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RADIOSENSITIZATION OF CANCER STEM CELLS: TARGETING TGFβ, NOTCH OR TELOMERASE TO IMPROVE TUMOR RESPONSE O RADIOTHERAPY

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ABSTRACT

Radiation resistant cancer stem cells are the main reason for treatment failure and tumor recurrence after cancer radiotherapy. Increasing biological evidences demonstrate that these cells possess the capacity to repair radiation induced DNA damage, protect themselves from radiation derived reactive oxygen species, survive and proliferate after several fractions of radiotherapy and finally, repopulate the heterogeneity of the tumor. Thus, targeting and eliminating these cells should be necessary to achieve cancer cure in radiotherapy. Three major approaches that specifically target radioresistant cancer stem cells have been recently investigated. First, inhibition of TGFβ, a major mediator of the tissue response to radiation, has been shown to induce radiosensitization of cancer stem cells by targeting the DNA damage response mechanism. Second, by preventing Notch activation during fractionated radiotherapy, cancer stem cells were depleted from their ability to repopulate the tumor after radiation. Finally, telomerase activity inhibitors have shown to specifically decrease the cancer stem cell population after radiotherapy. In the present review, we evaluate these radiosentitizing approaches and their possible effects when combined with fractionated radiotherapy as they promise to be a powerful tool in the battle against this cancer.

Keywords: Cancer Stem Cells (CSC), Reactive Oxygen Species (ROS), Double Strand Breaks (DSB), Extra Cellular Matrix (ECM), Anticancer Treatments

1. INTRODUCTION

Between 1991 and 2007, cancer mortality rates in the US decreased 22.2% for men and 13.9% for women (Siegel *et al*., 2011), thanks in part to the development of early detection techniques, like the discovery of the prsotate-specific antigen (Toubert *et al*., 1996) and the improvements in appropriate treatment and palliative techniques including surgery, radiotherapy and chemotherapy. But despite these encouraging trends, cancer is still one of the leading causes of death in developed and developing countries and behavioral trends indicate an alarming increase in the exposure to risk factors, specially for low-and middle-income countries (McCormack and Boffetta, 2011). During 2008, cancer alone was responsible for 13% of the total number of deaths worldwide, almost 7.6 millions and the incidence of new cases estimated for that year ascended to 12.6 millions (Boyle and Levin, 2008). More recently, 1.6 million new cases of cancer were estimated for 2011 in the US with a predicted mortality of almost 572,000 deaths (Siegel *et al*., 2011). In this context and even with the considerable reduction in mortality rates during the last twenty years, cancer is the leading cause of death among men and women younger than 85 in the US since 1999 (Landis *et al*., 1999).

It is also well established that despite the improvements achieved in the field of cancer therapy, the response to different treatments is still heterogeneous and unfortunately, the majority of the treated tumors maintain the capacity to reemerge after the treatment. This heterogeneity is not only observed in a population basis, but also in single tumors, animal models and even in cancer cells in culture and

indicates that the response of single cancer cells to anticancer treatments is heterogeneous.

A hierarchical organization of cancer cells subpopulations has been known for more than fifty years, when serial transplantation experiments demonstrated differential metastatic potential within the cells of a given tumor (Southam and Brunschwig, 1961). In this context, one of the most accepted theories that explains cancer cells heterogeneity in their resistance to anticancer therapies resides in the existence of a sub-population of cells within the tumor with specific capabilities that make them particularly resistant to both chemo and radiotherapy (Eramo *et al*., 2006; Liu *et al*., 2006; Ghods *et al*., 2007; Kang and Kang, 2007; Mimeault *et al*., 2007; Ma *et al*., 2008). These cells possess the capacity to self renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor (Clarke *et al*., 2006) and are known as Cancer Stem Cells (CSC) (Reya *et al*., 2001). However, the existence of CSCs is still controversial (Trott, 1994; Hill, 2006; Hill and Perris, 2007) mainly due to interpretation and nomenclature issues about what a stem cell truly is and to continuous discrepancies about what combination of surface biomarkers characterize and differentiate the CSC from other cancer cells (Baumann *et al*., 2008; Al-Assar *et al*. 2009; Alexander *et al*., 2009; McCord *et al*., 2009; Ropolo *et al*., 2009; Smith *et al*., 2011). In addition, few years ago (Mani *et al*., 2008) identified a connection between the stem cell signature and the epithelial to Mesenchymal Transition (EMT) program suggesting that differentiated cells may acquire stem capabilities. Some recent reports indicate the ability of single cancer cells to initiate tumors (Quintana *et al*., 2008) and this epithelial plasticity, although controversial since the EMT signature in differentiated cells differs from that executed in cancer cells when acquiring metastatic potential (reviewed in (Zeisberg and Neilson, 2009), has been suggested as a mechanism to initiate tumors for non cancer stem cells.

Is for all of those controversial issues that, for the purpose of this review, we will not get into the existence of single deterministic stem cells in the tumor or the plasticity capabilities of all cancer cells to become cancer stem cells and we will just focus our intent in the variability of responses to cancer therapy that are observed as a result of the intrinsic tumor heterogeneity and how to overcome them with novel therapeutic approaches. To do that, we will consider the existence of a population of cells within the tumor that, after a radiotherapy has killed most of the tumor volume, remain almost unaffected and, more importantly, retain the capacity to regrow into a fully developed cancer (Pajonk *et al*., 2010) (**Fig. 1**) and in the shake of simplicity, we will retain the traditional and most common nomenclature that entitles these cells as CSCs.

Radiotherapy and cancer radioresistance: Radiotherapy is one of the least expensive cancer treatments and one of the most effective in terms of patient cure and overall survival (Dunscombe *et al*., 2007). In high-income countries, radiotherapy should be used for direct and palliative treatment in 52% of all new cancer cases (Delaney *et al*., 2005; Kimm *et al*., 2005). This numbers vary depending on the tumor type, but make radiotherapy particularly required for the treatment of several solid cancers like breast, lung and prostate (83, 76 and 60% respectively) (Delaney *et al*., 2005). The principles in which radiation therapy bases its success and mechanisms of action when killing cancer cells were categorized by Whitners almost 40 years ago as the 4 R's of Radiobiology: Repair of DNA damage, Redistribution of cells in the cell cycle, Repopulation and Reoxygenation of hypoxic tumor areas (Withers, 1975).

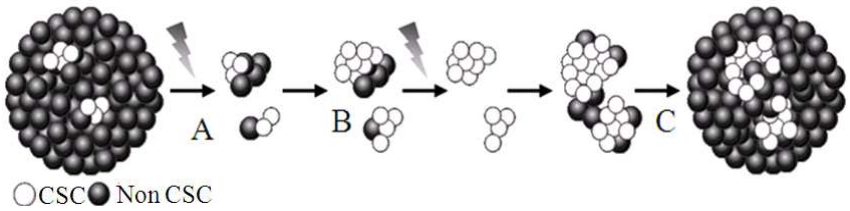


Fig. 1. Cancer recurrence after fractionated radiotherapy. (a) Radiation therapy induces DNA damage in cancer cells killing the majority of the tumor volume while acting as a selection mechanism for the intrinsically more resistant CSCs. (b) The surviving population of CSCs commence the repopulation of the tumor by reentering in cell cycle, proliferating and establishing an initial program of self renewal that increases their numbers. Further fractions of radiotherapy contribute to the selection of the CSC population and enrich the remaining tumor with symmetrically dividing CSC. (c) The increased population of CSC operates as seeds for proliferation, causing a rapid regrow of the tumor volume generally faster than the initial rate of the disease

The most studied and well known effect of radiation and radiotherapy is the induction of double strand breaks in the DNA of the target cells. In that regards, high dose radiation, like the one employed for radiotherapy, induces direct ionization of the DNA causing a number of Double Strand Breaks (DSB) in the genome of a single cell directly proportional to the dose (Olive *et al*., 1991). In addition, as a physical property of ionizing radiation, highly Reactive Oxygen Species (ROS) are generated by the ionization of water molecules. Those induce local radical scavengers, such as glutathione that contribute as well to cause double strand breaks in the DNA (Mitchell and Russo, 1987). As a response to the damage, the cells activate a DNA Damage Response (DDR) mechanism capable to repair a certain amount of these breaks. The principle by which radiation is effective against cancer cells is because it generates enough damage to override the DDR mechanism and induce cell death.

However the activation of the DDR mechanism in cancer cells populations is heterogeneous as evidenced by differential survival of certain subpopulations after radiotherapy. For example, in gliobastoma a population of $CD133^+$ putative cancer stem cells, was shown to be especially efficient in the repair of the radiation induced DSB as shown with the Alkaline Comet assay (Bao *et al*., 2006). Higher resistance to radiation was also found in breast cancer cell lines in mammosphere colony assays enriched for $CD24^{low}$ $CD44^{high}$ putative CSCs (Phillips *et al*., 2006), together with a more efficient repair of the DNA damage as evidenced by γH2AX foci (Diehn and Clarke, 2006; Phillips *et al*., 2006), a marker for the phosphorilation of the histone H2AX after focal recognition of a DSB in the DNA (Olive, 2004). Pointing in the same direction, breast $LIN^-CD24^+CD29^+$ mammosphere derived cells exhibited a distinct β-catenin and γH2AX activation patterns after radiation exposure, suggesting a possible role of the Wnt-β-catenin pathway in the DDR response to radiation induced DSBs (Chen *et al*., 2007; Woodward *et al*., 2007). In this context, inhibition of Chk1 phosphorilation, a downstream mediator of the γH2AX DDR pathway, has been shown to radiosensitize glioma and atypical teratoid/rhabdoid $CD133^+$ cells (Bao *et al*., 2006; Chiou *et al*., 2008; Ropolo *et al*., 2009). In addition to the DDR activation, autophagy has been proposed as an alternative mechanism for the acquisition of radiation resistance employed by $CD133^+$ cells and it has been shown that autophagy inhibition sensitized the cells to radiation reducing the sphere forming capacity of glioma CSCs (Lomonaco *et al*., 2009). Finally, described another mechanism of radiation resistance in breast cancer CSCs characterized by an antioxidant profile in which the CSCs showed increased expression of glutamate cystein ligase and glutathione synthetase. This translates into a more efficient scavenging of radiation induced ROS and results in less DSBs when compared to non CSCs irradiated at the same dose (Diehn *et al*., 2009).

Summarizing, CSCs exhibit an increased overall resistance to radiotherapy due to a more efficient activation of the DDR mechanism after radiation and to their capacity to minimize ROS induced DNA damage (**Fig. 2**). Fortunately, these characteristics also provide a new avenue for anticancer therapy and further evidences have demonstrated that, by specifically targeting them, there is an overall benefit in the tumors response to radiation.

TGFβ as a target for cancer stem cells Radiosensitization: One of the most interesting approaches to target the CSCs increased resistance to radiotherapy has been recently reported by three independent groups, in which breast cancer (Bouquet *et al*., 2011) and glioblastoma cell lines (Anido *et al*., 2010; Zhang *et al*., 2011) were radiosensitized by inhibition of Transforming Growth Factor β (TGFβ). Bouquet *et al*. (2011) reported in both colony forming assays and in vivo tumor control the inhibition of TGFβ with a small molecule or a neutralizing antibody increased the radiosensitivity of breast cancer. Interestingly, they showed that TGFβ inhibition reduces the efficiency of the DDR mechanism by specifically preventing the phosphorilation of ATM after radiation induced DNA damage (Bouquet *et al*., 2011). While the heterogeneity of the tumor populations response was not evaluated, (Anido *et al*., 2010) showed that inhibition of TGFβ specifically reduces the population of $CD44^+/Id1^+$ putative glioblastoma CSCs (Anido *et al*., 2010; Zhang *et al*., 2011) reported that glioblastoma $CD133^+$ cells in colony formation assays were specifically radiosensitize and suffered more DNA damage when the cultures were treated with the same inhibitors. Taken together, these studies indicate that the inhibition of TGFβ sensitize CSCs to radiotherapy by directly targeting their capacity to response to DNA damage (**Fig. 2**).

TGFβ is a family of cytokines involved in most biological processes including proliferation, migration, invasion, differentiation, angiogenesis, immune response and apoptosis (Moses *et al*., 2011). Just since its first role as an inhibitor of the mammary gland end buds development was identified by (Silberstein and Daniel, 1987) the controversy of its diametrically opposed roles arose as few months later, (Knabbe *et al*., 1987) showed that TGFβ was produced by the mammary gland to

contribute to hormone resistance (Knabbe *et al*., 1987). Since then, TGFβ has been associated with tumor suppressor functions, controlling proliferation, apoptosis and instability, as well as with tumor promotion ones such as migration, invasion and plasticity.

The main regulator of TGFβ is the restrain of the active cytokine from the Latent complex (LTGFβ) in which is secreted associated with the Latency Associated Peptide (LAP). This process of activation is the main controller for the bioactivity of TGFβ. The LTGFβ complexes are secreted to the Extra Cellular Matrix (ECM) which serves as a reservoir (Flaumenhaft and Rifkin, 1992). These can be then locally activated through a wide variety of mechanisms including integrins, metalloproteinases, elastase, plasmin, thrombospondin (reviewed in (Moses *et al*., 2011)) and, more interestingly, ionizing radiation and ROS (Barcellos-Hoff and Dix, 1996). Upon activation, TGFβ binds to the type II TGFβ receptor (TβIIR) promoting dimerization and transactivation of the type I TGFβ Receptor (TβIR), triggering a downstream phosphorilation signaling of the SMAD transcription factors cascade (Derynck and Zhang, 2003; Massague, 2008). Activated SMADs then translocate from the cytoplasm to the nucleus where they regulate transcription of various target genes (reviewed in (Moses *et al*., 2011)) that translate into the different range of responses observed in cancer cells. The diametrically opposed response observed to TGFβ activation can be explained by a molecular balance of transcription factor C/EBPβ isoforms, LIP and LAP (Gomis *et al*., 2006) that controls a key program for the induction of c-myc and repression of p15. As a result, cancer cells become insensitive to proliferative and instability controls while acquire the malignant characteristics of invasion, motility and plasticity (Massague, 2008).

In the light of these roles of TGFβ in cancer establishment and progression it would be expected that, in addition to the DNA damage radiosentitizing effect described above, the inhibition of TGFβ can result in an overall benefit for radiotherapy as several capabilities of both CSCs and non CSCs will be compromised. For example, by inhibiting TGFβ it would be expected that the invasive and metastatic potential of the tumors will be reduced. Interestingly, that effect was shown by (Bouquet *et al*., 2011) as they reported a significant decrease in the number of lung metastasis in a xenograft model for breast cancer after treatment with radiation and TGFβ inhibitors (Bouquet *et al*., 2011). Such effect can be attributed to a direct inhibition of the EMT program which increases cancer cells motility and invasion through a TGFβ dependent activation of the transcription factors Snail and Twist (Zeisberg and Neilson, 2009).

Additionally, inhibition of TGFβ can provide a new interesting approach in radiosentitizing tumors and CSCs through its effect on reoxygenation. Hypoxic cancer cells show increased resistance to radiation (Thomlinson and Gray, 1955) especially when they are under intermittent hypoxic conditions (Zolzer and Streffer, 2002), which can be explained by the intermittent angiogenesis induced in the CSCs perivascular niches through the expression of Hypoxia-Inducible Factor α (HIF-α) (Gustafsson *et al*., 2005; Pajonk *et al*., 2010). In contrast, persistent chronic hypoxia may increase radiosensitivity by decreasing RAD51-dependent DNA damage repair (Chan *et al*., 2008). A direct approach to induce chronic hypoxia is to prevent tumor angiogenesis. Interestingly, it has been shown that ionizing radiation may induce endothelial cell kill itself (Garcia-Barros *et al*., 2003; Imaizumi *et al*., 2010) and a synergistic effect between anti-angiogenic therapies and radiation has been observed (Seiwert and Cohen, 2008). TGFβ has been shown to stimulate angiogenesis through local transcriptional activation of Vascular Endothelial Growth Factor (VEGF) and Connective-Tissue Growth Factor (CTGF) (Sanchez-Elsner *et al*., 2001; Kang *et al*., 2003) and also by inducing endothelial cells to express monocyte chemoattractant Protein-1 (MCP-1) for the recruitment of vascular smooth muscle and mesenchymal cells toward the endothelium (Ma *et al*., 2007). Thus the power of inhibiting TGFβ in this scenario will reside in that, after radiotherapy, the surviving CSCs will be prevented for the possibility or reoxigenation and expansion, as angiogenesis will be blocked.

In essence, three major components of cancer malignancy can be targeted by inhibiting TGFβ in combination with radiotherapy. In addition to the specific radiosensitization effect caused in the CSCs, the tumor plasticity and angiogenesis capacity can be blocked resulting in an increased control of the tumor mass, essential for further targeting of the cancer cells in fractionated radiotherapy.

Targeting the cancer stem cell niche: The dependence of the CSCs to their local microenvironment is not only important for their response to reoxygenation and evidences accumulate showing that cancer cells are established in niches composed of several cell types that contribute to regulation and maintenance of the CSCs pool (Scadden, 2006; Gilbertson and Rich, 2007; Hambardzumyan *et al*., 2008). Is from these niches where, after radiotherapy has killed most of the tumor

volume, radioresistant cancer stem cells exit the quiescent state in which they reside and initiate the repopulation of the tumor (**Fig. 1**).

Different cell cycle stages are characterized by different resistance to radiation, being cells in mitosis the most sensitive ones (Pawlik and Keyomarsi, 2004). Although there is only direct evidence from hematopoietic stem cells (Hoey *et al*., 2009; Korkaya and Wicha, 2009), CSCs are believed to exist in a quiescent G0 state in their niches and they become recruited to enter in cell cycle in order to initiate proliferation and repopulation of the tumor after radiotherapy. The Notch pathway has been identified as one of the molecular mechanisms implicated in this transition (Wu *et al*., 2007; Campa *et al*., 2008). It has been described that multiple radiation fractions promote Notch activation above the levels observed for single doses (Phillips *et al*., 2006). This is concomitant with an increase in the number of cycling CSCs (Vlashi *et al*., 2009) being recruited from their niches to repopulate the tumor and opens a new window for radiosensitization.

The repopulation capacity of the cancer stem cells, recruited from their perivascular niches to actively proliferate again, is the main reason for the failure of radiation therapy (Withers *et al*., 1988; Bese *et al*., 2005). This imply that CSCs in radiation treated cancers not only have to exit their quiescent stage, but also initiate a new program of symmetric and asymmetric divisions to establish the tumor heterogeneity, usually with faster growth rate than that from non treated tumors. This is done through an increased initial frequency of symmetric divisions that leads to a higher number of CSCs in the niches early after radiation, which later on will switch to asymmetric divisions to generate the progeny of heterogeneous tumor cells. Interestingly, the mechanism that orchestrate the decision between symmetric and asymmetric divisions in both normal tissue stem cells and in CSCs, employs the Notch, Wnt and hedgehog pathways (Phillips *et al*., 2006; Xu *et al*., 2008; Bisson and Prowse, 2009). In that regards several reports have shown activation of the Notch and Wnt pathways after radiation (Phillips *et al*., 2006; Woodward *et al*., 2007; Scharpfenecker *et al*., 2009).

Activation of the Notch pathway requires cell to cell contacts, where binding between the Notch receptor and ligands of the Delta or Jagged family occur. After binding, the Notch receptor is divided and the extracellular part is internalized into the ligand cell, while the remaining Notch is cleaved in the receptor cell by γ-secretase. This cleavage induces the release of the Notch receptor intracellular domain which translocates into the nucleus and binds to CBF-1, transforming this factor from transcriptional repressor to activator. As a result, several products are expressed to promote progression into the S-phase (Weinmaster and Kopan, 2006), recruitment into cell cycle (Campy) and maintenance of the stem cell phenotype (Wu *et al*., 2007) (**Fig. 3**).

Thus, the activation of the Notch pathway in CSCs can be targeted during fractionation radiotherapy to prevent the repopulation of the tumor after each fraction of radiation. As a prove of principle, the potential benefit of inhibiting Notch has been demonstrated by knockdown of Notch1 or Notch2 and ex vivo irradiation of glioma cells before injection, which resulted into extended tumor latency more than either treatment alone (Wang *et al*., 2010). In a recent report (Liu *et al*., 2011) showed that Notch inhibition both by γ-secretase inhibition and by delta ligand specific blockade resulted in a synergistic tumor growth delay when combined with radiotherapy in colorectal carcinoma and head and neck cancer cells (Liu *et al*., 2011). Interestingly, the group also reported a significant anti angiogenic effect of the delta ligand blockade when combined with radiotherapy, indicating that specific inhibition of the Notch pathway may interfere not only with the recruitment and repopulation capacity of the CSCs, but also with their ability to recruit endothelial cells to promote angiogenesis.

Telomerase targeting and radiotherapy: In addition to the window opened for the combination of radiotherapy with inhibitors for the Notch pathway, the existence of a CSCs niche that operates as a seed for tumor repopulation, uncovers a new possibility for radiosensitization in fractionated radiotherapy: Telomere targeting and the inhibition of telomerase activity.

The terminal regions of linear genomes are called Telomeres (Blackburn, 1984). They are formed by multiple tandem repetitions of a six nucleotide sequence that confers chromosomal stability and protects from irregular recombination, degradation and end to end fusions (Artandi and Attardi, 2005). A whole protein complex composed by six telomere-associated proteins called Shelterin, contributes to the stability of these terminal regions by creating a two-loops system that protects the telomeres from being recognized as a double strand break by the DNA damage response mechanism-conformation known as capped telomere-(De Lange, 2005).

Telomeres are the main cause for the “mitotic clock for aging” (Blasco, 2005). During the cell cycle, prior to the cell division at the end of the S phase when the DNA is finishing its replication, the polymerase complexes are

unable to synthesize the end of the new strands of DNA, creating a gap in the 5' region (Olovnikov, 1973). This process will cause a progressive shortening of the telomeres in proliferating cells every time they divide (Harley, 1991) that will lead to the disruption of the protecting system enabled by the sheltering and the recognition of the telomeres as a DSB-uncapped telomeres-which will activate the cellular senescence pathway (Artandi and Attardi, 2005; De Lange, 2005). But this process can be bypassed by the expression of the telomerase protein complex. Telomerase is a DNA polymerase ribonucleoprotein complex Comprising an RNA subunit (TERC) and a Reverse Transcriptase component (TERT) that is express in normal stem cells and in embryonic tissues as a mechanism to prevent telomere attrition (Flores *et al*., 2006). The activation of the complex requires the interaction of its main subunits TERT and TERC with dyskerin 1 (Cohen *et al*., 2007), a protein for assembling and stabilizing the complex. Thus, the regulation of the telomerase is done through individual mechanisms that control the expression of its three major components and through the assembling process that binds them together (reviewed in (Flores *et al*., 2006; Collins, 2008).

In addition, the activity is mediated by the accessibility of the complex to the telomeres and as such, depends on the Shelterin complex conformation and its own regulation (De Lange, 2005; Seimiya *et al*., 2005). It is well known that telomerase is overexpressed in the majority of human cancers (Holt and Shay, 1999), where it acts as a mechanism to bypass telomere derived senescence and apoptosis. More importantly, ionizing radiation has been repeatedly shown to up regulate telomerase activity in several cancer cell lines *in vitro* (Finnon *et al*., 2000; Wang *et al*., 2000; Perez Mdel *et al*., 2002; Ram *et al*., 2009).

The differences between normal and cancer cells in terms of telomerase expression and telomere length provide a therapeutic opportunity for telomerase inhibition-based therapies. Cancer patients are less likely to develop resistance to telomerase-based therapies than to other cancer drugs and telomerase-based therapies are unlikely to cause tissue toxicity to normal non telomerase expressing cells (Harley, 2008). In addition, telomere dysfunction has been shown to enhance the radiosensitivity of cancer cells by decreasing the efficiency of the DDR mechanism in repairing the induced DNA (Wong *et al*., 2000). This characteristic has been employed to develop new agents that, by specifically targeting the telomeres conformation or the telomerase activity, increase radiosensitivity of cancer cells to radiation (Ayouaz *et al*., 2008; Wesbuer *et al*., 2010) (**Fig. 4**).

There are two different approaches for targeting telomerase activity: Direct enzyme inhibition and active telomerase immunotherapy. Different telomerase activity inhibitors have been proven to have anticancer properties in cell cultures *in vitro* as well as in animal models (Dikmen *et al*., 2005; Kelland, 2007). Furthermore, we have recently showed that inhibition of telomerase activity with small molecule inhibitors specifically enhances radiosensitivity of the AldH positive population of lung CSCs. We observed an increased tumor control *in vivo* after radiation and telomerase inhibition caused by the specific deletion of the AldH positive cells. Increased radiosensitivity of the AldH positive cells was also proven in vitro by sphere forming and colony survival assays (Serrano *et al*., 2011). As a different approach, cancer immunotherapies use synthetic TERT peptides to induce an immune response against cancer cells expressing TERT antigens. Recent results from phase I/II clinical trials have prove increased survival of patients with stage III non-small cell lung cancer when immunized with telomerase peptide vaccination in patients after having been treated with radiotherapy (Brunsvig *et al*., 2011). Although the specific response of the CSCs to the telomerase based immunotherapy was not evaluated, this results support the idea of a decrease in the proliferative capacity of the cancer cells after the combination of radiotherapy with the inhibition of telomerase. Finally, an example for direct telomere targeting for radiosensitization has been recently described by (Merle *et al*., 2011) in glioblastoma cells using a G-cuadruplex ligand that selectively binds to the spatial configuration of the telomeres causing instability and telomere uncapping. As a result, they showed a delay in the activation of the DDR mechanism by γH2AX and 53BP1 foci, resulting in enhanced radiosensitivity of GBM cells (Merle *et al*., 2011).

Given the specificity and selectivity of telomerase based therapies, the addition of a radiosensitization effect in the CSC population reinforce even more the potential in the combination of telomerase-telomere inhibition agents for controlling the tumor repopulation after radiotherapy. This way, the surviving cancer cells that remain after radiation will suffer from continuous telomere attrition under the telomerase inhibition treatment while proliferating to repopulate the tumor. This will finally cause telomere dysfunction and apoptosis, which contribute even further to control the tumor volume after several fractions of radiation.

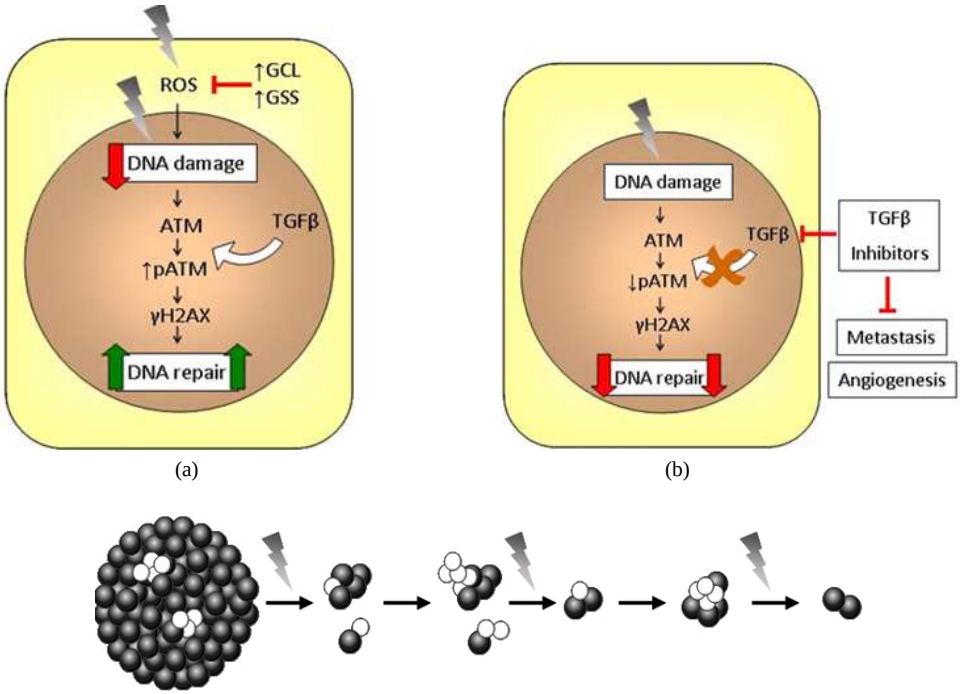
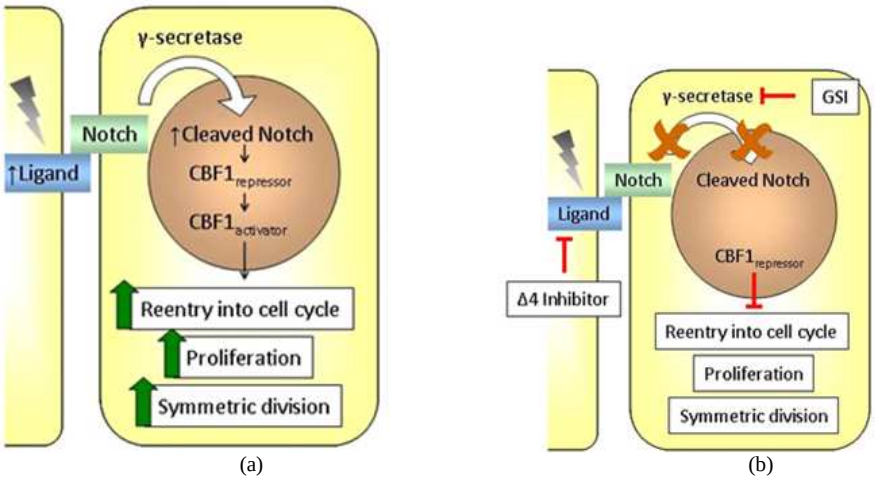
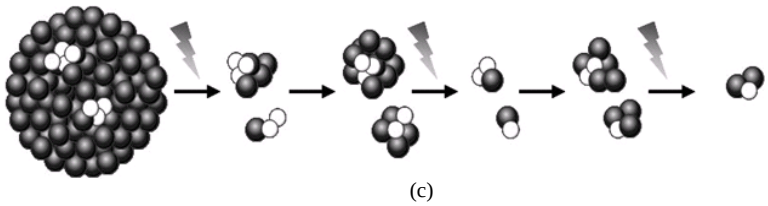


Fig. 2. TGFβ inhibition as a target for CSCs resistance to radiation induced DNA damage. (a) The increased radioresistance of CSCs is obtained by two intrinsic mechanisms: First, the DNA damage repair mechanism is generally activated more efficiently in CSCs than in non CSCs as evidenced by phosphorilation of ChK1 and ATM; Second, the overexpression of GCL and GSS facilitates the scavenging of radiation induced ROS, resulting in less DNA double strand breaks. (b) By targeting TGFβ with small molecule inhibitors or neutralizing antibodies cancer cells in general and CSCs in particular become more sensitive to radiation induced DNA damage due to a decrease in the phosphorilation of ATM and the consequent blockade of the DNA damage response mechanism. In addition, by inhibiting TGFβ, several other cancer cells capabilities such as angiogenesis and metastasis can be compromised resulting in an overall benefit greater than radiosensitization itself. (c) A tumor populations model representation for the effects of TGFβ inhibition in combination with fractionated radiotherapy. Due to the radiosentitizing effect of inhibiting TGFβ, the CSC subpopulation is no longer selected after radiotherapy, resulting in the eventual disappearance of the CSCs from the tumor after several fractions of radiation





(c)

Fig. 3. Notch inhibition and the CSCs niche. (a) While radiotherapy kills most of the tumor cancer cells, the surviving CSCs remain quiescent in their niches where the Notch ligand is overexpressed after radiation. Notch is expressed in the membrane of CSCs in their niches and interaction between Notch and its ligand induces the internalization of the cleaved Notch fraction which then activates CBF-1 to promote reentry in to cell cycle, proliferation and symmetric division. This is translated in an overall increase of radioresistant and tumor initiating CSCs after each fraction of radiation. (b) Blocking the Notch ligand or specifically inhibiting the γ-secretases prevents cleaved Notch from binding to CBF-1. Thus, CBF-1 remains as a transcriptional repressor preventing the maintenance of the stem cell phenotype. (c) In a model of fractionated radiotherapy, inhibition of the Notch pathway may result in an increased tumor control after each fraction of radiation, as the capacity of CSCs to proliferate in their niches in being compromise

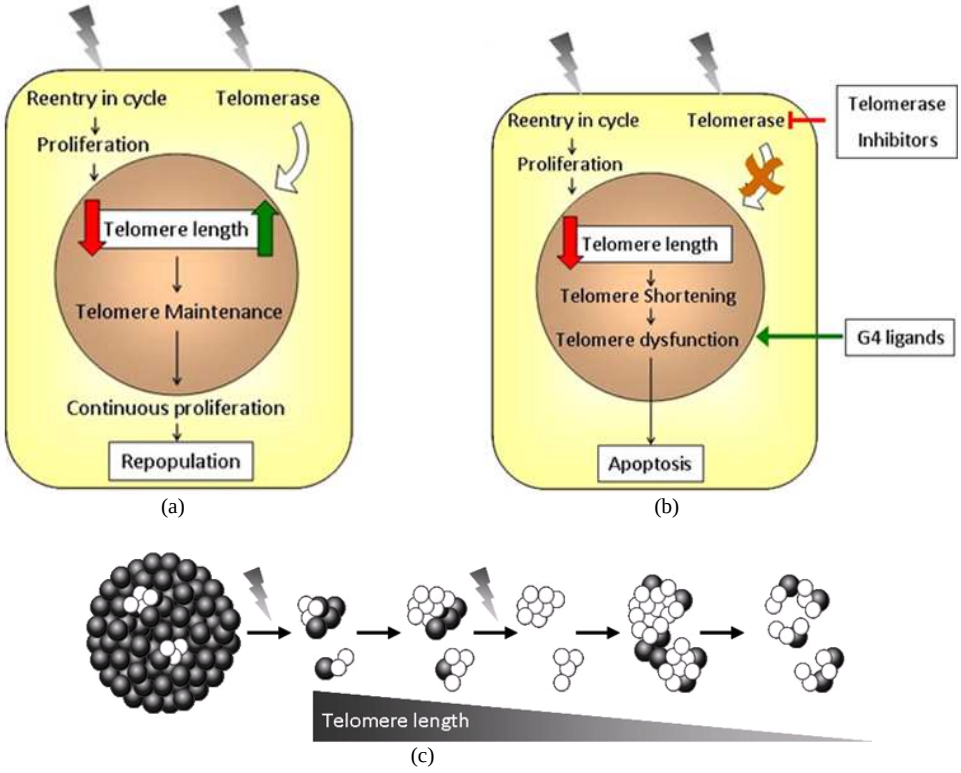


Fig. 4. Targeting the telomeres integrity for CSCs radiosensitization. (a) CSCs are stimulated to proliferate in their niches after radiotherapy has killed the radiation sensitive population of non CSCs. During the continuous cell divisions, overexpression of telomerase protects the telomeres from attrition and maintains a capped telomere conformation that facilitates further proliferation. (b) Inhibition of telomerase will cause continuous telomere shortening as the cells proliferate to repopulate the tumor after radiation. When the telomeres become critically short, the spatial protective conformation is compromised and the following uncapping induces apoptosis. The same effect can be triggered by specific agents that bind to the telomeric DNA G-cuadruplexes and destabilize the conformation to induce telomere dysfunction. (c) In a combination of radiotherapy with telomerase/telomere targeting agents, the surviving CSCs after radiation will suffer continuous telomere attrition and final apoptosis, as they proliferate in order to repopulate the tumor volume

2. CONCLUSION

While the existence of a subpopulation of cells within the tumor capable to survive radiotherapy and cause cancer recurrence is worrisome, the discovery of the molecular mechanisms employed by these so called cancer stem cells to bypass therapy have revealed a new promising avenue for directed targeting that just recently has started to be exploited. The idea underlying these recent discoveries is that, by targeting the CSCs with specific inhibitors, the efficiency of conventional fractionated radiotherapy will be significantly improved. Furthermore, as these CSCs are supposed to be the true initiators of the tumor recurrence, they should be the main target for cancer treatment and radiotherapy.

We have shown three different approaches for specific CSCs radiosensitization, targeting each of them a different characteristic of the CSC biology. All three have resulted in very promising preliminary data in vitro and in animal models and some of them, like the telomerase inhibitor GRN163L and the telomerase peptide vaccine GV1001, are already in different phases of clinical trials. Hopefully, in the years to come more approaches and compounds will show the light and will prove their efficiency, the clinic by being able to eliminate CSCs and tumors when combined with radiotherapy.

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