-grade glioma patients[30] and studies have identified different miR-21 key targets for glioma biology, such as *RECK*, *TIMP3*, *Spry2* and *Pdcd4* genes, which are suppressors of malignancy and inhibitors of matrix metalloproteinase.[16,31–33] Moreover, levels of expression of miR-21 have been associated to patients survival.[34]

Other studies indicate that knockdown of miR-21 in cultured glioblastoma cells triggers activation of caspases and leads to increased apoptotic cell death.[35] Corsten *et al.*[36] hypothesized that suppression of miR-21 might sensitize gliomas for cytotoxic tumor therapy. With the use of locked nucleic acid (LNA)-anti-miR-21 oligonucleotides and neural precursor cells (NPC) expressing a secretable variant of TRAIL (S-TRAIL), they showed that the combined suppression of miR-21 and NPC-S-TRAIL leads to a synergistic increase in caspase activity and a decreased cell viability in human glioma cells *in vitro* and *in vivo* in xenograft experiments. Interestingly, Papagiannakopoulos *et al.*[15] described that miR-21 targets multiple important components of the p53 tumor-suppressive pathways. They showed that downregulation of miR-21 in glioblastoma cells leads to repression of growth, increased apoptosis and cell cycle arrest, through the regulation of target proteins such as HNRPK and TAp63. Our study describes for the first time the direct link between miR-21, TAp63 and TRAIL sensitivity. We demonstrated that miR-21 targets the 3′UTR sequence of TAp63, and that transfection of miR-21 is able to downregulate TAp63 at both mRNA and protein levels. More importantly, we demonstrated that miR-21, through TAp63, is able to modulate TRAIL sensitivity, as the co-transfection of miR-21 and TAp63 cDNA renders the cells again responsive to TRAIL. TAp63 is a transcription factor that regulates the expression levels of different apoptosis-regulating genes, such as TRAIL receptors, bcl2l11 and Apaf1.[18] Thus, it is possible that those apoptosis-regulating molecules are regulated by miR-21 through TAp63.

Several studies link miR-30 to apoptosis and human cancer. Li *et al.*[37] demonstrated that miR-30 family members inhibited mitochondrial fission through the suppression of the expression of p53 and its downstream target Drp1, whereas, Joglekar *et al.*[38] demonstrated that miR-30 may have a role in epithelial-to-mesenchymal transition. Our recent data demonstrate that miR-30 targets the anti-apoptotic protein BIM, participating to gefitinib resistance in lung cancer.[39] MiR-30 has been also associated with stem cell properties. Yu *et al.*[40] described that miR-30 is reduced in breast tumor stem cells (BT-ICs), and demonstrated that enforced expression of miR-30 in BT-ICs inhibits their self-renewal capacity by reducing Ubc9, and induces apoptosis through silencing ITGB3. In our hands, miR-30 overexpression inhibits TRAIL-induced apoptosis in glioma cells by targeting caspase-3. In fact, modulating the expression of either miR-30 or caspase-3, we observed a modification of TRAIL sensitivity of glioma cells. The opposing results on the role of miR-30 on cell death may be ascribed either to different cell system (breast vs glioma), or to different type of cancer cell (stem vs differentiated cells). In favour of this hypothesis, many reports describe opposing role of miRs in a different cell contest.[28] Recently, miR-30d has been described to target caspase-3 in breast cancer cells, and thus to regulate apoptosis.[41] The seed sequence recognizing the 3′UTR of caspase-3 is highly homologous within the members of the miR-30 family (miR-30b/c/d) suggesting a more generalized role of miR-30 family members in the regulation of cell death and cancer progression.

In many experiments, we observed that there is a redundancy within miR-21 and miR-30 in the regulation of TRAIL sensitivity. Our data, either in primary or in established cell lines, demonstrates that it is sufficient that one of the two miRs is highly expressed in the cells, that apoptosis resistance will manifest. We have also observed that miR-30 has a predominant effect in contrasting TRAIL-induced apoptosis. This may be related to the effect of this miR in targeting one important component of the cell death machinery, that is, caspase-3.

In conclusion, our study analyzed microRNA expression pattern in TRAIL-resistant and TRAIL-sensitive glioma cells, and identified specific miRs and their targets involved in the regulation of the apoptotic programme. This may be of relevance for future cancer therapy improvement in glioma.

MATERIALS AND METHODS

Cell culture and transfection

U87MG, T98G, U251, TB10, CALU-1 and 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM). H460 were grown in RPMI. Media were supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin. LN229 and LN18 were grown in Advanced DMEM (Invitrogen, Milan Italy) + 2 mM Glutamine + 5% fetal bovine serum. For miRs transient transfection, cells at 50% confluency were transfected using Oligofectamine (Invitrogen) with 100 nM of pre-miR-30c, -30b, -125b, -146b, -181a, -21, miR-scrambled or anti-miR- (Applied Biosystems, Milan, Italy). For caspase-3 and TAp63 transient transfection, cells were transfected using Lipofectamine and Plus Reagent with 4 μg of caspase-3 cDNA (Origene, Rockville, MD, USA) or TAp63 cDNA for 24 h. TAp63 cDNA was obtained from Professor Viola Calabrò (Naples). SuperKiller TRAIL for cell treatment was purchased from Enzo Biochem (New York, NY, USA).

Primary cell cultures

Glioblastoma specimens were collected at neurosurgical Unit of Cardarelli hospital (Naples). All the samples were collected according to a prior consent of the donor before the collection, acquisition or use of human tissue. To obtain the cells, samples were mechanically disaggregated, then the lysates were grown in DMEM-F12 medium supplemented with 10% fetal bovine serum 1% penicillin streptomicyn and 20 ng/ml EGF (Sigma-Aldrich, Milan, Italy). To exclude a fibroblast contamination, cells were stained for GFAP, a protein found in glial cells.

Protein isolation and western blotting

Cells were washed twice in ice-cold phosphate-buffered saline, and lysed in JS buffer (50 mM HEPES pH 7.5 containing 150 mM NaCl, 1% Glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA, 1 mM Na_3VO_4 and 1 × protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (Bio-Rad, Milan, Italy) using bovine serum albumin as the standard, and equal amounts of proteins were analyzed by SDS–PAGE (12.5% acrylamide). Gels were electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). For immunoblot experiments, membranes were blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20, and incubated at 4 °C over night with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (GE Healthcare, Milan, Italy). Primary antibodies used were: anti-βActin from Sigma-Alrich; anti-caspase-8, 9 and 10 were from Cell Signalling Technology (Boston, MA, USA); anti-Caspase 3 and anti-TAp63 from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

miRNA microarray experiments

From each sample, 5 μg of total RNA (from T98G, LN18, TB10, LN229 cells) was reverse transcribed using biotin-end-labelled random-octamer oligonucleotide primer. Hybridization of biotin-labelled cDNA was performed on an Ohio State University custom miRNA microarray chip (OSU_CCC version 3.0), which contains 1150 miRNA probes, including 326 human and 249 mouse miRNA genes, spotted in duplicates. The hybridized chips were washed and processed to detect biotin-containing transcripts by streptavidin-Alexa647 conjugate and scanned on an Axon 4000B microarray scanner (Axon Instruments, Sunnyvale, CA, USA).

Raw data were normalized and analyzed with GENESPRING 7,2 software (zcomSilicon Genetics, Redwood City, CA, USA). Expression data were median-centered by using both the GENESPRING normalization option and the global median normalization of the BIOCONDUCTOR package (www.bioconductor.org) with similar results. Statistical comparisons were done by using the GENESPRING ANOVA tool, predictive analysis of microarray and the significance analysis of microarray software (http://www-stat.stanford.edu/~tibs/SAM/index.html).

RNA extraction and real-time PCR

Total RNAs (miRNA and mRNA) were extracted using Trizol (Invitrogen) according to the manufacturer's protocol. Reverse transcription of total miRNA was performed starting from equal amounts of total RNA per sample (1 μg) using miScript reverse Transcription Kit (Qiagen, Milan, Italy), for mRNASuperScript III Reverse Transcriptase (Invitrogen) was used. For cultured cells, quantitative analysis of Caspase-3, Tap63, β-Actin (as an internal reference), miR-30b/c, miR-21 and RNU5A (as an internal reference) were performed by real-time PCR using specific primers (Qiagen), miScript SYBR Green PCR Kit (Qiagen) and iQ SYBR Green Supermix (Bio-Rad), respectively. The reaction for detection of mRNAs was performed as follow: 95 °C for 15′, 40 cycles of 94 °C for 15′, 60 °C for 30′ and 72 °C for 30′. The reaction for detection of miRNAs was performed as follow: 95 °C for 15′, 40 cycles of 94 °C for 15′, 55 °C for 30′ and 70 °C for 30′. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For relative quantization, the $2^{(-\Delta CT)}$ method was used as previously described.[42] Experiments were carried out in triplicate for each data point, and data analysis was performed by using software (Bio-Rad).

Northern blot analysis

RNA samples (30 μg) were separated by electrophoresis on 15% acrylamide, 7 mol/l urea gels (Bio-Rad, Hercules, CA, USA) and transferred onto Hybond-N + membrane (Amersham Biosciences, Piscataway, NJ, USA). Hybridization was performed at 37 °C in 7% SDS/0.2 mol/l Na_2PO_4 (pH 7.0) for 16 h. Membranes were washed at 42 °C, twice with 2 × standard saline phosphate (0.18 mol/l NaCl/10 mmol/l phosphate (pH 7.4)), 1 mmol/l EDTA (saline–sodium phosphate–EDTA; SSPE) and 0.1% SDS and twice with 0.5 × SSPE/0.1% SDS. The oligonucleotides (PRIMM, Milan, Italy) used, complementary to the sequences of the mature miRNAs, were: miR-21-probe 5′-TCAACATCAGTCTGATAAGCTA-3′; miR-30c-probe 5′-GCTGAG AGTGTAGGATGTTTACA-3′. An oligonucleotide complementary to the U6 RNA (5′-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3′) was used to normalize the expression levels. Totally, 100 pmol of each probe were end labelled with 50 mCi [γ-32P]ATP using the poly-nucleotide kinase (Roche, Basel, Switzerland). Blots were stripped by boiling in 0.1% SDS for 10 min before re-hybridization.

Luciferase assay

The 3′ UTR of the human Caspase-3 genes was PCR amplified using the following primers: Caspase-3 forward: 5′-TCTAGAAGGGCGCCATCGCCAAG TAAGAAA-3′, Caspase-3 reverse: 5′-TCTAGACCCGTGAAATGTCATACTGA CAG-3′ and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega, Milan, Italy). A deletion was introduced into the miRNA-binding sites by using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA) using the following: primers: Caspase-3 mut forward 5′-GCAAAATTCTTAAGTATGTTATTTTCTGTTGAAATCAAAGGA AAATAGTAATGTTTTATACT-3′. Caspase-3mut reverse 5′-AGTATAAAACAT TACTATTTTCCTTTGATTTCAACAGAAAATAACATACTTAAGAATTTTGC-3′.

The 3′ UTR of the human *TAp63* gene was PCR amplified using the following primers: TAp63 forward: 5′-TCTAGAGCAAGAGATAAGTCTTT CATGGCTGCTG-3′, TAp63 reverse: 5′-TCTAGATGGAAATCCCACTATCCCA AG-3′, and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega). A deletion was introduced into the miRNA-binding sites by using the QuikChange Mutagenesis Kit (Stratagene) using the following: primers:TAp63 mut forward 5′-CTGGTCAAGGGCTGTCATTG CACTCCATTTTAATTT-3′ TAp63 mut reverse 5′-AAATTAAAATGGAGTGCAAT GACAGCCCTTGACCAG-3′.

Hek-293 cells were cotransfected with 1.2 μg of generated plasmid and 400 μg of a Renilla luciferase expression construct pRL-TK (Promega) with Lipofectamine 2000 (Invitrogen). Cells were harvested 24 h post transfection and assayed with Dual Luciferase Assay (Promega) according to the

manufacturer's instructions. Three independent experiments were performed in triplicate.

Cell death quantification

Cells were plated in 96-well plates in triplicate, stimulated and incubated at 37 °C in a 5% CO_2 incubator. SuperKiller TRAIL was used at final concentration of 50 or 100 ng/ml for 24 h. Apoptosis was analyzed via propidium iodide incorporation in permeabilized cells by flow cytometry. The cells (2×10^5) were washed in phosphate-buffered saline and resuspended in 200 μl of a solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 μg/ml propidium iodide (Sigma). Following incubation at 4 °C for 30 min in the dark, nuclei were analyzed with a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Milan, Italy). Cellular debris was excluded from analyses by raising the forward scatter threshold, and the DNA content of the nuclei was registered on a logarithmic scale. The percentage of elements in the hypodiploid region was calculated. Cell viability was evaluated with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol. Metabolically active cells were detected by adding 20 μl of MTS to each well. After 2 h of incubation, the plates were analyzed in a Multilabel Counter (BioTek, Milan, Italy).

Colony assay

Cells were transfected with miR-scrambled, miR-30b/c or miR-21 for 24 h, then were harvested and 2.4×10^4 cells were plated in six-well plates. After 24 h, cells were treated with 50 or 100 ng/ml of superKiller TRAIL for 24 h, as indicated. Cells were transferred to 100 mm dishes and let grown for 6 days. Finally, the cells were coloured with 0.1% crystal violet dissolved in 25% methanol for 20 min at 4 °C. Dishes were washed with water and then let dry on the bench, and then photographs were taken.

ACKNOWLEDGEMENTS

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PLOS ONE

miR-221/222 Target the DNA Methyltransferase MGMT in Glioma Cells

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Abstract

Glioblastoma multiforme (GBM) is one of the most deadly types of cancer. To date, the best clinical approach for treatment is based on administration of temozolomide (TMZ) in combination with radiotherapy. Much evidence suggests that the intracellular level of the alkylating enzyme O^6-methylguanine–DNA methyltransferase (MGMT) impacts response to TMZ in GBM patients. MGMT expression is regulated by the methylation of its promoter. However, evidence indicates that this is not the only regulatory mechanism present. Here, we describe a hitherto unknown microRNA-mediated mechanism of MGMT expression regulation. We show that miR-221 and miR-222 are upregulated in GMB patients and that these paralogues target MGMT mRNA, inducing greater TMZ-mediated cell death. However, miR-221/miR-222 also increase DNA damage and, thus, chromosomal rearrangements. Indeed, miR-221 overexpression in glioma cells led to an increase in markers of DNA damage, an effect rescued by re-expression of MGMT. Thus, chronic miR-221/222-mediated MGMT downregulation may render cells unable to repair genetic damage. This, associated also to miR-221/222 oncogenic potential, may poor GBM prognosis.

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Introduction

Glioblastoma multiforme (GBM) is the most common and deadly primary tumor of the central nervous system. Despite several therapeutic advances, the prognosis for GBM remains poor, with a median survival lower than 15 months [1,2]. Currently, first-line therapy for GBM comprises surgery with the maximum feasible resection, followed by a combination of radiotherapy and treatment with the alkylating agent temozolomide (TMZ), also referred to by its brand name Temodal [3,4,5]. TMZ is a methylating agent that modifies DNA in several positions, one of them being O^6-methylguanine MeG (O^6MeG) [6]. If the methyl group is not removed before cell division, this modified guanine preferentially pairs with thymine during DNA replication, triggering the DNA mismatch repair (MMR) pathway, DNA double-strand breaks, and, therefore, the apoptotic pathway [7,8]. O^6-methylguanine–methyltrasferase (MGMT) is a suicide cellular DNA repair enzyme ubiquitously expressed in normal human tissues. MGMT does not act as a part of a repair complex but works alone [9]. To neutralize the cytotoxic effects of alkylating agents, such as TMZ, it rapidly reverses alkylation at the O^6 position of guanine, transferring the alkyl group to an internal cysteine residue in its active site. In this form, the enzyme is inactive and, thus, requires *de novo* protein synthesis. In tumors, high levels of MGMT activity are associated with resistance to alkylating agents [10]. In contrast, epigenetic silencing of MGMT gene expression by promoter methylation

results in sensitization to therapy [11,12]. However, some studies have reported that MGMT promoter methylation does not always correlate with MGMT expression and with response to therapy [13,14]. Therefore, the existence of other mechanisms of MGMT regulation should be postulated.

MicroRNAs (miRs) are small regulatory molecules that have a role in cancer progression and in tumor therapy response [15,16]. By negatively regulating the expression of their targets, miRs can act as tumor suppressors or oncogenes [17]. miRs may also regulate DNA damage response and DNA repair, interfering with the response to chemotherapy or radiotherapy [18]. Several studies have indicated that the modulation of miR expression levels is a possible therapeutic strategy for cancer.

The paralogues miR-221 and miR-222 have frequently been found to be dysregulated in glioblastoma and astrocytomas [19,20,21,22]. Their upregulation increases glioma cell proliferation, motility, and *in vivo* growth in mouse models. miR-221/222 have also been shown to be implicated in cellular sensitivity to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-treatment [23,24,25]. In this manuscript, we provide evidence that miR-221 and miR-222 regulate MGMT expression levels in glioblastoma, increasing the response to TMZ, but due to their oncogenic potential, affect overall patient survival negatively.

Materials and Methods

Cell culture and transfection

U87MG, T98G, LN428, LN308, A172, and HEK-293 cells were grown in DMEM. LN229 were grown in Advanced DMEM (Gibco, Life technologies, Milan, Italy). T98G, U87MG, and LN229 were from ATCC (LG Standards, Milan Italy); LN428, LN308, and A172 were kindly donated by Frank Furnari (La Jolla University). Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) -5% FBS for LN229 -2 mM L-glutamine, and 100 U/ml penicillin/streptomycin. All media and supplements were from Sigma Aldrich (Milan, Italy). For overexpression of miRs, cells at 50% confluency were transfected using Oligofectamine (Invitrogen, Milan, Italy) and 100nM pre-miR-221 or pre-miR-222, a scrambled miR or anti-miR-221/222 (Applied Biosystems, Milan, Italy). For overexpression of MGMT, cells were transfected using Lipofectamine and Plus Reagent with 4 µg of MGMT cDNA (Origene, Rockville MD USA). Temozolomide was purchased from Sigma Aldrich (Milan, Italy).

Human Glioma samples

A total of 34 formalin-fixed, paraffin-embedded (FFPE) tissue samples were collected from the archives of the Department of Pathology, University Hospital of Kuopio, Finland. Permission to use the material was obtained from the National Supervisory Authority for Welfare and Health of Finland, and the study was accepted by the ethical committee of the Northern Savo Hospital District, Kuopio, Finland.

Primary cell cultures

Glioblastoma specimens were obtained as previously described [19]. Samples were mechanically disaggregated, and the lysates grown in DMEM-F12 medium supplemented with 10% FBS, 1% penicillin streptomycin, and 20 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, Milan, Italy). To determine the glial origin of the isolated cells, we stained the cultures for glial fibrillary acidic protein (GFAP), a protein found in glial cells.

Protein isolation and Western blotting

Cells were washed twice in ice-cold PBS and lysed in JS buffer (50 mM HEPES pH 7.5 containing 150 mM NaCl, 1% Glycerol, 1% Triton X100, 1.5mM $MgCl_2$, 5mM EGTA, 1 mM Na_3VO_4, and 1X protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (BioRad, Milan, Italy) using bovine serum albumin (BSA) as the standard, and equal amounts of proteins were analyzed by SDS-PAGE (12.5% acrylamide). Gels were electroblotted onto nitrocellulose membranes (GE Healthcare, Milan, Italy). For immunoblot experiments, membranes were blocked for 1 hr with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, and incubated at 4°C overnight with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (GE Healthcare, Milan, Italy). Primary antibodies used were: anti-β-actin from Sigma-Aldrich (Milan Italy); anti-caspase-3 and anti-PARP from Santa Cruz Biotechnologies (Santa Cruz, CA, USA), anti-γH2AX from Millipore (Milan, Italy), anti-p53, p^{ser15} p53, and phosphorylated-ATM from Cell Signaling Technology (Milan, Italy).

RNA extraction and Real-Time PCR

Cell culture: Total RNA (microRNA and mRNA) were extracted using Trizol (Invitrogen, Milan, Italy) according to the manufacturer's protocol.

Tissue specimens

Total RNA (miRNA and mRNA) from FFPE tissue specimens was extracted using RecoverAll Total Nucleic Acid isolation Kit (Ambion, Life Technologies, Milan, Italy) according to the manufacturer's protocol. Reverse transcription of total miRNA was performed starting from equal amounts of total RNA/ sample (1µg) using miScript reverse Transcription Kit (Qiagen, Milan, Italy), and with SuperScript® III Reverse Transcriptase (Invitrogen, Milan, Italy) for mRNA. Quantitative analysis of MGMT, β-actin (as an internal reference), miR-221, miR-222, and RNU5A (as an internal reference) were performed by RealTime PCR using specific primers (Qiagen, Milan, Italy), miScript SYBR Green PCR Kit (Qiagen, Milan, Italy), and iQ™ SYBR Green Supermix (Bio-Rad, Milan, Italy), respectively. The reaction for detection of mRNAs was performed as follows: 95°C for 15', 40 cycles of 94°C for 15″, 60°C for 30″, and 72°C for 30″. The reaction for detection of miRNAs was performed as follows: 95°C for 15', 40 cycles of 94°C for 15″, 55°C for 30″, and 70°C for 30″. All reactions were run in triplicate. The threshold cycle (CT) is defined as the fractional cycle number

at which the fluorescence passes the fixed threshold. For relative quantization, the $2^{(-\Delta CT)}$ method was used as previously described [26]. Experiments were carried out in triplicate for each data point, and data analysis was performed by using a Bio-Rad software (Bio-Rad, Milan, Italy).

Luciferase assay

The 3' UTR of the human MGMT gene was PCR amplified using the following primers: MGMT-Fw: 5'TCTAGAGTATGTGCAGTAGGATGGATG3'; MGMT-Rv: 5' TCCAGAGCTACAGGTTTCCCTTCC3', and cloned downstream of the Renilla luciferase stop codon in pGL3 control vector (Promega, Milan, Italy). A deletion was introduced into the miRNA-binding sites with the QuikChange Mutagenesis Kit (Stratagene, La Jolla CA USA) using the following primers: MGMT-mut Fw: 5' CTATATCCAAAAGGGAAACCTGTAGCTCTTGC 3'. MGMT-mut Rw: 5'- GCAGAGCTACACGTTTCCCTTTTGGATATAG 3'. HEK-293 cells were co-transfected with 1.2μg of plasmid and 400 μg of a Renilla luciferase expression construct, pRL-TK (Promega, Milan, Italy), with Lipofectamine 2000 (Invitrogen, Milan, Italy). Cells were harvested 24 hrs post-transfection and assayed with Dual Luciferase Assay (Promega, Milan, Italy) according to the manufacturer's instructions. Three independent experiments were performed in triplicate.

Cell death quantification

Cell viability was evaluated with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Milan, Italy) according to the manufacturer's protocol. Metabolically active cells were detected by adding 20 μL of MTS to each well. After 2 hrs of incubation, the plates were analyzed in a Multilabel Counter (BioTek, Milan, Italy). For caspase-3 inhibition experiments, ZVAD-Fmk was purchase from Calbiochem.

Comet assay

Alkaline comet assay was performed accordingly to manufacturer's instructions (Trevigen, Gaithersburg, Maryland, USA). Briefly, $12x10^4$ glioblastoma cell lines were transfected with miRs or MGMT cDNA and then treated with TMZ in 6-well plates. Cells were collected and then combined with LMAgarose. The mixture was applied to Comet slides and kept at 4°C in the dark for 10'. The slides were immersed in pre-chilled lysis buffer for 30 min. The slides were washed and then electrophoresis was carried out. The slides were fixed in 70% ethanol for 5 min and let dry overnight. SYBR green was added and comets were photographed at 100 x microscopes (Carl Zeiss Inc., NY, USA).

γH2AX flow cytometric analysis

Treated cells were fixed with 2% paraformaldehyde for 1 hr. Fixed cells were permeabilized with 0.1% Triton-X100/PBS for 5 min on ice. Blocking was done in PBS+2% BSA. Anti-phosphorylated H2Ax antibody(Ser139, γH2Ax, Millipore, Milan, Italy) was diluted in PBS and then FITC-conjugated goat anti-mouse antibody (Santa cruz Biotechnology, CA, USA) was used. Cells were analyzed with a Becton Dickinson FACScan flow cytometer.

Caspase Assay

The assay was performed using the Colorimetric CaspACE™ Assay System, (Promega, Milan, Italy) as reported in the instruction manual. Briefly, T98G cells were transfected with miR-221 and/or MGMT cDNA, plated in 96-well plates, and then treated with 300 μMol of temozolomide or with 10 μMol of ZVAD-Fmk. After treatments, 100 μl caspase-3/-7 reagent was added to each well for 1 hr in the dark. The plates were analyzed in a Multilabel Counter (BioTek, Milan, Italy).

MGMT Methylation Analysis

DNA methylation status in the CpG island of *MGMT* was established by PCR analysis of bisulfite modified genomic DNA, which induces chemical conversion of unmethylated, but not methylated, cytosine to uracil. DNA was extracted from cell lines using the DNeasy blood and tissue kit (Qiagen, Milan, Italy). DNA (1 μg) was modified with sodium bisulfite using the EZ DNA methylation-gold kit (Zymo Research, CA, USA) according to the manufacturer's instructions. Methylation-specific polymerase chain reaction (MSP) was performed with primers specific for either methylated or the modified unmethylated DNA. Primer sequences for the unmethylated reaction were 5'TTTGTGTTTTGATGTTTGTAGGTTTTTGT3' (forward primer) and 5'AACTCCACACTCTTCCAAAAACAAAACA3' (reverse primer), and for the methylated reaction they were 5'TTTCGACGTTCGTAGGTTTTCGC3' (forward primer) and 5'GCACTCTTCCGAAAACGAAACG3' (reverse primer.) The annealing temperature was 59°C. The cell line SW48 and *in vitro* methylated DNA (CpGenome Universal Methylated DNA, Millipore) were used as a positive control for the methylation of MGMT and DNA from normal lymphocytes used as a negative control. Controls without DNA were used for each set of methylation-specific PCR assays. The methylation-specific PCR product was loaded directly onto 2% agarose gels, stained with syber safe, and examined under ultraviolet illumination.

Colony Assay

Cells were transfected with scrambled miR or miR-221 for 24 hrs, harvested, and $2.4\ x10^4$ cells plated in 6-well plates. After 24 hrs, cells were treated with 300 μMol TMZ for 24 hrs, as indicated. Cells were transferred to 100-mm dishes and grown for 6 days. Finally, the cells were colored with 0.1% crystal violet dissolved in 25% methanol for 20 min at 4°C. Dishes were washed with water, left to dry on the bench, and then photographs taken.

Statistical analysis

Student's *t* test and nonparametric Mann-Whitney tests were used to determine differences between values for normally and, respectively, not normally distributed variables. A probability level <0.05 was considered significant throughout

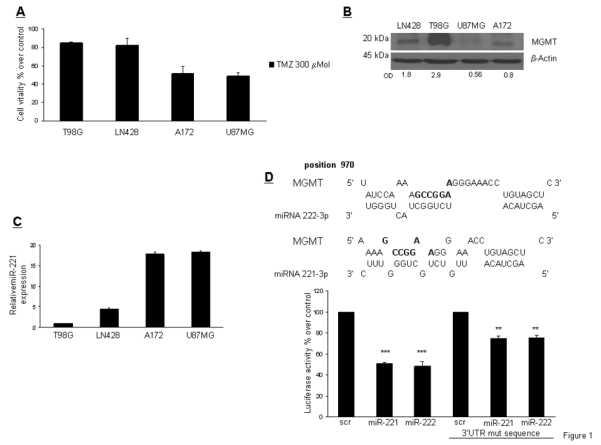

Figure 1. TMZ sensitivity and MGMT and miR-221/222 expression in glioma cells. (A) Glioma cells were treated with TMZ (300µMol) for 24 hr. Cell viability was evaluated with an MTT assay. (B) Western blot analysis of MGMT expression in glioblastoma cells. (C) Real time PCR of miR-221 expression in glioblastoma cells. (D) RNA Hybrid prediction analyzes of miR-222, miR-221, and MGMT 3' UTR. In bold are shown the mutated oligonucleotides. Luciferase activity of HEK-293 cells transiently co-transfected with the luciferase reporter containing wild-type MGMT-3'UTR or mutant MGMT-3'UTR in the presence of pre-miR-222, miR-221, or scrambled oligonucleotide. Representative of at least three independent experiments. *** p<0.001 versus control, ** p<0,0037 versus control.

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the analysis. Data were analyzed with GraphPad Prism (San Diego, CA, USA) for Windows.

Results

Sensitivity of human glioma cell lines to temozolomide

We analyzed the sensitivity to TMZ of human glioma cell lines by exposing the cells to 300 µMol TMZ for 48 hours and then assessing cell viability with the MTT assay (Figure 1A). We observed different TMZ sensitivities, which correlated with MGMT levels analyzed by Western blot (Figure 1B). We also observed an inverse correlation between the level of MGMT (Figure 1B) and miR-221 expression in glioma cell lines (Figure 1C). An RNA hybrid alignment bioinformatics search identified a possible binding site for miR-221/222 at position 970 of the 3' UTR of *MGMT*.

To examine whether miR-221/222 interfered with *MGMT* expression by directly targeting the predicted 3' UTR region, we cloned this region downstream of a luciferase reporter gene in the pGL3 vector. HEK-293 cells were co-transfected with the reporter plasmid plus the negative control miR (scrambled miR), miR-221, or miR-222. Only transfection of either miR-221 or miR-222 with the wild-type *MGMT*-3'UTR reporter plasmid led to a significant decrease of luciferase activity. On the contrary, co-expression of the scrambled miR had no effect (Figure 1D). In addition, miR-221/222's effect on the promoter of *MGMT* was reduced with the mutant *MGMT*-3'UTR reporter, in which the seed sequence was mutated. Together, these results demonstrate that miR-221/222 directly target *MGMT*-3'UTR, thereby reducing *MGMT* expression.

miR-221/222 target MGMT protein and mRNA

In order to establish a causal link between miR-221/222 and MGMT expression, we transfected T98G cells with either pre-miR-221 or pre-miR-222 for 72 hrs and then analyzed MGMT levels by Western blot and real time-PCR. Upon miR transfection, MGMT protein and mRNA were downregulated (Figure 2A). In contrast, MGMT expression was increased upon transfection with anti-miR-221 or -222 in U87MG cells (Figure 2B). Similarly, miR-221/222, induced downregulation of MGMT in LN428 cells, another TMZ-resistant glioma cell line (Figure 2C), and in A375 cells, a TMZ-resistant melanoma cell line (Figure 2D). Since *MGMT* expression is mainly dependent on the methylation status of its promoter [27], we determined if miR-221/222 acted by modulating *MGMT* promoter methylation. To this end, we performed a bisulfite modification assay by PCR using specific primers for both methylated and unmethylated *MGMT* promoter. As shown in Figure 2E, miR-221/222 expression in T98G cells, or anti-miR expression in U87MG cells, did not modify the methylation profile of the *MGMT* promoter.

miRs-221/222 modulate TMZ sensitivity in glioma cells

To verify if miR-221/222 play a role in the modulation of TMZ sensitivity because of their effects on MGMT expression, we characterized the viability of T98G, LN428, and A375 cells transfected with miR-221/222 and then treated with TMZ for 24 hrs. As shown in Figure 3A, miR-221/222 transfection increased the response to TMZ. These results were also confirmed by proliferation and colony assays (Figure 3B and 3C). To establish a causal link between miR-221 expression and MGMT downregulation, we performed a rescue experiment with simultaneous overexpression of miR-221 and MGMT cDNA in two different cell lines (T98G and LN428). As shown in Figure 3D, the effect of miR-221 on TMZ response was abolished by MGMT overexpression. We then verified in nine different glioblastoma primary cell lines and in six glioma cell lines any correlation between miR-221 expression and TMZ sensitivity. As shown, TMZ sensitivity positively correlated with the expression level of miR-221 (Figure 3E).

miR-221 promotes apoptotic cell death

In order to evaluate the mechanism of TMZ-induced cell death, we assessed the presence of apoptotic cells by PI staining and flow cytometry upon miR-221 transfection and TMZ treatment. We found that TMZ increased apoptotic cell death in miR-221-overexpressing cells compared with control cells. Interestingly, this effect was rescued by the co-expression of MGMT cDNA with miR-221 (Figure 4A). Caspase-3/7 activation assay further confirmed the involvement of the apoptotic machinery. As shown in Figure 4B, miR-221 expression increased caspase-3 activity upon

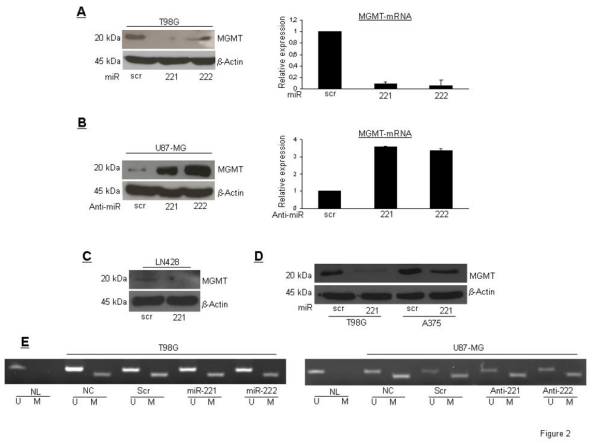

Figure 2. miR-221/222 target *MGMT*. (A) Western blot analysis and real time PCR of MGMT protein and RNA after miR-221/222 transfection of T98G cells. (B) Western blot analysis and real time PCR of MGMT protein and RNA after anti-miR-221 and -222 transfection of U87MG cells. (C) Western blot of MGMT expression upon miR-221 transfection of LN428 cells. (D) Western blot analysis of MGMT expression in T98G cells, as a control, and the melanoma cell line A375 upon miR-221 transfection. (E) Analysis of methylation status of MGMT promoter in T98G and U87MG upon miR- or anti-miR-221/222 transfection. U is for the un-methylated form, M for methylated form, NL is for normal lymphocytes, used as control.

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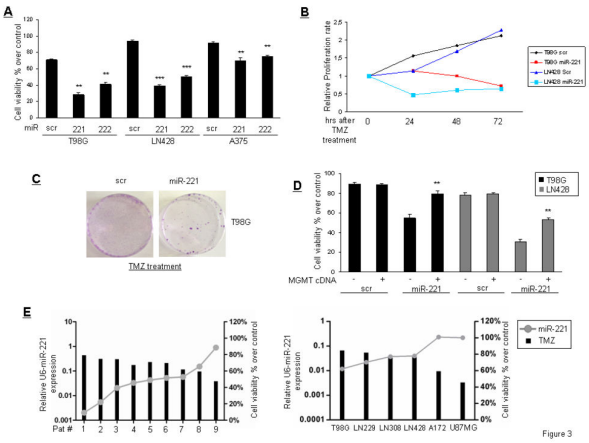

Figure 3. miR-221 modulates TMZ sensitivity. (**A**) Cell viability of T98G, LN428, and A375 cells transfected with miR-221 and miR-222 upon TMZ treatment (300 μMol) for 24 hrs. **p value<0.0082 versus scr column, ***p value<0.005 versus scr column. (**B**) Growth curve of T98G and LN428 cells transfected or not with miR-221 after 24 hrs of treatment with TMZ. (**C**) Colony assay of T98G and LN428 cells transfected with miR-221 and then treated for 24 hrs with TMZ (300 μMol). Cells were left to grow for 6 days after treatment removal. (**D**) MGMT expression rescues cell viability after TMZ treatment in T98G and LN428 cells overexpressing miR-221 **p value<0.0082 versus untransfected MGMT column. (**E**) Correlation between miR-221 expression and TMZ sensitivity in nine primary glioblastoma cell lines and in six glioblastoma cell lines.

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TMZ treatment, while the co-expression of MGMT cDNA with miR-221 abolished this effect. Simultaneous treatment with the caspase inhibitor ZVAD-fmk and TMZ was able to decrease caspase activity, confirming that TMZ induced cell death by a caspase-mediated mechanism. Caspase-3 activation, observed by Western blot in miR-221-transfected cells after 24 hrs of TMZ treatment, was rescued by MGMT cDNA (Figure 4C). Coherently, we observed an increase in cell viability after miR-221 transfection and simultaneous treatment with TMZ and ZVAD-fmk (Figure 4D).

miR-221 promotes DNA damage after TMZ treatment

MGMT activity repairs DNA by removing DNA adducts caused by TMZ treatment. The absence of MGMT increases cell death upon exposure to TMZ, but, as a long-term effect, may increase DNA damage, and thus the accumulation of mutations. We investigated whether miR-221 may increase DNA damage upon TMZ treatment by down-modulating MGMT expression. This was assessed by a comet assay, which quantifies double-stranded DNA (dsDNA) breaks, in T98G cells transfected with miR-221 or a scrambled sequence and then treated with TMZ at different times. We found that miR-221 produced a significant enhancement of dsDNA breaks (Figure 5A). To strengthen our hypothesis, we looked for the phosphorylation status of histone H2AX (γH2AX) at Ser139, which reflects dsDNA break formation. As shown in Figure 5B, miR-221 significantly increased γH2AX, as assessed by immunocytofluorescence (upper panel) or by Western blot (lower panel), suggesting that miR overexpression may induce DNA damage. This effect was even stronger in the presence of TMZ, but was rescued by MGMT cDNA (Figure 5B, middle panel). Furthermore, we also observed an increase of other DNA damage markers, such as P-ATM, P-p53^{ser15} and PARP cleavage, upon miR-221 transfection; this was even stronger upon treatment with both miR-221 and TMZ (Figure 5C). These effects were rescued by the simultaneous expression of MGMT with miR-221. Taken together, these data suggest that the targeting of *MGMT* by miR-221 increases DNA damage. This effect was amplified by TMZ treatment.

MGMT and miR-221 expression in glioblastoma patients

We then evaluated the expression of MGMT and miR-221 in human glioblastoma samples. Patients were clustered into two separate groups: a long survival (survival >15 months) group and a short survival (survival <15 months) group, according to common classification [2].

We first analyzed the methylation profile of the *MGMT* promoter, and then *MGMT* mRNA and miR-221 levels. We performed methylation-specific PCR (MSP) on 33 human

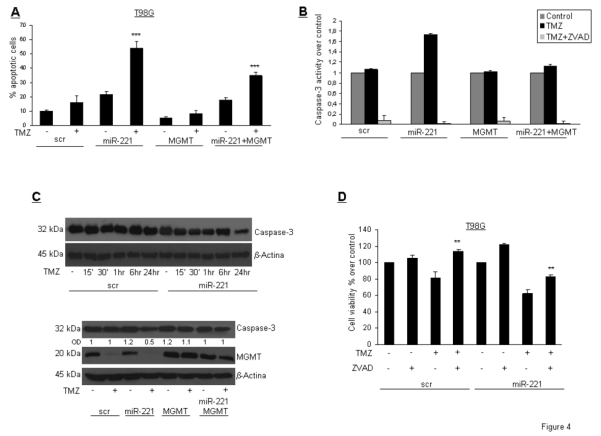

Figure 4. miR-221 promotes DNA damages upon TMZ treatment. (A) Apoptotic cell death assessed by FACS in T98G cells transfected with miR-221 or scrambled sequence and MGMT and treated with TMZ for 24 hrs. *** p value< 0.005 versus untrasfected MGMT column. (B) Active caspase-3 quantification in T98G cells as indicated and treated with TMZ for 24 hrs in the presence or absence of 3 hrs pre-treatment with ZVAD-fmk. (C) Upper panel Time course analysis of caspase-3 activation upon TMZ treatment in T98G cells transfected with miR-221 or with scrambled sequence. Lower panel Western blot analysis of caspase-3 activation after miR-221 and MGMT transfection. (D) Cell viability of T98G cells transfected with miR-221 or with scrambled sequence treated with TMZ for 24 hrs in the presence or absence of 3 hrs pre-treatment with ZVAD-fmk. ** p value< 0.0034 versus only treated TMZ column, Student's t test.

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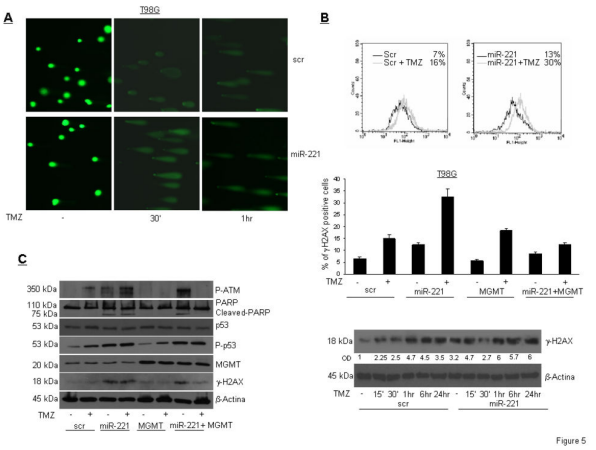

Figure 5. miR-221 promotes DNA damage. (A) Alkaline comet assay of T98G cells transfected with miR-221 and treated with TMZ for the indicated times. (B) Analysis of γH2AX in T98G cells transfected with scrambled control miR or miR-221, treated with TMZ in the presence or in the absence of MGMT cDNA, by immunocytofluorescence (upper and medium panel) or by Western blot (lower panel). (C) Western blot analysis of the indicated proteins upon transfection of T98G cells with miR-221 and MGMT cDNA and TMZ treatment for 24 hrs.

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glioblastoma paraffin-embedded tissues, and found 27 to be unmethylated and 4 to be methylated (samples 2, 21, 22, and 28) (Figure S1). For two samples (#31 and #32), it was not possible to define the *MGMT* promoter methylation profile. We then analyzed the effect of miR-221 on *MGMT* regulation among 15 unmethylated samples from which we obtained sufficient RNA for real time PCR analysis. We identified 4 long- (#1, #4, #10, and #14) and 11 short- (#6, #7, #8, #12, #13, #17, #18, #23, #25, #32, and #33) survival patients. We found that the short-survival group exhibited a higher miR-221 level and a lower MGMT level compared with the long-survival group (Figure 6 A,B). These data supports our in vitro evidence of an inverse correlation between miR-221 and MGMT expression. Furthermore, this observation identifies miR-221 as a negative prognostic factor for survival.

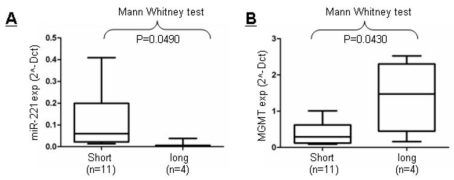

Figure 6. Association of miR-221 and MGMT expression. Mann–Whitney U test analysis was performed to evaluate the association between miR-221 and MGMT expression in long- and short -survival groups of patients. The expression of miR-221 (2^-Dct) (**A**-**B**) and MGMT (2^-Dct) are inversely correlated with patient survival (p < 0.0490 and p = 0.043, respectively).

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Discussion

Much evidence suggests that the intracellular level of the alkylating enzyme MGMT affects TMZ response in GBM patients [10,11]. Low levels of MGMT are associated with a better TMZ response, because in the absence of MGMT the cells are not able to repair the TMZ-induced base mismatch. Hence, double-strand DNA breaks, DNA mismatch repair, and the apoptotic pathway are activated. *MGMT* expression is regulated by the methylation of its promoter. *MGMT* promoter methylation lowers MGMT levels and accounts for a greater TMZ response when associated with radiotherapy. However, a fraction of patients with unmethylated *MGMT* show some TMZ response, suggesting that promoter methylation is not the only regulatory mechanism of MGMT expression [13,14].

In the present study, we addressed this specific issue by investigating the involvement of miRs in *MGMT* regulation. First, we characterized TMZ sensitivity in a subset of

glioblastoma cell lines and primary cells obtained from GBM patients. We found that the analyzed glioblastoma cell lines (T98G, LN428, U87MG, and A172) expressed different levels of miR-221/222 and displayed a consistent difference in MGMT expression. This inverse correlation was also observed in glioblastoma biopsies.

Bioinformatics identified a possible miR-221/222 binding site on *MGMT*. This was confirmed by a luciferase assay and overexpression experiments. The effect of miR-221/222 on MGMT levels was direct and not related to *MGMT* promoter methylation, since miR transfection did not alter the *MGMT* methylation profile. Instead, we found evidence that miR-221/222 regulated MGMT levels, leading to increased TMZ-induced apoptosis, reduced anchorage-independent growth, and reduced cell viability. Overexpression of MGMT cDNA with miR-221/222 rescued the effects on TMZ sensitivity. This result was not restricted to glioma cells, but was obtained also in other cancer cells sensitive to TMZ, such as human malignant melanoma.

It has been demonstrated that *MGMT* may be a target also of other miRs, such as miR-181, in GBM [28]. Zhang et al. demonstrated that miR-181d targets *MGMT* 3' UTR, and reported an inverse correlation between miR-181d and MGMT levels in human GBM samples, in particular in those samples in which the *MGMT* promoter was unmethylated [28]. However, the modest correlation between miR-181d and MGMT suggested that other miRs may regulate MGMT expression. Therefore, miR-221/222 may be part of this cohort.

MGMT expression may be regulated also thought the p53 pathway. Blough et al. provided evidence that p53 regulates MGMT expression in murine astrocytes, and presented data suggesting that p53 contributes to the regulation of MGMT gene expression in the human astrocytic glioma cell line SF767 [29].

In this manuscript, we demonstrate that miR-221 overexpression increases DNA damage in glioma cells. In fact, miR-221-overexpressing glioma cells exhibited an increase in DNA damage markers, such as P-ATM, P-p53, cleaved PARP, and γH2AX. These markers were activated even in the absence of TMZ, and became increased upon TMZ treatment. MGMT participates in the repair of DNA. Thus, miR-221/222 induces chronic MGMT downregulation, rendering the cells unable to repair DNA damage. It is well established that miR221/222 are oncogenic microRNAs that are upregulated in a number of human tumors [30,31,32]. In GMB tissue and cell lines, upregulated miR-222 and miR-221 expression correlated with the stage of the disease, cell motility, and TRAIL response [19,23,31,33]. We found that miR-221 is a negative prognostic factor, since it is up regulated in short-survival patients and is downregulated in long-survival ones. However, we did not observe the expected correlation between miR-221 expression and response to temozolomide/survival. Arguably, overall survival and therapy response have to be linked to other factors. It therefore seems that the pro-oncogenic effect of miR-221 is more powerful than its potentiation of the response to temozolomide.

The role of MGMT in DNA damage repair has been investigated also in animal models. Reduced expression of this repair enzyme has been thought to result in a spontaneous 'mutator' phenotype and to promote neoplastic lesions in the presence of either endogenous or exogenous sources of alkylation stress. Sakumi, et al. showed that Mgmt−/− mice develop thymic lymphomas and lung adenomas to a greater extent when exposed to methylnitrosourea (MNU), suggesting that the DNA repair methyltransferase protected these mice from MNU-induced tumorigenesis [34]. Sandercock et al. reported that MGMT-deficient cells exhibited an increased mutational burden, but only following exposure to specific environmental mutagens [35]. Takagi et al. demonstrated that mice with mutations in *Mgmt* as well as in the DNA mismatch repair gene *Mlh1* developed numerous tumors after being administered MNU. When exposed to a sub-lethal dose of MNU (1mM), the mutation frequency in $Mgmt^{-/-}/Mlh1^{-/-}$ cells was up to 12 times that of untreated cells; this effect was not present in control mice [36]. Walter et al. generated transgenic mice overexpressing MGMT in brain and liver, or in lung [37]. They found that expression of the transgene correlated with a reduced prevalence of MNU-induced tumors in liver and in lung and also with reduced spontaneous hepatocellular carcinoma. Reese et al. found that overexpression of MGMT decreased the incidence and increased the latency of thymic lymphoma induction in mice with both heterozygous and wild type p53 alleles [38]. This protective effect was described also by Allay et al., who reported that the incidence of lymphomas was much lower in MGMT transgenic mice compared with controls [39]. Those studies thus suggest that MGMT, other than being involved in the response to therapy, is also involved in DNA repair. Therefore, its inactivation may produce devastating effects on DNA integrity.

In summary, we have provided evidence of the existence of an adjunct mechanism of MGMT regulation, besides promoter methylation, involving miR targeting its 3' UTR. We have also shown that overexpression of miR-221/222 produces an increase in sensitivity to TMZ via a reduction in the level of MGMT. On the other hand, these miRs increase DNA damage, conferring oncogenic features to glioma cells. This may link miR-221/222 to poor GBM prognosis.

Supporting Information

Figure S1. Methylation-specific PCR analyses for MGMT methylation in glioblastoma human tumors. 33 glioblastoma samples were used for analysis. The SW48 cell line and *in vitro* methylated DNA (IVD) are shown as a positive control for methylation, normal lymphocytes (NL) as a negative control for methylation, and water (H2O) as a negative PCR control. U and M indicate the presence of unmethylated or methylated MGMT, respectively. Red colour is for methylated samples, green for unmethylated and orange for undetermined samples.
(TIF)

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Author Contributions

Conceived and designed the experiments: CQ GC. Performed the experiments: G. Roscigno CQ DM ADL G. Romano. Analyzed the data: DF ED MI GC. Contributed reagents/materials/analysis tools: ME YS PD. Wrote the manuscript: CQ GC.

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