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EXPRESSION AND ROLE OF BORIS/CTCFL IN HUMAN CANCER STEM CELLS

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A tutte le persone che mi sono sempre vicine

“Guarda verso il sole

e l’ombra ti cadrà alle spalle”

RESUME

Le cancer est défini comme la croissance incontrôlée des cellules dans le corps. Il est responsable de 20 % des décès en Europe. Plusieurs expériences montrent que les tumeurs sont issues et se développent grâce à un petit nombre de cellules, que l'on appelle cellules souches cancéreuses (CSC). Ces CSC sont également responsables de l'apparition de métastases et de la résistance aux médicaments anticancéreux. De ce fait, l'identification des gènes qui contribuent aux propriétés de ces CSC (comme la survie des tumeurs, les métastases et la résistance aux médicaments) est nécessaire pour mieux comprendre la biologie des cancers et d'améliorer la qualité des soins des patients avec un cancer. A ce jour, de nombreux marqueurs ont été proposés ainsi que de nouvelles thérapies ciblées contre les CSC. Toutefois, et malgré les énormes efforts de la recherche dans ce domaine, la quasi-totalité des marqueurs de CSC connus à ce jour sont aussi exprimés dans les cellules saines.

Ce projet de recherche visait à trouver un nouveau candidat spécifique des CSC. Le gène BORIS (pour Brother of Regulator of Imprinted Sites), nommé aussi CTCFL (CTCF-like), semble avoir certaines caractéristiques de CSC et pourrait donc devenir une cible prometteuse pour le traitement du cancer. BORIS/CTCFL est une protéine nucléaire qui se lie à l'ADN, qui est exprimée dans les tissus normaux uniquement dans les cellules germinales et qui est réactivée dans un grand nombre de tumeurs. BORIS est impliqué dans la reprogrammation épigénétique au cours du développement et dans la tumorigenèse. En outre, des études récentes ont montré une association entre l'expression de BORIS et un mauvais pronostic chez des patients atteints de différents types de cancers.

Nous avons développé une nouvelle technologie basée sur les Molecular Beacon pour cibler l'ARNm de BORIS et cela dans les cellules vivantes. Grâce à ce système expérimental, nous avons montré que seule une toute petite sous-population (0,02 à 5%) de cellules tumorales exprimait fortement BORIS. Les cellules exprimant BORIS ont pu être isolées et elles présentaient les caractéristiques de CSC, telles qu'une forte expression de hTERT et des gènes spécifiques des cellules souches (NANOG, SOX2 et OCT4). En outre, une expression élevée de BORIS a été mise en évidence dans des populations enrichies en CSC ('side population' et sphères). Ces résultats suggèrent que BORIS pourrait devenir un nouveau et important marqueur de CSC. Dans des études fonctionnelles sur des cellules de cancer du côlon et du sein, nous avons montré que le blocage de l'expression de BORIS altère largement la capacité de ces cellules à former des sphères, démontrant ainsi un rôle essentiel de BORIS dans l'auto-renouvellement des tumeurs. Nos expériences montrent aussi que BORIS est un facteur important qui régule l'expression de gènes jouant un rôle clé dans le développement et la progression tumorale, tels le gène hTERT et ceux impliqués dans les cellules souches, les CSC et la transition épithélio-mésenchymateuse (EMT). BORIS pourrait affecter la régulation de la transcription de ces gènes par des modifications épigénétiques et de manière différente en fonction du type cellulaire.

En résumé, nos résultats fournissent la preuve que BORIS peut être classé comme un gène marqueur de cellules souches cancéreuse et révèlent un nouveau mécanisme dans lequel BORIS jouerait un rôle important dans la carcinogénèse. Cette étude ouvre de nouvelles voies pour mieux comprendre la biologie de la progression tumorale et offre la possibilité de développement de nouvelles thérapies anti-tumorales et anti-CSC avec BORIS comme molécule cible.

SUMMARY

Cancer is defined as the uncontrolled growth of cells in the body. It causes 20% of deaths in the European region. Current evidences suggest that tumors originate and are maintained thanks to a small subset of cells, named cancer stems cells (CSCs). These CSCs are also responsible for the appearance of metastasis and therapeutic resistance. Consequently, the identification of genes that contribute to the CSC properties (tumor survival, metastasis and therapeutic resistance) is necessary to better understand the biology of malignant diseases and to improve care management. To date, numerous markers have been proposed to use as new CSC-targeted therapies. Despite the enormous efforts in research, almost all of the known CSCs markers are also expressed in normal cells.

This project aimed to find a new CSC-specific candidate. BORIS (Brother of Regulator of Imprinted Sites) or CTCFL (CTCF-like) is a DNA binding protein involves in epigenetic reprogramming in normal development and in tumorigenesis. Recent studies have shown an association of *BORIS* expression with a poor prognosis in different types of cancer patients. Therefore, BORIS seems to have the same characteristics of CSCs markers and it could be a promising target for cancer therapy. BORIS is normally expressed only in germinal cells and it is re-expressed in a wide variety of tumors.

We developed a new molecular beacon-based technology to target *BORIS* mRNA expressing cells. Using this system, we showed that the BORIS expressing cells are only a small subpopulation (0.02-5%) of tumor cells. The isolated BORIS expressing cells exhibited the characteristics of CSCs, with high expression of *hTERT* and stem cell genes (*NANOG, SOX2* and *OCT4*). Furthermore, high BORIS expression was observed in the CSC-enriched populations (side population and spheres). These results suggest that BORIS might be a novel and powerful CSCs

marker. In functional studies, we observed that BORIS knockdown significantly impairs the capacity to form spheres in colon and breast cancer cells, thus demonstrating a critical role of BORIS in the self-renewal of tumors. The results showed in the functional analysis indicate that BORIS is an important factor that regulates the expression of key-target genes for tumor development and progression, such as *hTERT*, stem cells, CSCs markers and EMT (epithelial mesenchymal transition)-related marker genes. BORIS could affect the transcriptional regulation of these genes by epigenetic modification and in a cell type dependent manner.

In summary, our results support the evidence that BORIS can be classified as a cancer stem cell marker gene and reveal a novel mechanism in which BORIS would play a critical role in tumorigenesis. This study opens new prospective to understand the biology of tumor development and provides opportunities for potential anti-tumor drugs.

Keywords: BORIS/CTCFL, cancer stem cells, cancer-testis antigens, epigenetic reprogramming, Molecular Beacon technology, hTERT telomerase, stem cell genes.

ABBREVIATIONS

5-FU	5-Fluorouracil
ABC	ATP-binding cassette
ABCB5	ATP-binding cassette sub-family B member 5
ABCG2	ATP-binding cassette sub-family G member 2
ALDH1	Aldehyde dehydrogenase 1
all-ATRA	Trans retinoic acid
AML	Acute myeloid leukemia
APC	Adenomatous polyposis coli
bFGF	Basic fibroblast growth factor
BMPs	Bone morphogenic proteins
BORIS	Brother of the regulator of imprinting sites
CDH1	E-cadherin
CMV	Cytomegalo-virus
CSC	Cancer stem cell
CTA	Cancer testis antigen
CTCFL	CCCTC-binding factor-like
Cy3	Cyanine 3
DAPI	4',6-diamidino-2-phenylindole
DEAB	Diethylamino-benzaldehyde
DKK	Dickkopf protein
DMEM	Dulbecco's modified Eagle's medium
DOX	Doxycycline

ECSA	Embryo cancer sequence A
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
eGFP	Enhanced Green Fluorescent Protein
EpCAM	Epithelial cellular adhesion molecule
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
G418	Geneticin
Hh	Hedgehog
iPS	Induced pluripotent stem
MB	Molecular beacon
miRNAs	MicroRNAs
mRNA	Messenger RNA
MTT	3-(4,5-dimethyl-2-thiazol)-2,5-diphenyltetrazolium bromide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium Iodide
qRT-PCR	Quantitative reverse transcriptase PCR
RPMI	Roswell Park Memorial Institute
SA-β-gal	Senescence-associated β-galactosidase
SFRP	Soluble Frizzled-related protein
shRNA	Small hairpin RNA or short hairpin RNA
siRNA	Small interference RNA
SLUG	SNAI2

SNAIL	SNAI1
SP	Side population
tRFP	Turbo red fluorescent protein
TWIST	Twist-related protein 1
ZF	Zinc finger

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1. INTRODUCTION

1.1. Cancer and cancer stem cells

Recent evidences support the view that cancers are complex tissues where aberrant cell growth is driven by a small population defined as cancer stem cells (CSCs) (Reya, Morrison et al. 2001; Jordan, Guzman et al. 2006; Dalerba, Cho et al. 2007). CSCs are characterized by stem cell proprieties and allow tumor metastasis and drug resistance. Their presence may explain tumor relapses and the failure of cancer treatment (chemotherapy, radiotherapy). Numerous markers have been proposed as possible new CSCs-targeted therapies. Despite the enormous efforts in research, almost all of known CSCs markers are also expressed in normal cells.

The following section will introduce the principal features of cancer and describe the CSCs, in particular their definition, discoveries and their characteristics. Then, the use of specific markers for the identification and isolation of CSCs and the development of novel CSC-targeted therapies to overcome tumor resistance and relapse will be discussed.

1.1.1. Introduction to cancer and cancer stem cells

Cancer is one of the major causes of death in the world. The World Health Organization estimated that, in 2008, cancer was a leading cause of around 13% of all deaths. The most common cancer worldwide is lung cancer followed by breast, colorectal, stomach and prostate cancers (http://globocan.iarc.fr). Tumor incidence is different between men and women. In particular, lung cancer is the most common in men with 16% of the total number of new cases, while in women breast cancer is the most frequent with 23% of the total number of new cases (http://globocan.iarc.fr).

Cancer cells acquire functional capabilities allowing their growth and dominance in a local tissue environment by a multistep process named tumorigenesis. During tumor progression, different tumor types gain distinct features as proliferation, survival, resistance to apoptosis and dissemination. Genomic instability, random mutations and epigenetic changes are the main events whereby cancer cells acquire these capabilities and are essential hallmarks for tumor development and progression (Hanahan and Weinberg 2011). Recently, it has been shown that also normal cells (such as endothelial, immune and mesenchymal cells) are recruited at the tumor site and can actively participate in tumorigenesis (Hanahan and Weinberg 2011). Thus, cancer is considered as a complex system compounds of many different cell types enable to interact together.

To explain the heterogeneity and growth of tumors, two models have been proposed (Figure 1). The first was the stochastic model, which predicts that all tumor cells have the potential to become tumorigenic and stochastically a fraction of cells proliferate to tumor growth, while the other tumor cells differentiate. The second model proposes that tumors are hierarchically structured similar to normal tissues, in which only a subset of tumor cells is capable to drive indefinitely the tumor growth and the other progenitor cells have limited growth potential. In this latter model, the tumorigenic cells have been termed “cancer stem cells” (CSCs) due to the analogy to stem cells, which control the maintenance of adult tissues. CSCs have been proposed to be responsible for the development and the progression of tumors. The clonal evolution theory assumes that in both models, the tumor cells can accumulate mutations which confer survival advantage and competition with the other non-mutated cells (Greaves and Maley 2012).

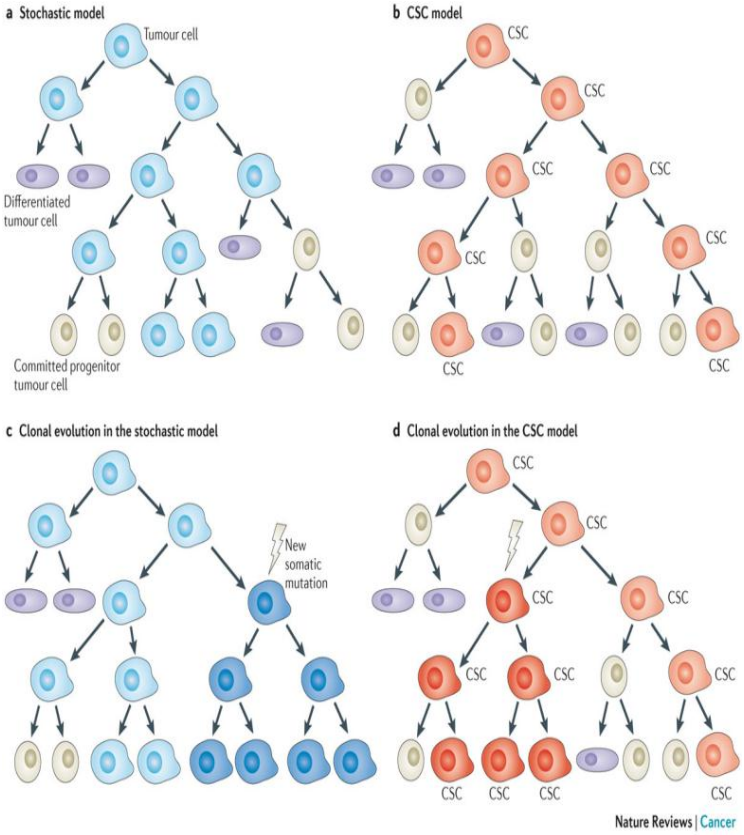

Figure 1. Models to explain the heterogeneity of tumors. a) In the stochastic model of tumor growth, all tumor cells have the potential to become tumorigenic and stochastically a fraction of cells proliferate and the other tumor cells differentiate. b) In the cancer stem cell (CSC) model of tumour growth, only a subset of tumor cells is capable to drive indefinitely the tumor growth and the other committed progenitor cells have limited growth potential and could differentiate. c, d) In both models, the clonal evolution proposes that new somatic mutations can generate clonal diversity (Beck and Blanpain 2013).

In recent years, with the research advances in stem biology and the development of new technologies to measure the CSCs properties, the CSCs theory has gained validation (Reya, Morrison et al. 2001; Pardal, Clarke et al. 2003). The experimental demonstrations of CSCs in different tumors support the concept of stem cell theory in tumor biology (Bonnet and Dick 1997; Al-Hajj, Wicha et al. 2003; Singh, Hawkins et al. 2004; Li, Heidt et al. 2007; Ricci-Vitiani, Lombardi et al. 2007; Zhang, Balch et al. 2008).

Although the term “cancer stem cell” is referred to stem cells, the cells having the property to originate the tumors could be stem cells, progenitor cells or differentiated cells, depending on the tumor type (Clarke, Dick et al. 2006). Cancer stem cells, which are also

named tumor-initiating cells, are characterized by three distinctive properties: 1) capacity to originate the tumors and drive tumor proliferation, 2) ability of long-term self-renewal, that is the ability to create infinite copies of themselves, 3) capacity to divide into differentiated tumor cells, which are non-CSC progeny (Schatton, Frank et al. 2009) (Figure 2).

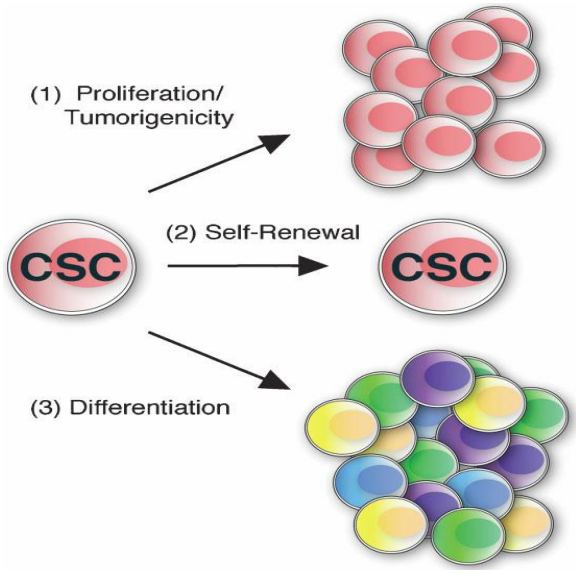

Figure 2. The principal features of cancer stem cells. CSCs are defined by three proprieties: (1) enhanced proliferation and tumorigenic growth, (2) self-renewal capacity, (3) ability to differentiate in non-CSC population (Schatton, Frank et al. 2009).

CSCs have the capability of both symmetrical and asymmetrical cell division (Clarke, Dick et al. 2006). The symmetrical division into two identical daughter CSCs explains the self-renewal ability while the asymmetrical division into one daughter cell and one progenitor differentiated cell explains the differentiation ability of CSCs.

The hypothesis that tumors could evolve from a subset of cell population with stem cell characteristics was first demonstrated in hematological malignancies. It has been identified a subpopulation of tumor cells in acute myeloid leukemia (AML) that was able to give rise to leukemic growth in severe immodeficient mice (Lapidot, Sirard et al. 1994; Bonnet and Dick 1997). This $CD34^{+}CD38^{-}$ subpopulation represents a small fraction of the

leukemia population that shows self-renewal and differentiation capabilities, which allows to recapitulate the entire hierarchy of human leukemia in mice. Following these studies in hematological malignancies, a number of studies have also identified CSCs in solid tumors. For example, $ESA^{+}CD44^{+}CD24^{-/low}$ breast tumor cells demonstrated to form tumors when injected into mammary fat pads of NOD/SCID mice, while cells lacking these markers were not capable to form tumors (Al-Hajj, Wicha et al. 2003). Different reports have identified CSCs in solid tumors based on other surface markers in a variety of organs such as brain, pancreas, colon and ovaries (Bonnet and Dick 1997; Al-Hajj, Wicha et al. 2003; Singh, Hawkins et al. 2004; Li, Heidt et al. 2007; Ricci-Vitiani, Lombardi et al. 2007; Zhang, Balch et al. 2008). Other phenotypic markers such as aldehyde dehydrogenase (ALDH) activity (Ginestier, Hur et al. 2007) and side population (SP) (Kondo, Setoguchi et al. 2004) have been demonstrated to identify CSCs. The most common CSCs markers used so far will be described in detail in the following section.

The existence of this small population of tumor cells that drives tumor development, progression and metastasis could explain the recurrent failures of the conventional anticancer therapies. The conventional anticancer drug discovery has focused on the cytotoxic agents which inhibit metabolic pathways crucial for cell division. These drugs have demonstrated to have significant cytostatic or/and cytotoxic activity *in vitro* on tumor cell lines and to cause tumor regression in cancer xenograft mouse model. However, experimental evidences support the hypothesis that some cancer cells can survive and adapt to the selective pressure imposed by the drugs, becoming resistant to the treatment. This adaptation could involve mutations, epigenetic reprogramming and changes in the local tissue environment and it could be the cause of the restoring of the cancer growth and consequently cancer relapse. These evidences suggest the existence within a tumor of a

subpopulation of cancer cells that have the ability to adapt to their microenvironment and become resistant to drug treatment. This cell subpopulation could be represented by CSCs, since there have been a number of studies showing that these cells are resistant to standard chemotherapies. For example, high level of $CD44^{+}CD24^{-/low}$ cells were found in biopsy samples of human breast cancer patients treated with standard chemotherapy (Li, Lewis et al. 2008). CD133+ human colon tumor cells were more resistant to apoptosis after treatment with oxaliplatin or 5-fluorouracil (Todaro, Alea et al. 2007). Therefore, some properties of CSCs, as expression of drug resistance transporters and quiescence, could make them resistant to the conventional cytotoxic drugs.

Recent studies have correlated the acquisition of CSCs properties with the epithelial-mesenchymal transition (EMT) process (Mani, Guo et al. 2008; Morel, Lievre et al. 2008; Singh and Settleman 2010). EMT is a morphogenic cellular program in which epithelial cells acquire a mesenchymal phenotype characterized by dramatically alteration of their shape and increase of motility (Thiery 2002). EMT is considered the first step in the metastatic process, as cancer cells acquire the capability to migrate, invade and disseminate (Biddle, Liang et al. 2011; Dave, Mittal et al. 2012). For example, acquisition of EMT and CSC marker expression was observed in mammary epithelial cells transformed by mutant *Ras* expression (Morel, Lievre et al. 2008). Moreover, it has been shown that the overexpression of some genes, such as *NANOG, CD44, TWIST, hTERT*, leads to reprogram the non-CSCs or bulk tumor cells in cells having CSCs characteristics (Jeter, Liu et al. 2011; Scaffidi and Misteli 2011; Su, Lai et al. 2011; Paranjape, Mandal et al. 2012).

In 2006, Takahashi and Yamanaka have demonstrated that a differentiated fibroblast cell could be reprogrammed into an induced pluripotent stem cell (iPS) by a defined set of transcription factors (Takahashi and Yamanaka 2006). Oct4, Sox2, Nanog, Klf4 and c-Myc are

the transcription factors whereby pluripotency was induced. These transcription factors are highly expressed in embryonic stem cells and they represent the key regulators of embryonic stem cell identity. Their expression was observed in different human cancer types (Gidekel, Pizov et al. 2003; Li, Eishi et al. 2004; Rodriguez-Pinilla, Sarrio et al. 2007; Santagata, Ligon et al. 2007), where their role in tumorigenesis was established (Suvà 2013). Moreover, an overexpression of Oct4, Sox2, Nanog and c-Myc was associated with poorly differentiated aggressive tumors, such as basal-like subtype ER-negative breast cancer, poorly differentiated glioblastoma and bladder carcinoma (Ben-Porath, Thomson et al. 2008). All these evidences reveal a link between these genes, associated with embryonic stem cell identity, and the stem cell-like phenotype observed in tumors. Indeed, a number of studies showed an overexpression of these pluripotent stem cell genes in CSCs (Apostolou, Toloudi et al. 2012; Akhavan-Niaki and Samadani 2013; Giampieri, Scartozzi et al. 2013; Wang, Liu et al. 2013).

Based on the model of cancer as a hierarchical disease, the CSCs are biologically diverse from the bulk tumor cells. The molecular pathways involved in survival and response to injury could be different in CSCs compared to non-tumorigenic cells. To achieve permanent cure and prevent tumor relapse, CSCs that drive the tumor growth must be eradicated, in addition to eliminating the bulk tumor cells. Therefore, it is crucial to understand the biology of CSCs in order to develop more effective targeted anti-CSCs treatments.

The major obstacle to develop CSC-targeted therapies becomes from the fact that the CSCs retain features of the pathways involved in normal development, such as pathways that regulate self-renewal in normal stem cells (Hope, Jin et al. 2004). Therefore, drugs that target specifically process involved in survival or self-renewal may be effective against CSCs

but they could also affect their normal counterparts. Moreover, progenitor or normal stem cells could be more sensitive to chemotherapy compared to CSCs. Indeed, it is possible that CSCs acquire genetic and epigenetic modifications which allow them to bypass the tumor-suppressing process that normal cells possess in response to DNA damage, such as senescence and apoptosis. Therefore, therapies with drugs that induce senescence or apoptosis may provide an advantage to the growth of CSCs (Bao, Wu et al. 2006). More effective drugs should target pathways important for CSCs survival but not for the growth of normal stem cells.

1.1.2. Cancer stem cells markers

Currently, the most common method to identify and isolate the CSCs is based on the expression of cell surface proteins. A list of the major CSC markers identified in solid tumor types is reported on Table 1 (Medema 2013).

Table 1. CSCs markers of different types of solid tumors. (Medema 2013).

Breast	Colon	Glioma	Liver	Lung	Melanoma	Ovarian	Pancreatic	Prostate
ALDH1	ABCB5	CD15	CD13	ABCG2	ABCB5	CD24	ABCG2	ALDH1
CD24	ALDH1	CD90	CD24	ALDH1	ALDH1	CD44	ALDH1	CD44
CD44	β-catenin activity	CD133	CD44	CD90	CD20	CD117	CD24	CD133
CD90	CD24	α6-integrin	CD90	CD117	CD133	CD133	CD44	CD166
CD133	CD26	nestin	CD133	CD133	CD271		CD133	α2β1-integrin
Hedgehog-Gli activity	CD29						c-Met	α6-integrin
α6-integrin	CD44						CXCR4	Trop2
	CD133						Nestin	
	CD166						Nodal-Activin	
	LGR5							

However, most of the CSCs marker identified so far exhibit broad expression because they are also expressed by normal stem cells, rendering them non-CSCs specific. As an example, CD44 is a membrane glycoprotein involved in cell–cell interactions, cell adhesion and migration (Goodison, Urquidi et al. 1999), it is expressed in normal epithelial tissues and marks also CSCs of breast and other type of tumors. In addition, CD44 is amplified in different isoforms. The variants that identify specifically CSCs is still unknown (Fox, Fawcett et al. 1994). One widely used CSCs marker is CD133 (prominin-1), a transmembrane glycoprotein discovered in haematopoietic and neural stem cells. It has been shown that $CD133^+$ CSCs have strong resistance to chemotherapy and radiotherapy (Todaro, Alea et al. 2007). Monoclonal antibodies against two epitopes of CD133 protein were used to treat human melanoma cells and a cytotoxic effect was found in these cells (Rappa, Fodstad et al. 2008). CD133 is considered as a potential target of CSCs. Indeed, it has been found that $CD133^+$ CSCs isolated from colon cancer were able to initiate tumor growth in immunodeficient mice (Todaro, Alea et al. 2007). Nevertheless, CD133 could not be used as an efficient CSCs marker because is also expressed in a large variety of normal (Mizrak, Brittan et al. 2008).

To identify and isolate CSCs, it has been proposed to use a combination of markers. As reported above, human breast cancer cells with $CD44^+CD24^-$ phenotype have been observed to be tumorigenic (Al-Hajj, Wicha et al. 2003). $CD44^+CD24^+ESA^+$ human pancreatic cancer cells have been shown to be tumorigenic (Li, Heidt et al. 2007). $CD45^-CD90^+CD44^+$ was recommended to be a good marker combination for human liver tumor (Yang, Ngai et al. 2008). However, the use of marker combination makes more difficult the development of specific CSCs–targeted therapies.

Another marker highly expressed in CSCs, especially in breast CSCs, is ALDH1 (aldehyde dehydrogenase 1) (Ginestier, Hur et al. 2007). ALDH1 is an isoenzyme of ALDH family, $NAD(P)^+$-dependent enzymes able to catalyze the oxidation of aldehyde in carboxylic acid (Ma and Allan 2011) and expressed in both normal stem cells and CSCs. It functions as drug detoxifying enzyme, and is thus responsible for chemoresistance (Sun and Wang 2010; Marcato, Dean et al. 2011). The ALDH-activated fluorescent substrate (Aldefluor assay) is a suitable marker widely used to detect cells having high ALDH activity (Ma and Allan 2011). In human breast cancer cells, an $ALDH^{hi}CD44^+$ subpopulation was identified to be resistant to chemotherapeutic drugs (doxorubicin/paclitaxel) and radiotherapy. Furthermore this subpopulation was observed to be sensitized to treatment after exposure with some ALDH inhibitors, as DEAB (diethylamino-benzaldehyde) and ATRA (all-trans retinoic acid) (Croker and Allan 2012). However, ALDH1 could not be a good target for CSCs because the inhibition of ALDH1 led to a significant up-regulation of stem cell genes (Ginestier, Hur et al. 2007).

The ABC (ATP-binding cassette) drug transporters are highly expressed in normal stem cells and also in many CSCs. They have the ability to efflux dyes out of the cells, protecting them from xenobiotic toxins (Haraguchi, Utsunomiya et al. 2006; Remsberg, Lou et al. 2007). This capacity is usually used in protocols where Hoechst dye is pumped out by cells expressing these pumps, which identifies a dye-free cell subpopulation named side population (SP) (Vermeulen, Sprick et al. 2008). The major responsible of this phenotype is ABCG2 (also named BCRP) protein, a member of ABC transporters protein family. ABCG2 is responsible of multidrug resistance, thus is considered as a potential CSCs marker (Hirschmann-Jax, Foster et al. 2004; Kondo, Setoguchi et al. 2004; Chiba, Kita et al. 2006). SP cells have shown different properties of CSCs, such as self-renewal, tumorigenicity, expression of stem cell genes and other CSC markers (Wu and Alman 2008). Furthermore,

they can be isolated from tumor cell lines (Kondo, Setoguchi et al. 2004). It has been shown that another ABC transporter family (ABCB5) has a functional role on CSCs in colorectal cancer, as its depletion decreased the tumorigenic capacity (Wilson, Schatton et al. 2011).

Another important property of stem cells, which is widely used to enrich CSCs, is the ability to form spheroid colonies called spheres (Dontu, Abdallah et al. 2003). The spheres can be obtained using *in vitro* assay based on serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) where cells from tumor specimens or cell lines are seed at low density (Dontu, Abdallah et al. 2003). The sphere-derived cells have shown to be tumorigenic and have drug resistance property (Qiu, Wang et al. 2012). However, the results obtained using *in vitro* assay should be confirmed by *in vivo* assay, as they do not show the tumor formation ability of CSCs. The transplantation in animal models is considered the gold standard assay which measures the self-renewal ability of CSCs and tumor propagation (Clarke, Dick et al. 2006). Typically, tumor cells isolated with specific CSC marker or from primary human tumors are transplanted into immunocompromised mice (normally NOD/SCID). Then, the mice are monitored at different time points to follow the tumor formation.

In summary, mostly all the known CSCs markers are used to isolate an enriched populations that are also expressed by normal progenitor and stem cells. Therefore, they do not represent effective markers for the development of specific anti-CSC drugs.

1.1.3. Signaling pathways involved in cancer transformation

To identify functional CSC markers, tumor research has focused on molecular signaling pathways which have important roles in normal stem cells, such as self-renewal, proliferation and differentiation. Wnt/β-catenin, Hedgehog and Notch signaling pathways

are the most characterized and they have shown to be responsible for the formation of cells with properties of CSCs (Merchant and Matsui 2010; Maugeri-Sacca, Zeuner et al. 2011) (Figure 3).

The Wnt signaling pathway is involved in embryogenesis, development, cell proliferation, survival and differentiation process (Klaus and Birchmeier 2008). Wnt ligand (secreted glycoprotein) initiates Wnt pathway binding to cell membrane receptors, resulting in the activation of target genes. The tissues where Wnt signaling is most involved are skin, intestine and mammary gland (Katoh and Katoh 2007). Aberrant activation of Wnt pathway was shown in many tumors (Barker, Ridgway et al. 2009; Gaston-Massuet, Andoniadou et al. 2011).

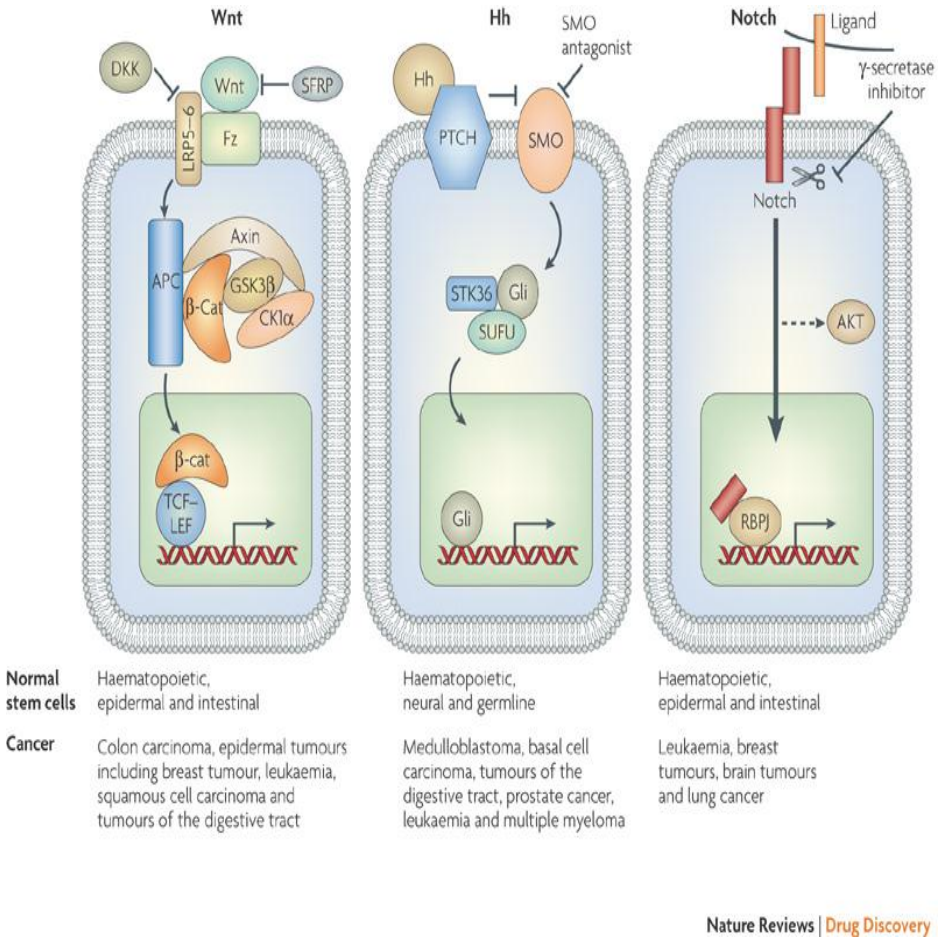

Figure 3. Signaling pathways which regulate self-renewal during normal stem cell development and cancer transformation. Wnt/β-catenin, Hedgehog and Notch pathways. (Zhou, Zhang et al. 2009).

Deregulation of Wnt pathway was observed after oncogenic mutation of β-catenin and APC (adenomatous polyposis coli) genes, resulting in neoplastic transformation (Reya and Clevers 2005). In several tumors, such as chronic myelogenous leukemia and squamous cell carcinoma, the Wnt pathway is required for the self-renewal of CSCs (Zhao, Blum et al. 2007; Malanchi, Peinado et al. 2008). It has been discovered that Wnt signaling can be inhibited by secreted proteins, as the Soluble Frizzled-related protein (SFRP) and Dickkopf protein (DKK), which act at cell membrane inhibiting the Wnt pathway through its receptors (Kawano and Kypta 2003). Small molecules antagonist of TCF- β-catenin complex factor have been described (Lepourcelet, Chen et al. 2004).

Hedgehog (Hh) signaling is another pathway important for development during embryogenesis and for the maintenance of stem cells (Varjosalo and Taipale 2008). This pathway is activated when Hh protein binds to PTCH, a transmembrane protein, resulting in the activation of target genes. Recently, it has been observed that the Hh pathway is critical for the maintenance of the proprieties of CSCs in different human tumors, such as pancreatic, gastric, and colorectal (Merchant and Matsui 2010; Song, Yue et al. 2011; Tang, Fu et al. 2012). A small molecule inhibiting the co-receptor SMO (smoothened homologue) was reported (Sanchez, Hernandez et al. 2004) and also pharmacological inhibitors that display efficacy in animal models of basal cell carcinoma, small cell lung and pancreatic tumors (Berman, Karhadkar et al. 2003; Thayer, di Magliano et al. 2003; Romer, Kimura et al. 2004). However, the results vary among the different models and these agents are not so effective (Fan, Pepicelli et al. 2004; Sasai, Romer et al. 2006).

Notch signaling pathway is involved in cell-cell communication and also in embryonic development (Wang, Li et al. 2008). It is activated by four receptors (Notch 1, 2, 3 and 4) and five Notch ligands (Delta-like 1-4 and Jagged 1,2) (Wang, Li et al. 2008). The deregulation of

Notch signaling plays different roles in tumorigenesis depending on the tumor type: oncogenic in cervical, lung, colon, head and neck, prostate, pancreatic tumors, while it could act as tumor suppressor in hepatocellular carcinoma, small cell lung cancer and skin cancer (Radtke and Raj 2003; Lobry, Oh et al. 2011). Notch pathway was targeted to eliminate CSC cells (Pannuti, Foreman et al. 2010; Wang, Ahmad et al. 2011). In glioblastoma, γ-secretase inhibitors (GSIs) were used to block Notch signaling, leading reduction of neurospheres growth, tumorogenecity and expression of CSC markers (Fan, Khaki et al. 2010). Another report shows the use of GSI MRK-003 in breast cancer, resulting in inhibition of self-renewal and proliferation of CSCs (Kondratyev, Kreso et al. 2012). However, GSIs are non-selective CSC-targeted drugs because they block the cleavage of all four Notch ligands and also other γ-secretase substrates (Wang, Ahmad et al. 2011).

There are growing evidences that Wnt, Hedgehog and Notch pathways interact with other signals, such as bone morphogenic proteins (BMPs) and growth factors produced by CSCs, tumor bulk cells and their microenvironments (Ayyanan, Civenni et al. 2006). For example, in prostate and esophageal cancer, Hedgehog activity is associated to ABC drug transporters expression (Sims-Mourtada, Izzo et al. 2007) and in colon cancer Wnt is linked to CD133 and CD44 (Katoh and Katoh 2007; Van der Flier, Sabates-Bellver et al. 2007).

Although, the dysregulation of these signaling pathways has been found in CSCs, the genes involved in these pathways are normally expressed in stem cells. Therefore, all the drugs against these pathways can target both CSCs and normal stem cells, with adverse effects. Advances in research are needed to improve their specificity against CSCs.

1.1.4. microRNAs

An altered expression of microRNA (miRNA) has been observed in CSCs (Zimmerman and Wu 2011). MiRNAs are short RNAs that post-transcriptionally regulate expression through complementary binding of mRNA sequences. They commonly led to gene silencing through target mRNA degradation or translational repression. They normally regulate different process, such as self-renewal, differentiation and cell division (Zimmerman and Wu 2011). Many studies have shown a deregulation of miRNAs in cancer, in which miRNAs can act as tumor suppressor or oncogene (Hatfield and Ruohola-Baker 2008). For example, miRNA-34a is down-regulated in several tumors and it can target p53 tumor suppressor gene as well as oncogenes, as c-Met, Notch-1-2, and CDK6 (Hermeking 2010). MiRNA-34a could induce the differentiation of CSCs in glioblatoma cells and brain tumors (Li, Guessous et al. 2009; Guessous, Zhang et al. 2010). Furthermore, miRNA-34a was observed to act as negative regulator of $CD44^+$ prostate cancer cells (Liu, Kelnar et al. 2011), suggesting that miRNA-34a could be used to target CSCs of prostate cancer. In head and neck cancer cells, miRNA-21 and miRNA-205 were highly expressed (Hatfield and Ruohola-Baker 2008). Interestingly, it has been observed that some specific miRNAs promote EMT process and metastasis. For example, the re-expression of miRNA-21 in MCF7 cell line leads to the acquisition of EMT phenotype, the upregulation of CSC markers (ALDH1 and $CD44^+CD24^-$) and the increase of sphere formation capacity (Han, Liu et al. 2012). Recently, nanoparticles load with an oligonucleotide anti-miRNA (AMO-loaded SLNs) that specifically suppresses miRNA-21 functions were tested in lung tumor A549 cells. These AMO-loaded SLNs lead to the decrease of proliferation, migration and invasion (Shi, Zhong et al. 2012). Therefore, the development of therapies targeting miRNAs could become an attractive proposition to target CSCs.

1.2. BORIS/CTCFL

BORIS (Brother of the regulator of imprinting sites) or CTCFL, CCCTC-binding factor (CTCF)-like, is a zinc finger (ZF) DNA-binding protein. BORIS is a mammalian paralog of CTCF with which it shares a 11 ZF domain (Loukinov, Pugacheva et al. 2002). It is a member of cancer testis antigen (CTA) gene family, as it is expressed in germ cells and aberrantly re-expressed in cancer (Kalejs and Erenpreisa 2005).

1.2.1. BORIS gene and protein

Human *BORIS* gene is located at chromosome 20q13.2, which is a chromosomal region amplified in many cancers (Tanner, Tirkkonen et al. 1994; Cuthill, Agarwal et al. 1999). Human *BORIS* transcript consists of 11 exons. Its expression is controlled by three alternative promoters, corresponding to transcription start sites at −1447, −899 and −658 bp upstream of the first ATG and designated promoter A, B and C respectively (Renaud, Pugacheva et al. 2007). Each of these promoters has putative binding sites for specific transcription factors, such as CREB for promoter A, NF-kB, N-myc for promoter B, WT1 and EKLF for promoter C (Renaud, Pugacheva et al. 2007). It has been also reported that BORIS can be expressed in 23 different isoforms which are translated in 17 different proteins, each isoprotein has a unique combination of ZF domains and N- and C- termini, according to promoter usage (Pugacheva, Suzuki et al. 2010). Human BORIS is 75 kDa protein that shares 70% of homology with the CTCF protein in the 11 ZF domains but differs in the N- and C- termini (Klenova, Morse et al. 2002; Loukinov, Pugacheva et al. 2002). The N-termini and full length protein interact with PMRT7-DNA methylase, hystones H1, H3, H2A (Jelinic, Stehle et al. 2006) and with the promoter of cerebroside sulfotransferase (CST) (Pugacheva, Suzuki et al. 2010).

1.2.2 BORIS functions in normal tissue

In normal human tissues, BORIS expression was firstly described to be restricted to testis, where it is involved in the spermatogenesis (Loukinov, Pugacheva et al. 2002). It was detected in primary spermatocytes, at early stage of spermatogenesis. In contrast, in spermatids and spermatozoa, *BORIS* is apparently not expressed (Klenova, Morse et al. 2002). *BORIS* expression was found to be associated with a concomitant erasure of DNA methylation marks. Although some studies indicate that in testis tissue, BORIS regulates gene expression and meiosis events, the function of BORIS in spermatogenesis is not fully understood. It has been shown that BORIS-knockout mice have small testis and defective spermatogenesis, despite they are fertile. In addition, inactivation of BORIS led to a reduction of *CST* expression, which has an important role in meiosis, and to a significant delay of sperm production (Suzuki, Kosaka-Suzuki et al. 2010). Recently, it was reported that BORIS regulates also other genes important in male germ cell development in mice, such as *Gal3st1, Prss50* and *Stra8* (Sleutels, Soochit et al. 2012). It has been observed that BORIS has an important role on DNA methylation of male germline imprinted genes, specifically on the methylation of the *H19* imprinting control region (Jelinic, Stehle et al. 2006). BORIS interacts with PRMT7, a methlytransferase protein and stimulates its histone-methyltransferase activity on histones H2A and H4. Moreover, it has been shown that BORIS preferentially binds to the paternal *H19-ICR* region which is differentially methylated, while CTCF binds to the unmethylated maternal allele (Nguyen, Cui et al. 2008). This result shows a regulatory mechanism whereby BORIS preferentially binds to methylated CTCF DNA-binding sites.

In mouse testis, it has been observed that a miRNA-709 specifically targets BORIS. Indeed, after x-ray radiation the miRNA-709 was up-regulated, resulting in the inhibition of *BORIS* expression (Tamminga, Kathiria et al. 2008). It was suggested that *BORIS* inhibition

may be act as protective mechanism to prevent aberrant erasure of DNA methylation in the treated testis. This finding also suggests that BORIS could be a target of other miRNAs that could allow the re-activation of BORIS in malignant disease.

Additionally, *BORIS* expression was detected also in ovary, precisely in oocytes and 4-cell embryos (Monk, Hitchins et al. 2008). The same report shows the co-localisation of BORIS with other proteins involved on self-renewal, as ECSA (embryo/cancer sequence A), OCT4 and NANOG in cultured embryonic stem cells. This suggests a role of BORIS in epigenetic reprogramming events relating the pluripotency and thus, a possible role of BORIS as a gene involved in the epigenetic modification that could lead to CSCs phenotype.

1.2.3. BORIS functions in cancer

In different tumors, a reactivation of *BORIS* expression was observed. Transcription activation was found in several cancer cell lines and in about 70% of primary tumors (de Necochea-Campion, Ghochikyan et al. 2011). However, contradictory results were reported concerning *BORIS* expression in some types of tumors. For example, *BORIS* expression was observed in different breast tumor cell lines and in primary breast tumors (D'Arcy, Pore et al. 2008) while, another report shows the absence of *BORIS* expression in the same breast cell lines and breast carcinoma (Hines, Bazarov et al. 2010). Furthermore, *BORIS* expression was detected in melanoma cell lines but not in primary melanoma (Kholmanskikh, Loriot et al. 2008). There are some reasons that could explain these discrepancies. A validated good antibody against BORIS is still not available and the main commercial antibodies lead to a high number of false-positive and false-negative results. Additionally, as BORIS is known to be translated in 23 isoforms (Pugacheva, Suzuki et al. 2010), expression analysis with different set of primers may produce inconsistent reproducible data.

Due to its restricted expression in normal germinal tissues and its re-expression in a wide variety of tumors, BORIS belongs to cancer testis antigen (CTA) family. CTAs are normally expressed in germ cells and aberrantly expressed in a wide variety of human tumor (Caballero and Chen 2009). They are mainly regulated by epigenetic mechanism, such as DNA methylation (De Smet, Lurquin et al. 1999; Meklat, Li et al. 2007). So far, almost all identified CTAs are silenced by methylation of CpG island promoters in normal somatic tissue and are reactivated by demethylation during spermatogenesis (Lim, Kim et al. 2005). CTAs are considered promising targeted molecules for anti-cancer vaccines because of their restricted expression in normal tissues, their high immunogenicity and their re-expression in tumors (Simpson, Caballero et al. 2005; Akers, Odunsi et al. 2010). Encouraging results have been obtained in clinical trials using vaccines targeting the CTA genes MAGE-A3 or NY-ESO-1, CTAs genes (Simpson, Caballero et al. 2005; Odunsi, Qian et al. 2007; Atanackovic, Altorki et al. 2008). However, due to the low frequency and heterogeneous expression of CTAs genes in human tumors, there are some limitations with this approach (Woloszynska-Read, Mhawech-Fauceglia et al. 2008).

The immunogenicity of BORIS was confirmed when in sera of breast cancer patients anti-BORIS antibodies were detected (USA Patent 7785814 B2). Several vaccines based on truncated mouse BORIS were generated and tested (Loukinov, Ghochikyan et al. 2006; Ghochikyan, Mkrtichyan et al. 2007; Mkrtichyan, Ghochikyan et al. 2008). Vaccination with BORIS-based vaccine in poorly immunogenic and aggressive 4T1 adenocarcinoma mice model resulted in inhibition of tumor growth and reduction of tumor metastasis compared to control mice (Mkrtichyan, Ghochikyan et al. 2011).

In Korean patients, a susceptibility to breast cancer was correlated to allelic variations in the minisatellite of BORIS (BORIS-MS2), which is located upstream of BORIS gene (Yoon,

Kim et al. 2010). Consequently, this report suggests that BORIS-MS2 short rare alleles may be used as risk factor for breast cancer.

Several studies have demonstrated a direct role of BORIS in the regulation of the expression of some CTAs genes. Indeed, overexpression of BORIS in normal cells allows *MAGEA1* expression through its promoter demethylation (Vatolin, Abdullaev et al. 2005). In lung tumor, *MAGEA1* was found to be regulated by binding of BORIS together with CTCF to *NY-ESO-1* promoter (Hong, Kang et al. 2005). In other two reports, a correlation between BORIS and expression of CTA genes in non-small cell lung cancer and head and neck squamous cell carcinoma it has been shown (Glazer, Smith et al. 2009; Smith, Glazer et al. 2009). Interestingly, in a variety of tumors, BORIS was implicated in the coordinated promoter demethylation and transcriptional re-activation of putative oncogenes, which are epigenetically silenced in somatic cells (Smith, Glazer et al. 2009) .

BORIS is the unique gene of the CTA family with a somatic counterpart gene, CTCF. BORIS and CTCF share the same ZF DNA-binding domain (Loukinov, Pugacheva et al. 2002). CTCF is a highly conserved gene, while BORIS is less conserved across species and it is detected only in amniotes because during the evolution its protein arose later (Hore, Deakin et al. 2008). In contrast to BORIS, CTCF is ubiquitously expressed and it has been shown that CTCF is a multifunctional chromatin factor that plays as a tumor suppressor gene (Dunn and Davie 2003; Moon, Filippova et al. 2005; Herold, Bartkuhn et al. 2012). CTCF was found localized in the nucleolus compartment and BORIS was found in both nucleolus and cytoplasm compartments (Rosa-Garrido, Ceballos et al. 2012). CTCF and BORIS are both insulator proteins. BORIS is present in euchromatin domains and in the sites of RNA transcription, but it is not present in highly condensed chromatin, suggesting a role of BORIS in the unfolding of the chromatin before the transcription (Rosa-Garrido, Ceballos et al.

2012). BORIS could regulate gene expression through histone modification. Indeed, it has been shown that binding of BORIS to some CTAs leads to an enrichment of modification of two histones, H3K9 and H3K4 (Bhan, Negi et al. 2011). In another study, the binding of BORIS led to some modifications in the local chromatin organization, allowing altered *Rb2/p130* expression (Fiorentino, Macaluso et al. 2011). Apparently, the binding of BORIS could lead to the conformational modification of chromatin from a close state to an open state, allowing the transcriptional activation of some important genes for tumorigenesis.

The important role of BORIS in the immortalization process during tumorigenesis through transcriptional regulation of *hTERT* telomerase gene has been reported (Renaud, Loukinov et al. 2011). Telomerase is a specialized DNA polymerase complex responsible for adding telomeric DNA repeats to the ends of chromosomes. hTERT is the catalytic subunit of the telomerase complex and is the limiting factor for telomerase activation (Bodnar, Ouellette et al. 1998; Counter, Meyerson et al. 1998). In human, telomerase is generally absent in somatic cells but remains active in germ cells, progenitor cells and some adult stem cells. It has been shown that telomerase is reactivated in a majority (approximately 90%) of tumors (Kim, Piatyszek et al. 1994). In clinical studies, its reactivation is associated with poor outcomes in different types of cancer (Gertler, Rosenberg et al. 2004; Domont, Pawlik et al. 2005; Tabori, Ma et al. 2006; Terrin, Rampazzo et al. 2008). The enhancement of telomerase activity is considered one of the hallmarks of cancer and is essential for cellular immortalization and malignant transformation (Hanahan and Weinberg 2011). Interestingly, the transcriptional regulation of *hTERT* is controlled by CTCF and this regulation is cell type dependent (Renaud, Loukinov et al. 2005). In somatic cells, CTCF inhibits *hTERT* expression by binding to the *hTERT* promoter. In about 85% of tumor cells, a hypermethylation of the CTCF binding site was observed within the hTERT promoter. This hypermethylation blocks

CTCF binding and the CTCF repressive effect, leading to *hTERT* expression (Renaud, Pugacheva et al. 2007). Recently, we have reported that in testicular and ovarian tumor cells, BORIS binds to the CTCF binding site, resulting in *hTERT* activation (Renaud, Loukinov et al. 2011). In addition, ectopic BORIS expression in normal cells led to an up-regulation of *hTERT* expression and an increasing of cell passages number (Renaud, Loukinov et al. 2011). All these data revealed an important role of BORIS in the immortalization process during tumorigenesis.

Recent reports have highlighted the association of *BORIS* expression with poor prognosis in different types of cancers. In ovarian cancers, *BORIS* expression was correlated with advanced stage and decreased survival (Woloszynska-Read, James et al. 2010). It has been demonstrated that BORIS was involved in proliferation and invasion of esophageal squamous cell cancer and BORIS-positive tumors had a poor overall survival (Okabayashi, Fujita et al. 2012). In hepatocellular carcinoma, a correlation between *BORIS* expression and poor overall survival as well as with the CSCs marker CD90 has been observed (Chen, Huang et al. 2013). All these findings recognize an important role for BORIS in cancer and suggest that BORIS could play a key function in CSCs.

Interestingly, an overexpression of BORIS after treatment with apicidin and docetaxel drugs in metastatic breast tumor cells has been reported, suggesting a potential role of BORIS on favoring an antitumor immune response (Buoncervello, Borghi et al. 2012).

Collectively all these observations show the structural complexity of *BORIS* gene and its important role in cancer. However, all its functions of BORIS in malignant disease are not fully understood. Therefore, a better understanding of biological functions of BORIS in tumor disease was the final goal of this study.

AIM OF THIS WORK

The goal for this study was to better understand the function of BORIS in tumor cells. BORIS is a DNA binding protein that is expressed in normal tissues only in germinal cells (testis and oocytes) and is re-expressed in a wide variety of tumors. Recent studies have shown an association of *BORIS* expression with a poor prognosis in different type of cancer patients. Although the expression of BORIS in tumors is well documented, there are questions not yet answered:

1) How frequently is BORIS expressed?
2) Is BORIS expressed at basal level in almost all the tumor cells or rather is it expressed in a restricted cell population with a high significant expression level?
3) What is the role of the BORIS-expressing cell population in tumor development?
4) Is the role of BORIS different according to the cell type?

Our hypothesis is that BORIS is mainly expressed in the cancer stem cells (CSCs). This restricted expression of BORIS in CSCs may lead to epigenetic reprogramming of several genes and therefore, BORIS could play an essential role in tumor development.

To address the first two questions, we developed a new technology system to target *BORIS* mRNA expressing cells. Using this system, we were able to quantify the estimated frequency of the BORIS-expressing cells. To address the last two questions, we set up to use this system to isolate the BORIS-high expressing cells and to assess their expression profile. We evaluated the expression of *hTERT* and stem cell genes in order to identify an association of BORIS with the known characteristics of CSCs. We further investigated the BORIS expression in the CSCs-enriched populations (side population and spheres). To validate our main hypothesis, we finally analyzed the role of BORIS in the self-renewal of tumors using a functional assay approach in which BORIS was stably silenced or induced.

2. BORIS/CTCFL promotes *hTERT* expression and acquisition of stem cell-like traits in embryonic cancer cells

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2.1. Abstract

BORIS/CTCFL is a member of cancer testis antigen family normally expressed in germ cells. In tumors, it is aberrantly expressed although its functions are not completely well-defined. To better understand the functions of BORIS in cancer, we selected the embryonic cancer cells as a model. Using a molecular beacon (MB), which specifically targets *BORIS* mRNA, we demonstrated that BORIS positive cells are a small subpopulation of tumor cells (3-5% of total). The BORIS-high expressing cells, isolated by flow cytometer sorting, expressed higher telomerase *hTERT* mRNA compared to the BORIS-low and the entire bulk tumor cells. Interestingly, the isolated BORIS-high cells also displayed significant high expression of stem cells genes (*NANOG, OCT4, SOX2*) and cancer stem cells (CSCs) markers genes (*CD44* and *ALDH1*). In order to define the functional role of BORIS, stable BORIS-depleted cells were generated. BORIS silencing strongly down-regulated the expression of *hTERT,* stem cell genes and CSC markers genes. Moreover, the BORIS knockdown increased cellular senescence in embryonic cancer cells, revealing a protective role of BORIS on senescence biological program likely by transcriptional regulation of telomerase. Our data indicate an association of BORIS expressing cells subpopulation with stem cell-like traits, demonstrating the critical role played by BORIS on embryonic neoplastic disease.

2.2. Introduction

Brother of the regulator of imprinting sites (BORIS) also designed as CTCFL, CCCTC-binding factor-like, is a DNA-binding protein with functions in cancer not fully understood. CTCF is a highly conserved, ubiquitously expressed, multifunctional chromatin factor that plays a role as a tumor suppressor gene (Dunn and Davie 2003; Moon, Filippova et al. 2005; Herold, Bartkuhn et al. 2012). BORIS is a mammalian paralog of CTCF with whom it shares 11 zinc-finger domains but differs at N- and C- termini, within zinc-finger domains BORIS and CTCF exhibit 70% of homology. In normal tissues, *BORIS* expression is restricted to germ cells, where it is involved in epigenetic reprogramming (Klenova, Morse et al. 2002; Loukinov, Pugacheva et al. 2002). Indeed, *BORIS* is expressed in spermatocytes during male germ line development, in absence of CTCF. In tumors, BORIS is aberrantly expressed and its transcription was found in several cancer cell lines and 70% of primary tumors (de Necochea-Campion, Ghochikyan et al. 2011). Due to its restricted expression in normal germinal tissues and its re-expression in a wide variety of tumors, BORIS belongs to cancer testis antigen (CTA) family. It has been shown that BORIS induced expression of other CTA genes, as MAGE-A1, NY-ESO-1 (Hong, Kang et al. 2005; Vatolin, Abdullaev et al. 2005) and SPANX (Kouprina, Noskov et al. 2007) but not in all tumors (Kholmanskikh, Loriot et al. 2008; Woloszynska-Read, James et al. 2010). In addition, we previously showed that BORIS activated *hTERT* expression by binding to the first exon of the *hTERT* gene (Renaud, Loukinov et al. 2011) in embryonic and ovarian tumor cells. Furthermore, in studies of exogenous BORIS expression in normal BORIS-negative cells, we demonstrated that these transfected cells exhibited high levels of *hTERT* mRNA. All these results revealed an important role of BORIS in the immortalization process during tumorigenesis. Interestingly, current reports

show a correlation between *hTERT* expression and stem cell like properties (Hiyama and Hiyama 2007; Schepers, Vries et al. 2011; Shay and Wright 2011; Paranjape, Mandal et al. 2012; Kim, Yoo et al. 2013).

An important question not yet answered is how frequently *BORIS* is expressed among cancer cells within a tumor. The molecular beacon (MB) imaging technology is an approved method to detect and also to visualize mRNA expression (Monroy-Contreras and Vaca 2011). MBs are oligonucleotides structured as stem-loop hairpin with at one end, a fluorescence quencher and at the opposite end, a fluorescent dye also called fluorophore. Due to their specific structure, MBs in presence of their target complementary sequences are able to bind them and emit fluorescence signals, otherwise in absence of their targets do not emit fluorescence. To explore the frequency of BORIS positive cells within tumor cell lines, we first designed a *BORIS* mRNA-targeting MB and analyzed BORIS expression in human embryonic and ovarian tumor cell lines, respectively NCCIT and OVCAR3. After verifying that BORIS-MB enable FACS sorting of BORIS-positive cells, we showed that the isolated BORIS-high fraction cells expressed high *hTERT* mRNA level. A correlation with the stem cell like properties was observed in the BORIS-high isolated cells from NCCIT embryonic cancer cells. We further confirmed this result by BORIS silencing studies and finally demonstrated that BORIS is associated with increase expression of *hTERT* and acquisition of stem cell-like traits in embryonic cancer cells. Moreover, we showed that BORIS protects from senescence process apparently through *hTERT* regulation. Altogether, our data confirm a direct role of BORIS in embryonic neoplastic disease.

2.3. Materials and methods

2.3.1. Cells

The human cell lines (BJ, foreskin fibroblast; HeLa, cervical adenocarcinoma; NCCIT, embryonic carcinoma; OVCAR3, ovary carcinoma) were purchased from the American Type Culture Collection (ATCC). The cells were cultured at 37°C with 5% CO_2 either in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) for HeLa and BJ cells, or in RPMI-1640 medium (Gibco, Invitrogen) for NCCIT and OVCAR-3, supplemented with 10% of heat inactivated fetal bovine serum (FBS; Invitrogen) and 1% of Penicillin-Streptomycin (Gibco, Invitrogen).

2.3.2. Molecular beacon (MB) design

Sequences of BORIS-MB1 and BORIS-MB2 were designed using Beacon Designer (Premier Biosoft). *BORIS* mRNA secondary structures were predicted using mFOLD software (mFOLD, http://www.bioinfo.rpi.edu/applications/mfold/) and specificity was determined by a BLAST search (NCBI). The target sequence of BORIS-MB1 is located on exon 2 and that of BORIS-MB2 is located on exon 11 of *BORIS* mRNA. These location were chosen since they are outside the zinc-finger domains and do not cross-hybridize with the CTCF homology regions. In addition, previous study has shown that the starting and ending regions of mRNA are the more accessible for MBs hybridization (Rhee, Santangelo et al. 2008). The RANDOM-MB that was used as negative control does not match with any mammalian sequences (Rhee, Santangelo et al. 2008). Sequences were the following: BORIS-MB1 5'-CGCTGTCTCTGCACACTCCGTCTTCAGCG-3'; BORIS-MB2 5'-CAGCCATTCCTCTTTGACTCTGGCTG-3' and RANDOM-MB 5'-CGACGCGACAAGCGCACCGATACGTCG-3' (underlined bases indicating

those complementary to the target). A fluorophore (Cy3 or ATTO647) was 5'-conjugated and a Black Hole Quencher (BHQ-2) was linked to the 3'-end. The MBs were purchased from Sigma with high-pressure liquid chromatography purification method.

2.3.3. In vitro determination of MB specificity

Oligos were designed to be specific of the MBs target (BORIS-MB1 specific target: 5'-AAGACGGAGTGTGCAGAGAGA-3'; BORIS-MB2 specific target: 5'-CAGCCAGAGTCAAAGAGGAA-3' and RANDOM-MB specific target: 5'-TATCGGTGCGCTTGTCG-3'). A non-specific oligo was also designed (non-specific target: 5'-CGATGCCGAACCAATTCTCCAC-3'). To test the specificity in solution, 200 nM of MB was mixed or not with 1 µM of oligo in 10 µL of Opti-MEM medium (Invitrogen).

The emission fluorescence profiles were obtained after heating the MB-target oligo mix to a progressive temperature elevation ranging from 15 to 80°C using 1°C steps. Fluorescence signal was acquired at the end of each increasing degree and detected on the Cy3 channel using a Rotor Gene 6000 Real-Time PCR system (Corbett Life Science).

2.3.4. MB delivery and cell fluorescence imaging

Cells were detached using 0.05% trypsin-EDTA (Invitrogen) and resuspended in serum-free DMEM medium at the concentration of 10^6 cells/ml. Firstly, Cy3-BORIS-MB or Cy3-RANDOM-MB (200 nM) was incubated at room temperature in presence of 1 µl/ml of Lipofectamine RNAiMAX siRNA transfection reagent (Invitrogen) using Opti-MEM medium. The Lipofectamine RNAiMAX reagent was used as delivery vehicle since in our conditions it gave less background compared to other reagents such as Streptolysin (data not shown). After 10 min, the transfection mix was added to the suspended cells and together incubated for 1

hour at 37°C. Hoechst 33342 (Invitrogen) was added at concentration of 5 µg/mL during the last 10 min of incubation. Then, cells were washed using PBS and resuspended in PBS with 5 mM EDTA. Transfected cells were cytocentrifugated onto glass slide using a cytospin centrifuge and examined under fluorescent microscope (Axioplan2 Imaging, Zeiss). The fluorescence signal of Cy3-coniugated MB was analyzed using the red channel and Hoechst 33342 fluorescence emission was observed under blue channel.

2.3.5. FACS analysis and sorting using MB

For FACS analysis and cell sorting, we used MBs conjugated with ATTO 647, a dye characterized by its high photostability (Arden-Jacob, Frantzeskos et al. 2001). Cells were prepared and incubated with MBs as described above (except that Hoechst 33342 was not added) and were directly analyzed using Gallios flow cytometer (Beckman Coulter). At least 10,000 events were collected and analyzed by Kaluza Software. The BORIS-high and BORIS-low population were sorted after exclusion of dead cells by Propidium Iodide (PI) staining using FACSAria I (Becton Dickinson) instrument at the Flow Cytometry Facility of UNIL (University of Lausanne, Switzerland). Ranges of 2×10^4 - 9×10^4 BORIS-high cells and 2×10^5 - 9×10^5 BORIS-low cells were sorted.

2.3.6. BORIS knockdown by inducible shRNA lentiviral system

Stable cell lines with inducible expressing shRNAs targeting human BORIS mRNA were generated using the doxycycline-inducible shRNA lentiviral system, pINDUCER (Meerbrey, Hu et al. 2011). The lentiviral vector pINDUCER11 constitutively expresses the eGFP fluorescent reporter protein, which enables to track cells transduced by the virus. This vector also contains a cassette with a doxycycline-inducible promoter that controls the transcription of a

tRFP reporter gene together with the shRNA, which allows detection of cells with doxycycline activated shRNA transcription (Meerbrey, Hu et al. 2011). Four different shRNAmiR (shRNA) specifically targeting *BORIS*, and not its parolog CTCF (BORIS-sh1: 5'-ATTCACCAAGATCAAAGAACTC-3', BORIS-sh2: 5'-GTTCTCACAGTTTCAAATTCAA-3', BORIS-sh3: 5'-TTCATCCCGACTGTTTACAAAT-3', BORIS-sh4: 5'TCCGACAGAAGCAACTTCTAAA-3') and a control shRNA with scrambled sequence (CTR sh: 5'CAGAGCTAACTCAGATAGTACT3') were synthetized (Sigma). They were PCR amplified and cloned into the pINDUCER11 backbone using *EcoRI* and *XhoI* restriction enzymes. The sequences of all constructs were verified by sequencing. Lentivirus were generated by co-transfection of the appropriate shRNA constructs along with the packaging vectors (pMD2G-VSVg, pCMV-dR8.74) into HEK-293T cells using FuGENE 6 reagent according to the manufacturer's protocol (Roche Diagnostics). Viral supernatants were harvested 48 hours after transfection, filtered through a 0.45 µm pore filter, ultracentrifugated for 1.5 hours at 19,500 rpm in a Beckman SW28 rotor and resuspended in RPMI medium. The viral suspension combined with 8 µg/ml polybrene (Sigma) was used to infect target cells (NCCIT). Twenty-four hours post infection the medium was replaced and stably infected cells were eGFP-sorted using FACSAria I instrument (Becton Dickinson) at the Flow Cytometry Facility of UNIL. Induction of shRNA expression was obtained by addition to the medium of 2 µg/ml of doxycycline (Sigma). To maintain the knockdown, doxycycline-containing medium was refreshed every 3 days.

2.3.7. Ectopic BORIS transfection in HeLa cells

The day prior transfection, Hela cells were seeded at a density of 2 x 10^5 cells/well in 12-well plates. Cells were transfected with 3 µg of the previously described pCMV-BORIS vector (Renaud, Loukinov et al. 2011) using the Lipofectamine 2000 transfection reagent

(Invitrogen) following the manufacturer's instructions. Cells were harvested 2 days post-transfection for cell fluorescence imaging.

2.3.8. Quantitative RT-PCR

Total RNA was isolated using the RNeasy mini kit (Qiagen) including on-column DNAse treatment according to the manufacturer's instructions. RNA concentration was determined using Nanodrop 2000 (Thermo Scientific) and Qubit Fluorescent Technology (Invitrogen).

A major limiting step was the low amount of total RNA isolated from cell sorting. To solve this technical limitation, we applied a method already described and validated (Peixoto, Monteiro et al. 2004; Noutsias, Rohde et al. 2008). Firstly, 200 ng of total RNA were retrotranscribed using random hexamers and Superscript III reverse transcriptase (Invitrogen). Then 2 µl of cDNA were used for a preamplification reaction consisting on a multiplex PCR made with a mix of primers (Table 1) at 0.1 µM final concentration, 0.5 unit of Platinum Taq DNA Polymerase (Invitrogen), 1X PCR buffer, and 2 mM $MgCl_2$.

For preamplification, PCR cycling conditions were: one denaturation step at 95°C for 5 min followed by 15 cycles of amplification (45 sec at 95°C, 30 sec at 60°C, 1 min at 72°C). Finally, for quantitative PCR, the preamplification reaction was 20-fold diluted and 2 µl of this dilution were used as template. Reaction was complemented with 0.5 units Platinum Taq DNA Polymerase (Invitrogen), 1X PCR buffer, 2.5 mM $MgCl_2$, 2.5 µM SYTO9 green (Invitrogen) and 0.1 µM of each gene specific primer (Sigma). PCR conditions were: 95°C for 5 min followed by 40 times: (5 sec at 95°C, 30 sec at 60°C, 45 sec at 72°C). Melting curve analysis was also performed at the end of the cycling to check PCR homogeneity. Cycling and fluorescence acquisition were done in Rotor Gene 6000 Real-Time PCR system (Corbett Life

Science). Relative expression levels were determined with the comparative ΔΔCt method in Rotor-Gene 6000 software using *GAPDH* as reference gene.

The human specific primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/) and OLIGO Primer Analysis software. Specificity was verified using BLAST search (NCBI). The designed primer pairs cross intron-exon boundaries to avoid genomic DNA contamination. BORIS primers were chosen (between exon 8 and 9) in base of results obtained previously (Pugacheva, Suzuki et al. 2010) and amplify the most abundant *BORIS* isoforms. For each set of primers, standard curves were performed and efficiencies were determined. PCR products were loaded on agarose gel to verify the size of amplified products.

Table 1 Primer sequences for qRT-PCR analysis

Gene	Forward primer	Reverse primer
BORIS	5' GCCCTCATTCAGCACCAGAAAAC 3'	5' CTCCAGTGTGGGTACGAATGTGA 3'
CTCF	5’ GTGGCAGGGCATTCAGAACAG 3’	5’ CGATGCCGAACCAATTCTCCAC 3’
hTERT	5’ TGACACCTCACCTCACCCAC 3’	5’ CACTGTCTTCCGCAAGTTCAC 3’
ALDH1	5’ GCAACTGAGGAGGAGCTCTG 3’	5’ AAGCATCCATAGTACGCCAC 3’
BMI1	5’ GCTAAATCCCCACCTGATGT 3’	5’ GGTCTCCAGGTAACGAACAA 3’
NANOG	5’ ATACCTCAGACTCCAGCAGA 3’	5’ TCTGGAACCAGGTCTTCACC 3’
OCT4	5’ GGTATTCAGCCAAACGACCA 3’	5’ TTCTCTTTCGGGCCTGCACG 3’
SOX2	5’ CCTGGCATGGCTCTTGGCTC 3’	5’ TGGAGTGGGAGGAAGAGGTA 3’
CD44	5’ TAAGGACACCCCAAATTCCA 3’	5’ ACTGCAATGCAAACTGCAAG 3’
GAPDH	5’ AAGGTGAAGGTCGGAGTCAAC 3’	5’ GAGTTAAAAGCAGCCCTGGTG 3’

2.3.9. DNA methylation analysis

DNA was extracted using DNeasy kit (Qiagen). A range of 200 ng (from sorted cells) and 500 ng of DNA was used to bisulfite reaction using EpiTect Bisulfite kit (Qiagen) according to the manufacturer’s instructions. The modified DNA was used to amplify a 123 bp fragment of the BORIS promoter.

Assay was designed using the PyroMark Assay Design Software 1.0 (Qiagen). This assay allowed sequencing of 50 bp (from position -968 to -918) inside the promoter B of *BORIS* (Renaud, Pugacheva et al. 2007) and included 10 CpG. To perform sequencing, 3 µl of bisulfite treated DNA were first amplifyied by PCR. Sequences of the PCR primers were: BORIS-pyro Forward 5' TGGTTTGTGGGTTTTGT 3' and BORIS-pyro BIO Reverse 5' CCCTTCACCCCCCCTCTTT 3'. PCR conditions were as follow: 95°C for 5 min; 45 cycles of 95°C for 30 s, 58°C for 15 s and 72°C for 1 min; and a final extension step at 72°C for 10 min. Then, purification and subsequent processing of the biotinylated single-stranded PCR fragment were performed according to the manufacturer's recommendations. Pyrosequencing of this PCR fragment was performed on a PyroMark Q24 instrument using Pyro Gold Q24 Reagents (Qiagen). The pyrosequencing primer (5' GTGTTGTAGTTTATAGT 3') was used at a final concentration of 0.3 µM. Resulting data were analyzed and quantified with the PyroMark Q24 software (Qiagen) which calculates the methylation percentage for each CpG site, allowing quantitative comparisons.

2.3.10. Cell proliferation assay

Cell proliferation was assessed by MTT assay. MTT (3-(4,5-dimethyl-2-thiazol)-2,5-diphenyltetrazolium bromide, Sigma) reagent was used according to the manufacturer's instructions. Briefly, stably infected cells were seeded at a density of 25 x 10^3 cells/well in 24-well/plates with doxycycline-containing medium. After 3 days, cells were incubated with MTT reagent (200 µg/ml final concentration) for 3 hours at 37 °C. Then, cells were lysed adding isopropanol/HCl for 10 min and the plates were gently shaken for 5 min. Absorbance values were determined using a microplate reader (Synergy Mx, BioTek) at 570 nm. Each experiment was performed in triplicate and 2-3 independent experiments were conducted.

2.3.11. Apoptosis analysis

Apoptosis was measured in triplicates using Annexin V Apoptosis Detection Kit (BD bioscience) according to the manufacturer's protocols. Briefly, 5 x 10^4 cells/well were seeded in 12-well/plates and were grown in presence of doxycycline until confluence (5-7 days). The floating cells as well as trypsinized cells were collected, washed with PBS and resuspended in 100 µl Binding Buffer. Then, 5 µl of Annexin V-V500 and 5 µl of 7AAD were added and incubated with the cells for 30 min at room temperature. After addition of 400 µl of Binding Buffer the samples were immediately analyzed by Gallios flow cytometer (Beckman Coulter). At least 5 x 10^4 events were counted for all samples. The percentage of apoptotic cells was estimated after gating on eGFP and tRFP (transduced and doxycycline-induced, respectively) positive cells.

2.3.12. Western blot analysis

Whole cell lysates were obtained using RIPA buffer (Sigma) in presence of protease inhibitor cocktail (Sigma) and quantified using the BCA assay (Thermo Scientific). Thirty micrograms of protein were loaded on a 10% SDS-polyacrylamide gel, followed by blotting on a nitrocellulose membrane using a semi-dry transfer apparatus (BIO RAD). Non-specific binding was blocked by overnight incubation in 5% non-fat dried milk in TBST buffer (0.1% Tween 20 in TBS) at 4°C. The membranes were then probed with monoclonal mouse anti-human BORIS/CTCFL antibody (produced and kindly provided by Dr Dmitri Loukinov, NIH/NIAD) used at 1:1000 dilution in 1% blocking buffer (1% low-fat dried milk in TBST buffer) and incubated at room temperature for 1.5 hours. As loading control, mouse anti-human β-actin antibody (Sigma) at 1:5000 dilution in 1% blocking buffer was used and

incubated at room temperature for 45 min. The membranes were washed 3X with TBST and incubated at room temperature for 1 hour with horseradish peroxidase (HRP)-labeled rabbit anti-mouse IgG (Sigma) diluted at 1:5000 in 5% blocking buffer. After 3X washing with TBST, the membranes were developed using WesternBright Quantum (Advansta) and visualized with Fusion FX Chemiluminescence System (Vilber Lourmat).

2.3.13. Senescence-associated β-Galactosidase staining

Senescence-associated β-galactosidase (SA-β-gal) staining was performed using β-galactosidase staining kit (BioVision), according to the manufacturer's instructions. Briefly, 5 x 10^4 cells/well were seeded in 12-well/plates in presence of doxycycline and were grown until confluence (5-7 days). Then, cells were rinsed with PBS, fixed for 15 min and incubated with freshly prepared SA-β-Gal staining solution at 37°C for 24 hour. After washing with PBS, SA-β-gal activity was observed using inverted microscope (Nikon) by detection of blue stained cells. At least 10 separate fields were selected. For each field the number of blue stained cells and the number of total cells were counted. Results are expressed as percentage of SA-β-gal-positive cells calculated as: (number of blue cells/number of total cells) x 100.

2.3.14. Telomerase activity

Telomerase activity was measured using TRAPEZE RT telomerase detection kit (Millipore). This assay quantifies telomerase activity by SYBR Green real-time quantitative PCR (Wege, Chui et al. 2003). Briefly, cells were lysed in 200 µl of CHAPS buffer and protein concentrations were determined with Nanodrop 2000. Aliquots of cell lysate (1.5 µg of protein/sample) were used. Inactivated samples, no-template reactions, and positive control

were also assayed for quality control. A standard curve was prepared by serial dilution of TSR8 control template following manufacturer's instructions. Real-time amplifications were performed using Platinum Taq DNA Polymerase (2 unit/sample, Invitrogen). Cycling and fluorescence acquisition were done in Rotor Gene 6000 real-time PCR system (Corbett Life Science). Telomerase activity was calculated by comparing the average Ct values from each sample against the standard curve generated by the TSR8 control template.

2.3.15. Statistical analysis

Statistical significance was evaluated using two-tailed student t-test analysis. P-value <0.05 was considered statistically significant.

2.4. Results

2.4.1. In vitro validation of Molecular beacons (MBs)

Two different MBs specific to *BORIS* mRNA (BORIS-MB1 and BORIS-MB2) were designed as described in the Material and Methods. The hybridization temperatures and specificity of the BORIS-MBs, and that of a RANDOM-MB which does not have any complementary target in human genome, were first tested in solution. The fluorescence emission of these MBs was monitored at temperatures ranging from 25 to 80°C, under different conditions: MB alone, MB in presence of the specific target, in presence of a non-specific target or in presence of a plasmid containing BORIS cDNA (pCMV-BORIS). As shown in Figure 1, noticeable fluorescence signal was detected only when the MBs were mixed with their specific targets. Optimal fluorescence emission with acceptable signal-to-background ratio (> 4) was observed below 40°C. The assay also showed that BORIS-MBs discriminate single and double stranded structures, since they do not emit fluorescence when incubated with the pCMV-BORIS plasmid. These results demonstrated that MBs specifically hybridize to their target sequences and strongly suggested that these MBs would be able to specifically bind mRNA (and not DNA). Therefore, MBs could emit strong fluorescence signal under *in vivo* conditions (at 37°C).

2.4.2. Detection of BORIS mRNA using BORIS-MB

We first verified whether BORIS-MBs could be able to distinguish positive and negative BORIS expressing cells in living cells. Quantitative RT-PCR detected strong levels of *BORIS* mRNA in NCCIT and OVCAR-3 cell lines, whereas this level was low in Hela cells and not detectable in BJ cells (Figure 2A), in accordance to previous studies (Renaud, Loukinov et al.

2011). Therefore, to study the specificity of MBs, NCCIT was used as a positive control cells and BJ as a negative control. NCCIT cells were transfected with BORIS-MB1 or BORIS-MB2 and fluorescence emissions were measured by flow cytometry. As shown in Figures 2B and C, both MBs showed an increase of fluorescence. However, since BORIS-MB1 provided higher (5 fold) mean fluorescence signal compared to BORIS-MB2, this MB was selected for the subsequent analysis. From here onward BORIS-M1 is referred as BORIS-MB. As expected, when BORIS negative BJ cells were transfected with BORIS-MB, they did not show any fluorescence signal (Figure 2D).

To further challenge the specificity of the BORIS-MB, HeLa cells that expressed *BORIS* mRNA at low level, were transiently transfected with the pCMV-BORIS expression plasmid. As expected, the transfected cells presented higher fluorescence signal compared with the non-transfected cells (Figure 2E). All together, these results confirmed the capacity of the BORIS-MB to reliably and specifically detect *BORIS* mRNA in living cells.

Hence, the cell lines were transfected with the BORIS-MB to visualize *BORIS* mRNA expression by fluorescence imaging. According to the qRT-PCR results, fluorescence of the BORIS-MB was nicely detected in NCCIT and OVCAR-3 cells (Figure 2F), but remarkably, only a subset of cells were fluorescent (from 3 to 5% BORIS positive cells of total cells). While in Hela few cells were positive (about 0.5%) and no fluorescent cells were observed in BJ cell line. This experiment demonstrated that within tumor cell lines, *BORIS* mRNA is not present at the same level in all cells but rather occurs at higher level only in a subset of cells.

2.4.3. Isolation of cell population expressing high levels of BORIS mRNA using BORIS-MB

NCCIT cell line was used for the isolation of BORIS high-expressing cells. FACS sorting was performed after cell transfection with BORIS-MB. The brightest and the lowest (8.4 ± 1.5 and 41.5 ± 7.2 % of total cells, respectively; mean ± SD) BORIS-expressing cells were sorted (Figure 3A).

BORIS expression analysis of the sorted subpopulations showed that *BORIS* mRNA level of BORIS-high fraction was 20 fold higher compared to the non-sorted cells (Figure 3B) while BORIS-low fraction expressed lower *BORIS* mRNA. This result demonstrated the efficiency of the sorting method and the successful enrichment of a cell population that highly expressed *BORIS* mRNA.

2.4.4. Cells expressing high and low levels of BORIS mRNA harbors similar methylation pattern of BORIS promoter B

In a previous study, it has been shown that *BORIS* expression is controlled by three alternative promoters, corresponding to transcription start sites at −1447, −899 and −658 bp upstream of the first ATG and designated promoters A, B and C, respectively (Renaud, Pugacheva et al. 2007). Interestingly, it has been observed that in tumors, demethylation of BORIS promoter B, is generally correlated with the expression of *BORIS,* which is not the case for promoters C and A (Renaud, Pugacheva et al. 2007). Consequently, we interrogated the presumed correlation between methylation of promoter B and *BORIS* expression in the sorted cells. BORIS methylation level of this promoter was measured by pyrosequencing, after bisulfite modification of DNA extracted from BORIS-high and -low cell fractions. Pyrosequencing results indicated that in both fractions CpGs were heavily methylated (85-100 % methylation) and no differences were detected (Figure 4A). This confirmed that in

NCCIT cells, *BORIS* is not expressed from promoter B and suggested that the different expression of *BORIS* among the sorted fractions is not guided by DNA methylation status of promoter B but rather involves other levels of control.

2.4.5. BORIS-high cells express higher mRNA levels of hTERT as well as stem cell genes and cancer stem cell markers genes

We previously established that BORIS binds *hTERT* promoter at the same site of CTCF and can activate *hTERT* transcription in NCCIT cells (Renaud, Loukinov et al. 2011). To further investigate the correlation between *BORIS*, *hTERT* and *CTCF* expression, BORIS-low and BORIS-high populations were sorted as mentioned above, and expression of these genes markers was evaluated by qRT-PCR. Interestingly, three independent sorting experiments showed that *hTERT* mRNA level was significant higher (from 1.5 to 3.8 fold) in the BORIS-high population compared to the BORIS-low population. This result confirms the positive correlation between *BORIS* and *hTERT* expression, whereas no difference was observed for *CTCF* (Figure 4B).

Expression of *hTERT* has been frequently associated with expression of stemness-related markers (Hiyama and Hiyama 2007; Schepers, Vries et al. 2011; Shay and Wright 2011; Paranjape, Mandal et al. 2012; Kim, Yoo et al. 2013). Consequently, considering our results, we also investigated if this correlation could exist with *BORIS* expression. To assess this, a panel of representative genes considered as stemness markers (*NANOG, OCT4, SOX2* and *BMI1*) (Suvà 2013) or as specific cancer stem cell (CSC) markers (*ABCG2, CD44* and *ALDH1*) (Medema 2013) were added to the qRT-PCR analysis. Interestingly, it emerged that the BORIS-high/hTERT-high population also expressed higher levels of stem cell like markers. Indeed, mRNA levels of mostly all these genes were significantly higher in BORIS-high

population compared to BORIS-low population and non-sorted cells (between 2 and 6 fold in average), at exception of *BMI1* and *ABCG2* genes (Figure 4B).

2.4.6. Knockdown of BORIS down-regulates expression of CTCF, hTERT, stem cell and CSC markers genes

To investigate more directly the functions of BORIS, stable cell lines with inducible expressing shRNAs, targeting human *BORIS* mRNA, were generated using a lentiviral system. Four different BORIS shRNA lentivirus (BORIS sh-1, sh-2, sh-3 and sh-4) and a lentivirus carrying a scrambled sequence (CTR sh) were produced and tested. A significant reduction of *BORIS* mRNA was observed in all BORIS-depleted NCCIT-derived cells, compared to control cells (Figure 5A). The western-blot analysis indicated a remarkable protein decreasing in BORIS sh-3 and sh-4 cells (Figure 5B). Therefore, all following knockdown experiments were performed using these BORIS sh-3 and sh-4 NCCIT-derived cells. The capacity of doxycycline-induction to maintain BORIS-knockdown overtime was also verified. As shown in figure 6A, *BORIS* mRNA levels were significantly reduced during 1 month, even though the knockdown slightly relapsed the last week. Interestingly, *CTCF* expression was dramatically decreased (Figure 6B). As expected, *hTERT* expression was down-regulated compared to control (Figure 6C) and this down-regulation was even stronger after 3 weeks. This observation was consistent with the correlation observed in the sorting experiment (Figure 4A) and was further confirmed by telomerase activity analysis. Indeed, we observed that telomerase activity was also decreased, especially after 3 and 4 weeks of BORIS silencing (Figure 6D). Notably, absence of *BORIS* triggered a dramatic down-regulation of the expression of stem cell and CSC marker genes (between 75% and 99%), at exception of CD44 (Figure 6E). This down-regulation was consistent up to the third week (data not shown).

All these results firstly confirmed that BORIS transcriptional regulates *hTERT* and strongly suggested that BORIS could also affect the transcription of stem cell and CSC marker genes, which play an important role during tumorigenesis.

2.4.7. Knockdown of BORIS inhibits cell proliferation through cellular senescence

We also investigated the impact of BORIS silencing on cell survival of embryonic tumor cells. Cell proliferation was measured each week during 1 month by MTT assay (Figure 7A). An inhibition of cell growth was observed starting from the second week, with 26% of reduction to the fourth week, with 40% of decrease compared to control. The analysis of apoptosis showed that the percentage of apoptotic cells (late apoptotic AnnexinV$^+$/7AAD$^+$ and early apoptotic AnnexinV$^+$/7AAD$^-$) was not significantly different between BORIS knockdown cells and control cells (Figure 7B). This result suggested that the decreasing of the observed cell growth is not due to cell apoptosis, therefore we further explored the causes of proliferation defects. As BORIS knockdown led to a reduction of *hTERT* expression, a possible alteration of cellular senescence was additionally investigated. Interestingly, analysis of senescence-associated β-galactosidase showed that the percentage of senescent cells was 2 fold higher in BORIS silenced cells compared to control cells (Figure 7C). This result confirmed that BORIS directly affects hTERT telomerase activity.

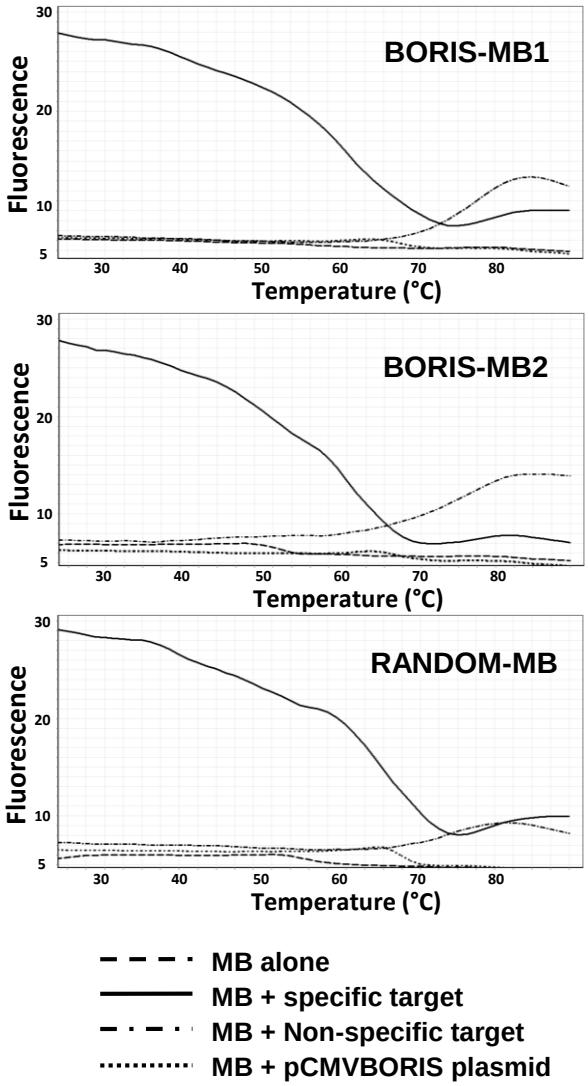

Figure 1. Fluorescence emission profile of MBs. Representative fluorescence emission profile of BORIS-MB1, BORIS-MB2 and RANDOM-MB. All the MBs were 5'-end Cy3-coniugated. All thermal profiles indicate the MBs (200 nM) alone (dash line) and MBs mixed with specific target (solid line), with non-specific target (dash-dot line) and with plasmid (pCMV-BORIS, dot line). The targets were used at the final concentration of 1 µM. The samples were analyzed immediately by Rotor Gene 6000 Real-Time PCR system and the fluorescence was measured at each temperature (from 25°C to 80°C) using filter for Cy3.

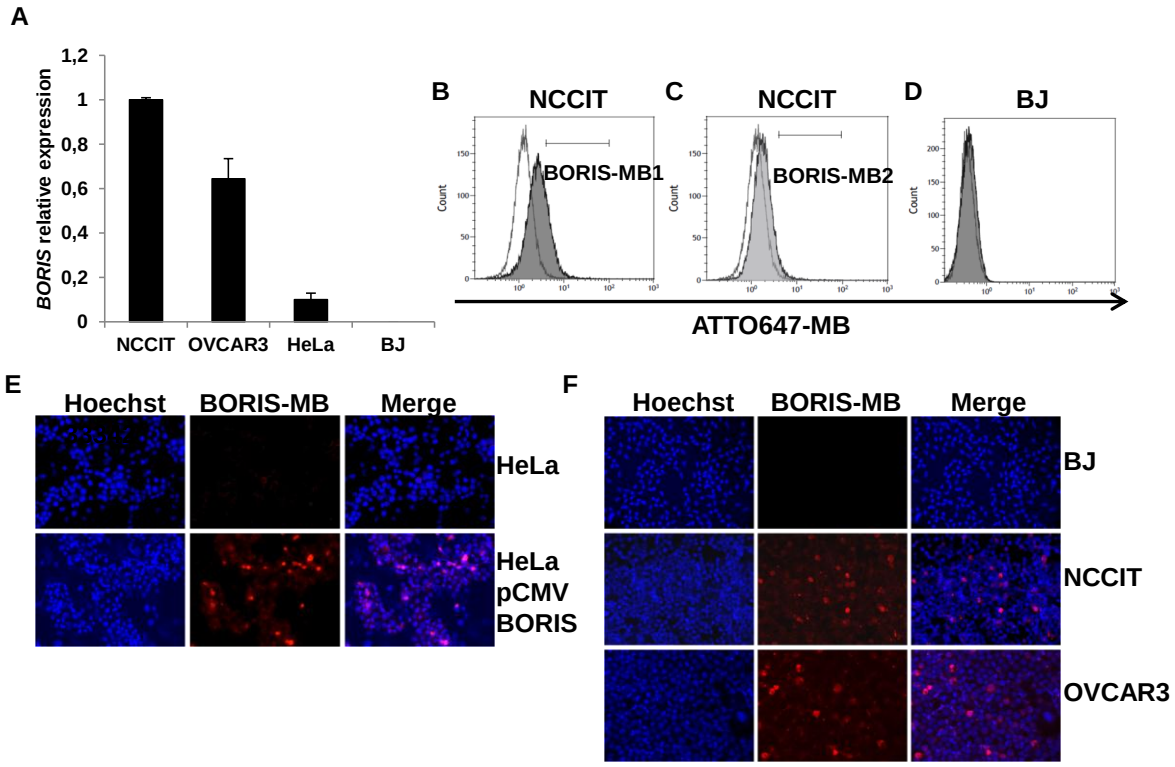

Figure 2. Detection of *BORIS* mRNA by MB. (A) *BORIS* expression in human cell lines. Total RNA from human tumoral cell lines, NCCIT (embryonic), OVCAR3 (ovarian), HeLa (cervical) cells, and normal BJ (fibroblast) cells were isolated. mRNA levels of *BORIS* were analyzed by qRT-PCR. Results were normalized to *GAPDH* and are shown relative to NCCIT cells. BJ and NCCIT were considered as negative and positive controls, respectively. Error bars represent the mean ± SD (n=3). (B) Fluorescent signals measured by flow cytometry of NCCIT cells transfected with ATTO647-BORIS-MB1 (dark grey peak) and with ATTO647-RANDOM-MB (white peak). (C) Fluorescent signals measured by flow cytometry of NCCIT cells transfected with ATTO647-BORIS-MB2 (weak grey peak) and with ATTO647-RANDOM-MB (white peak). (D) Fluorescent signals measured by flow cytometry of BJ cells treated with ATTO647-BORIS-MB1 (from here onward referred to BORIS-MB) and with ATTO647-RANDOM-MB (white peak). (E) *BORIS* expression in HeLa cells using BORIS MB. Representative images of HeLa cells transiently transfected with the BORIS expression vector, pCMV BORIS (bottom) and non-transfected control cells (top). 20X magnification. (F) *BORIS* expression in human cell lines as detected using BORIS MB. Representative images of BJ, NCCIT and OVCAR3 cells, 20X magnification. For fluorescence imaging, 1 x 10^6 cells were incubated at 37°C for 1 hour in serum-free DMEM medium with Cy3-BORIS MB (200 nM). Hoechst 33342 5 µg/mL was added during the last 10 min of incubation. Then, cells were cytocentrifugated onto glass slide using a cytospin centrifuge and the slides were analyzed by fluorescence microscopy.

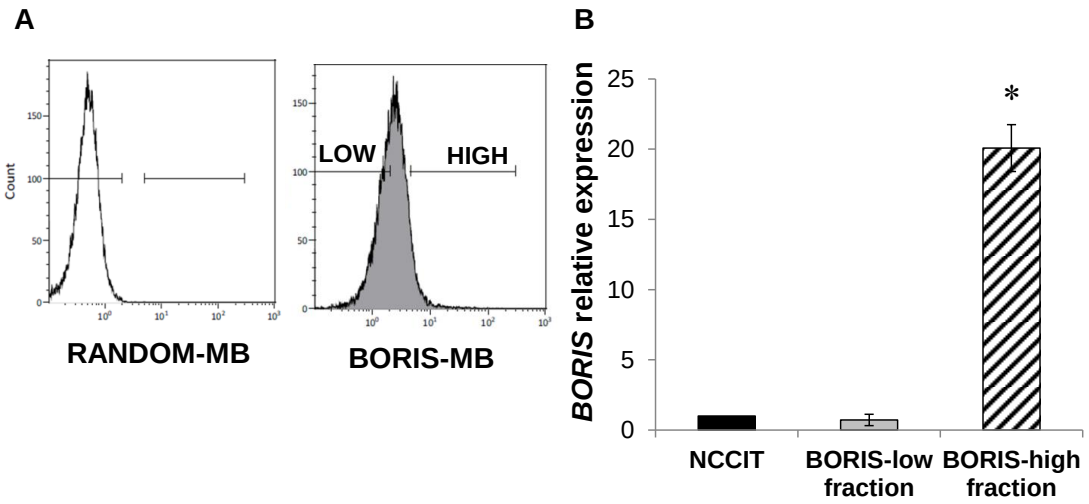

Figure 3. Isolation of BORIS high expressing cells using BORIS-MB. (A) NCCIT cells were transfected with ATTO647-RANDOM-MB (white peak) and ATTO647-BORIS-MB (grey peak). The two subpopulations, BORIS-low and BORIS-high cells were selected by comparing the fluorescent signal of RANDOM-MB to that of BORIS-MB. After exclusion of dead cells by PI staining, the two fractions were sorted. (B) *BORIS* expression of the isolated BORIS-low and BORIS-high fractions were analyzed by qRT-PCR. The results were normalized to *GAPDH* and related to NCCIT non-sorted cells. Error bars represent the mean ± SD of 3 independent experiments.

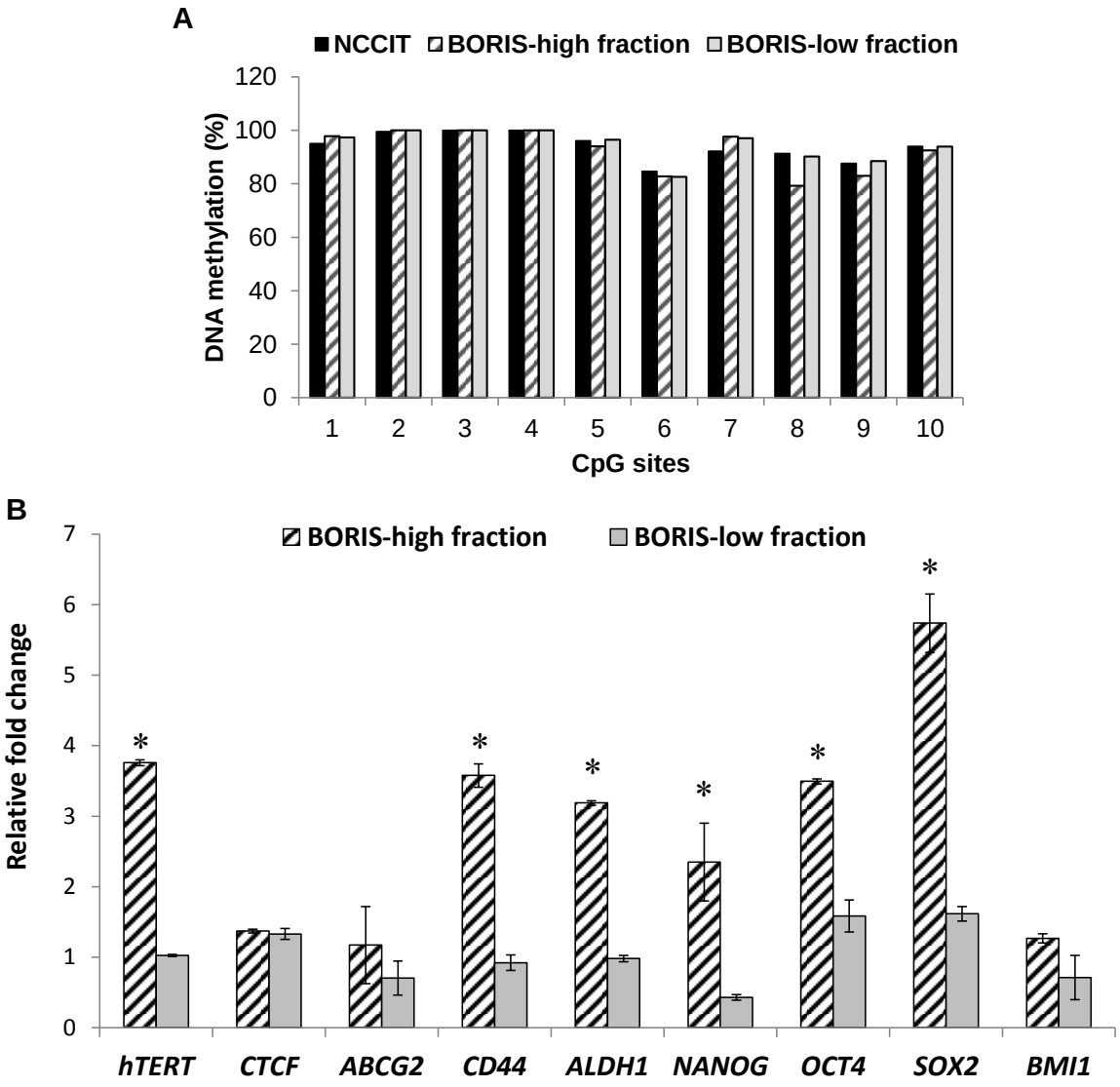

Figure 4. Analysis of the isolated BORIS-high cells reveals higher expression of *hTERT*, stem cells and cancer stem cells (CSCs) marker genes compared to BORIS-low and non-sorted cells. (A) Methylation analysis of 10 CpG islands within the BORIS promoter region (B promoter). The representative graphic shows the percentage of methylation of each CpG island for the isolated BORIS-high, -low and non-sorted NCCIT cells. (B) Expression analysis of the isolated BORIS-high and BORIS-low fractions. The indicated genes were analyzed by qRT-PCR. The results were normalized to *GAPDH* and relative to NCCIT non-sorted cells. Graphic shown one representative experiment out of 3 independent experiments (the trend was similar in all independent experiments). Asterisks indicate statistically significant difference ($p<0.05$) between BORIS-high fraction and BORIS-low fractions and non-sorted cells.

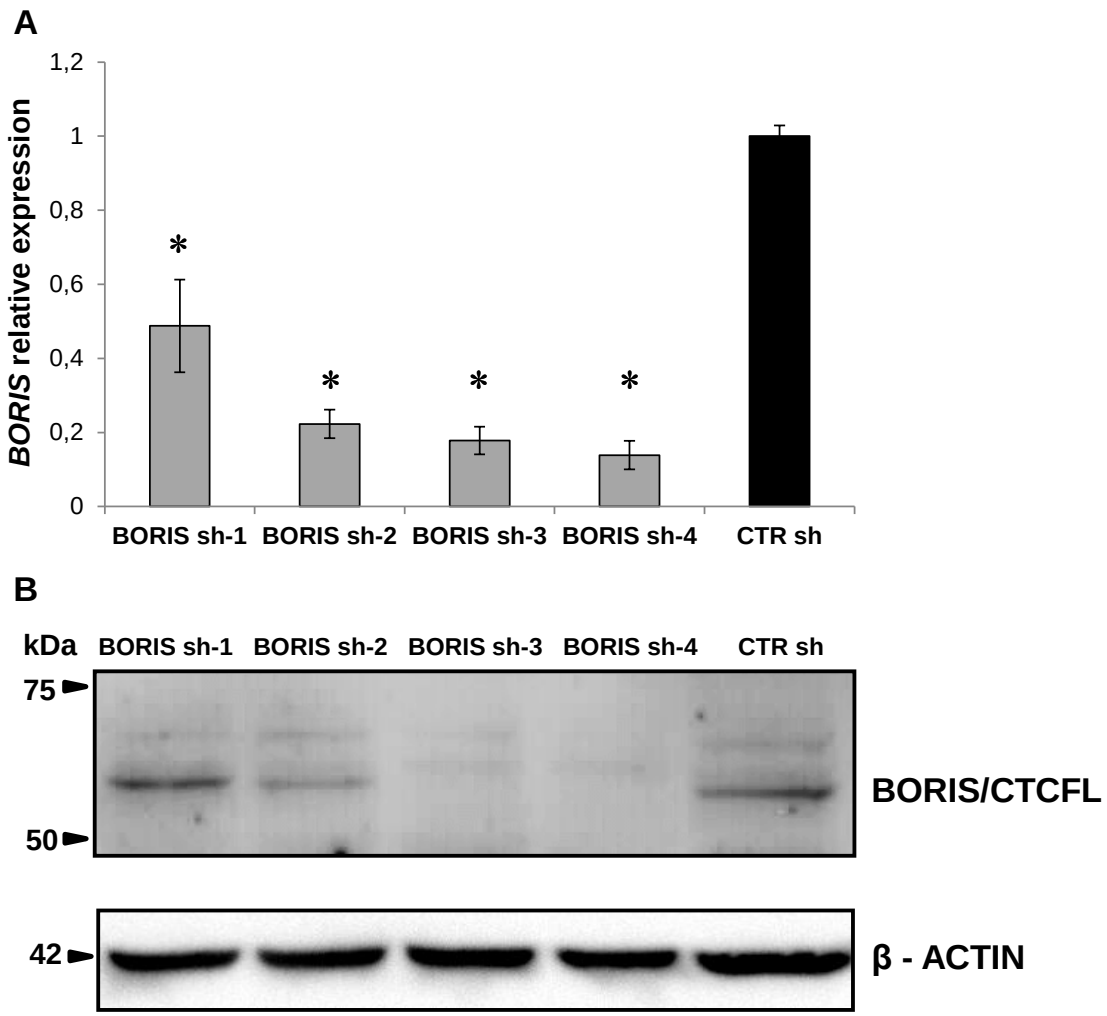

Figure 5. BORIS knockdown using lentivirus with doxycycline inducible BORIS-specific shRNA. Four different BORIS shRNA lentiviral vectors (BORIS sh-1, sh-2, sh-3 and sh-4) and the vector carrying a scrambled sequence (CTR sh) were tested. NCCIT were transduced with the indicated lentivirus and sorted for eGFP marker expression. Then, cells were cultured with doxycycline-containing medium and after 3 days were analysed. (A) BORIS mRNA levels were analysed by qRT-PCR, normalised to *GAPDH* and compare to that of CTR sh. Error bars represent the mean ± SD of 3 independent experiments. Asterisks indicate $p<0.05$. (B) Representative western blot analysis. BORIS and β-actin (as a loading control) protein levels were determine by western blot. For both analysis (qRT-PCR and western blot) the knockdown was especially noticed with the BORIS sh-3 and sh-4 compared to the CTR shRNA cells.

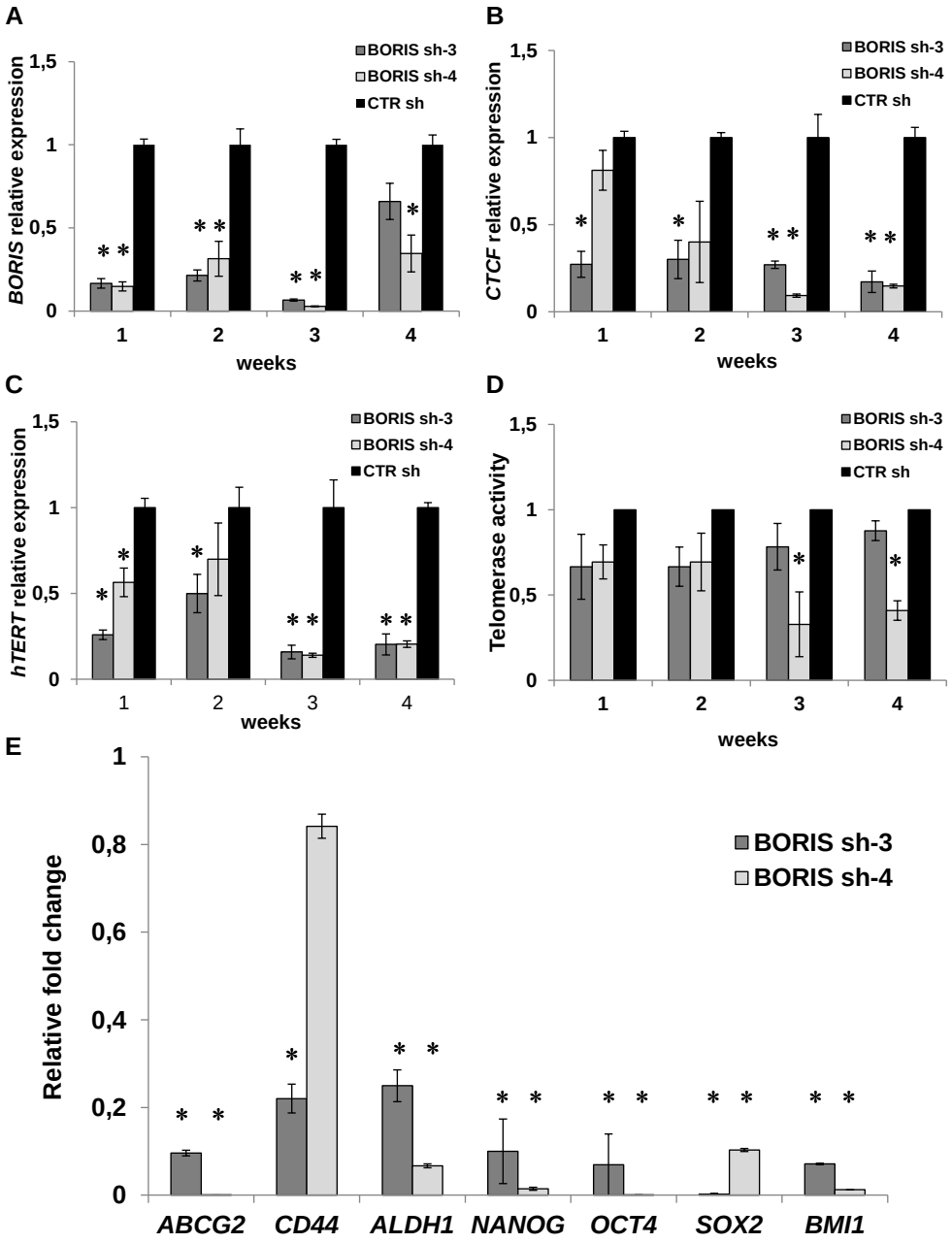

Figure 6. Knockdown of BORIS resulted in decreasing of *hTERT* expression and telomerase activity but also decrease of expression of stem cell and CSCs genes. NCCIT cells were engineered to stably exhibit knocked-down *BORIS* mRNA. BORIS sh-3, sh-4 and CTR sh (control with scrambled sequence) lentivirus were used to infect NCCIT cells. Each transduced cells were cultured with doxycycline to induce BORIS shRNA expression. Doxycycline-containing medium was replaced every 3 days. At each week over 1 month, RNA was isolated from BORIS sh-3, sh-4 and CTR sh of transduced NCCIT cells. mRNA levels of (A) *BORIS*, (B) *CTCF* and (C) *hTERT* were analysed by qRT-PCR. Results were normalized with *GAPDH* and are shown relative to that of control cells (CTR sh) at each week. Error bars represent the mean ± SD of 2 independent experiments. (D) Telomerase activity was measured at each week by real-time quantitative PCR using TRAPEZE RT Telomerase Detection Kit. Values of telomerase activity of BORIS sh-3, sh-4 NCCIT-derived cells are shown relative to that of control cells at each week. Error bars represent the mean ± SD of 2 independent experiments. Asterisks indicate $p<0.05$. (E) BORIS sh-3, sh-4 and CTR sh NCCIT-derived cells were cultured with doxycycline and after 7 days RNA was analysed by qRT-PCR. mRNA levels of the indicated genes are shown relative to that of control cells (CTR sh) after normalization with *GAPDH*. Error bars represent the mean ± SD of 2 independent experiments. Asterisks indicate statistically significant difference ($p<0.05$) between BORIS sh-3, sh-4 and CTR sh cells.

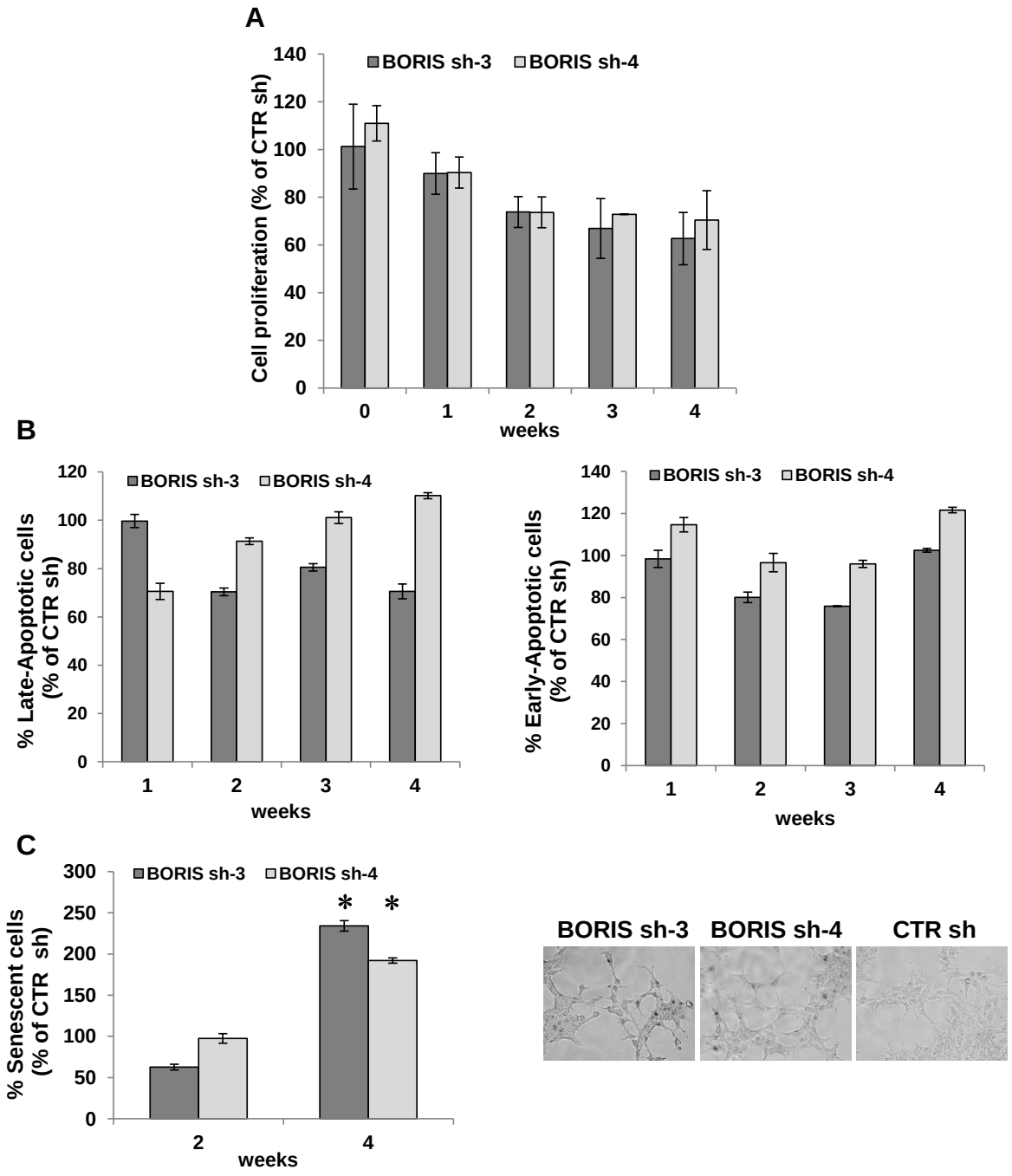

Figure 7. Knockdown of BORIS impairs cell senescence. (A) Cell proliferation over 1 moth of dox-induced BORIS shRNA cells were analysed by MTT assay. Results of the two specific BORIS-shRNA (BORIS sh-3 and sh-4) NCCIT-derived are indicated as a percentage compare to the cell proliferation of control cells (scrambled shRNA, CTR sh). Error bars represent the mean ± SD of 3 independent experiments. (B) After dox-induction of the BORIS specific shRNA in NCCIT cells, apoptosis was tested at each week using Annexin V Apoptosis Detection Kit. Results show the percentage of apoptotic cells (late apoptotic AnnexinV$^+$/7AAD$^+$ and early apoptotic AnnexinV$^+$/7AAD$^-$) of BORIS sh-3 and sh-4 cells compared to the control cells. Error bars represent the mean ± SD of 2 experiments. (C) The senescence-associated β-galactosidase (SA-β-gal) staining was performed using β-galactosidase staining kit. SA-β-gal were analysed after 2 and 4 weeks of dox-induction of the BORIS specific shRNA in NCCIT cells. Results show the percentage of senescent cells of BORIS sh-3 and sh-4 NCCIT-derived cells compared to the control cells. Error bars represent the mean ± SD of 2 experiments. Asterisks indicate $p< 0.05$. Representative images were shown after 1 month of BORIS knockdown.

2.5 Discussion

Our studies showed that in embryonic tumor cell line, BORIS positive cells represent only a subset of the tumor cell population. Indeed, all the tumor cells did not express a similar level of *BORIS* mRNA but a small fraction of them, about 3 to 5 %, showed remarkably higher *BORIS* mRNA levels compared to the rest of bulk tumor cells. This observation was confirmed by performing experiments with *BORIS* mRNA-targeting MB. Molecular beacon technology provides a powerful tool to discriminate target sequences, with very high specificity. Since their discovery (Tyagi and Kramer 1996), the MBs have been seen to distinguish target sequences differing only by a single nucleotide. Due to the characteristics of exceptional specificity and high sensibility, MBs have found a wide range of applications in biological sciences. MBs were used as taqman probes in real time quantitative PCR, for detection of mutations, SNP and allele, as indicators of contaminating infectious agents and also for *in vivo* detection of mRNA (Li, Zhou et al. 2008). Furthermore, MBs are capable to bind target sequences without modify them. To date, there is no yet a validated antibody for *in vivo* detection of BORIS, therefore we used the MB technology to visualize *BORIS* mRNA positive cells. Recently, two publications demonstrated the use of MBs in FACS sorting (King, Liszewski et al. 2011; Larsson, Lee et al. 2012). However, they showed different delivery systems of MBs to enter into the cells. One group used electroporation with a dual-FRET MB (King, Liszewski et al. 2011) and the other group described a delivery system with a cationic lipid vehicle (Larsson, Lee et al. 2012). Instead, we used the RNAiMAX Transfection Reagent, a cationic lipid formulation that was designed specifically for delivery single strand nucleotides (siRNA and miRNA). Additionally, we used for the sorting experiments, BORIS-MB conjugated with ATTO647, a fluorophore which confers a high photostability of

fluorescence signal (Arden-Jacob, Frantzeskos et al. 2001). Analysis of *BORIS* expression demonstrated that we successfully enriched the cell population that highly expresses *BORIS* mRNA (Figure 3B). In our experiments we used NCCIT cell line, classified as germ-cell tumor or embryonic cancer cells (Teshima, Shimosato et al. 1988). Due to its higher *BORIS* expression compared to the other tumor cell lines, the NCCIT cell line provides a good model for our studies, especially for the feasibility of isolation of BORIS positive cells by MB technology.

It has been previously showed that BORIS modulates the transcription of *hTERT* telomerase gene in NCCIT and in OVCAR3 and it has opposite effects compared to its paralog CTCF (Renaud, Loukinov et al. 2011). To further investigate the correlation between BORIS, hTERT and CTCF, we analyzed their expression in the isolated BORIS-high and BORIS-low expressing cells. The results confirm the positive correlation between *BORIS* and *hTERT*, whereas no correlation was observed with *CTCF*. In the same previous work, it has been found that ectopic *BORIS* expression, in normal BORIS negative cells, allowed to expand the *in vitro* lifespan increasing cell passages, this finding could be explained by the expression of high levels of *hTERT* mRNA in these BORIS-transfected cells. Induction of *hTERT* expression and telomerase activity are well established as hallmarks of cancer and are prerequisite to cellular immortalization and malignant transformation (Hanahan and Weinberg 2011). Our previous data revealed an important role of BORIS in immortalization during tumorigenesis. Here, we further confirm this role of BORIS, since the isolated BORIS-high cells expressed significant higher *hTERT* compared to counterpart BORIS-low cells and the entire bulk tumor cells.

In human, telomerase is generally absent in somatic cells but remains active in germ cells, progenitor cells and some adult stem cells (Shay and Wright 2011). It has been shown

that telomerase is reactivated in a majority (approximately 90%) of tumors (Kim, Piatyszek et al. 1994) and in clinical studies, its reactivation is associated with poor outcomes of different tumors (Gertler, Rosenberg et al. 2004; Domont, Pawlik et al. 2005; Tabori, Ma et al. 2006; Terrin, Rampazzo et al. 2008). In addition, current literature supports the evidence that CSCs express telomerase and its inhibition suppresses the self-renewal of CSCs (Marian, Cho et al. 2010; Marian, Wright et al. 2010; Vicente-Duenas, Barajas-Diego et al. 2012). All these discoveries, together with our observations indicate that BORIS could play an important and direct role in tumor malignancies by up-regulation of the *hTERT* telomerase gene. Consistent with this, we notably observed that the BORIS-high/hTERT-high isolated cells expressed also high levels of the most important stem cell markers. The embryonic carcinoma cells that we investigated, indeed, provide a good model system to study the stem cell concept of cancer. In fact, they are stem cells derived from a teratocarcinoma and are also the malignant transformed embryonic stem cells (Przyborski, Christie et al. 2004). Therefore, they show gene expression profiles close to those of human embryonic stem cells (Sperger, Chen et al. 2003). Hence, we analyzed the association of BORIS-high/hTERT-high cells with the expression of the key-regulator genes of embryonic cells (NANOG, SOX2 and OCT4) and with some of the most known specific markers of CSCs (Medema 2013). Interestingly, a correlation of BORIS-high/hTERT-high isolated cells with stem cell markers was observed. These findings were confirmed by BORIS silencing studies. Stable BORIS knockdown NCCIT-derived cells were generated by an efficient system of inducible-shRNA lentivirus (Meerbrey, Hu et al. 2011). After BORIS silencing, a significant decreasing of *hTERT* expression was observed, as well as a down-regulation of telomerase activity, which is strictly regulated by *hTERT* gene transcription. The decreasing involved also *CTCF* expression, which is in accordance with our previous results (Renaud, Loukinov et al. 2011) and thus, confirms a

role of BORIS in the transcriptional regulation of *CTCF*. Such correlation between CTCF and BORIS was not observed in the expression analysis of BORIS-high isolated cells. This discrepancy could be due to the different experimental conditions. Indeed, in the expression analysis of the BORIS-high isolated cells, *CTCF* was analyzed at steady state, while in BORIS silencing studies, what we observed is the result of cellular and genetic modifications.

Importantly, after BORIS knockdown a decreasing of expression of stem cell and CSC marker genes (*NANOG, OCT4, SOX2, BMI1, ABCG2, CD44* and *ALDH1*) was also observed. These results highlight the importance of BORIS in malignant disease and its possible critical role on cancer development and progression. Previous works have already showed a correlation of BORIS with stem cells. One group has observed the co-localization at the protein level of BORIS with ECSA, OCT4 and NANOG in cultured embryonic stem cells (Monk, Hitchins et al. 2008). Another group has detected BORIS expression in ECSA-expressing lung tumors (John, Caballero et al. 2008). Here, we additionally showed the molecular function of BORIS in embryonic cancer cells and all these data strongly suggest that BORIS may play a key role in the regulation of stem cell genes.

Cell proliferation analysis through one month of BORIS silencing revealed that the depletion of BORIS led to cell growth inhibition and an increase of cellular senescence in embryonic cancer cells. Cellular senescence is defined as the irreversible arrest of cell growth that is activated after alterations of telomeres or in response to different forms of stress (Campisi and d'Adda di Fagagna 2007). Of note, cellular senescence is considered as a potent tumor suppressive mechanism, a protective barrier against neoplastic expansion (Sager 1991). Senescent cells cannot divide, even if they continue to be metabolically and synthetically active (Campisi 2001). Senescent cells also show changes in chromatin organization and gene expression (Campisi 2013). In our studies, the cellular senescence was

measure by the most widely used senescence associated marker, the β-galactosidase activity (Dimri, Lee et al. 1995). The increasing of senescent cells after BORIS knockdown could be caused by the simultaneous telomerase inhibition. Consistent with the finding that inhibition of telomerase has been shown to initiate telomere shortening followed by cell senescence and cell death by apoptosis (Hahn, Stewart et al. 1999; Zhang, Mar et al. 1999). Future studies, analyzing more in details the effects of telomerase inhibition, as a result of BORIS depletion, will be needed to better understand the functions and regulation of BORIS in cancer cells.

The present study provides some evidences that, in embryonic tumors, BORIS is expressed only in a small subset of tumor cells and this subpopulation plays an important role in cancer development and progression, since BORIS directly regulates the expression of stem cell and CSCs genes.

3. Expression and different role of BORIS/CTCFL in human cancer stem cells

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Manuscript in preparation

3.1. Abstract

Cancer stem cells (CSCs) are cancer cells characterized by stem cell properties and represent a small population of tumor cells that drives tumor development, progression, metastasis and drug resistance. To date, the molecular mechanisms that generate and regulate CSCs are not well-defined. BORIS (Brother of Regulator of Imprinted Sites) or CTCFL (CTCF-like) is a DNA-binding protein that is expressed only in germ cells in normal tissues and is re-activated in tumors. Recent evidences have highlighted the correlation of BORIS/CTCFL expression with poor overall survival of different cancer patients. Previously, we showed an association of BORIS expressing cells with stem cell-like traits, demonstrating the critical role played by BORIS in embryonic neoplastic disease. Here, we studied the role of BORIS in BORIS-low expressing cells, and especially in cervical, breast and colon tumor cell lines. Interestingly, BORIS was found highly expressed in all the analyzed CSC-enriched populations (Side Population and spheres) of cervical, colon and breast tumor cells. BORIS silencing studies showed a decrease of sphere formation capacity in breast and colon tumor cells. On the contrary, BORIS induction showed an increasing of sphere formation in colon tumor cells. Importantly, BORIS-silencing and BORIS-induction led to down-regulation and up-regulation, respectively, of *hTERT,* stem cell and CSC markers genes of cervical, colon and invasive breast cells. However, a completely different behavior of the non-invasive breast cells (MCF7) was observed after BORIS silencing; indeed, these cells acquired an epithelial mesenchymal transition (EMT) phenotype. In this study, we demonstrated that BORIS is associated with CSC-enriched populations of several epithelial tumor cell lines and has different critical roles depending on the tumor origins.

3.2. Introduction

Enormous evidences support the view that human cancer could be considered as a stem cell disease (Reya, Morrison et al. 2001; Jordan, Guzman et al. 2006; Dalerba, Cho et al. 2007). The cancer stem cells theory assumes that cancers are viewed as complex tissues where aberrant cell growth is driven by a small population of cells defined as cancer stem cells (CSCs) or tumor-initiating cells. The CSCs are characterized by distinct proprieties: uncontrolled proliferation capacity, ability to self-renewal and ability to differentiate into a non-CSC progeny (Schatton, Frank et al. 2009). The first observations of CSCs was performed in human acute myeloid leukemia (Bonnet and Dick 1997) and consequently developed in different types of human solid tumors, as breast (Al-Hajj, Wicha et al. 2003), brain (Singh, Hawkins et al. 2004), colon (Ricci-Vitiani, Lombardi et al. 2007), colorectal (Dalerba, Dylla et al. 2007), pancreatic (Li, Heidt et al. 2007) and ovarian (Zhang, Balch et al. 2008) tumors. It has been shown that many patients, especially with solid tumors, do not respond to the conventional therapies, such as chemotherapy and radiotherapy, and after an initial remission, tumors relapse. The reasons of such failure could be explained by the drug- and radio- resistance of CSCs. In addition, it has been demonstrated that CSCs are more frequent in highly aggressive and refractory tumors (Al-Hajj, Wicha et al. 2003; Singh, Hawkins et al. 2004). Therefore, it becomes extremely important to identify the CSC populations and their markers to develop CSC-targeted therapies to overcome the resistance of CSCs to the conventional anti-cancer drugs. Using experimental approaches, the CSCs of many tumor types have been characterized phenotypically and several CSC markers have been identified (Schatton, Frank et al. 2009; Zhou, Zhang et al. 2009). However, most of the identified markers are not fully specific to CSCs because are also expressed in normal cells, and

generally, the use of multiple markers is required. Therefore, much efforts on cancer research will be necessary to optimize targeting CSCs therapies. There are several approaches to enrich the CSCs population, which are used mainly for *in vitro* analyses and screening methods. One approach is based on the selection of a cell subpopulation that is able to efflux dyes. The efflux of Hoechst 33342, a DNA-binding dye, is a capacity of CSCs which express genes encoding the ATP-binding cassette (ABC) drug transporters, such as ABCG2, and it is a feature of also stem cells from different origin (Hirschmann-Jax, Foster et al. 2004; Kondo, Setoguchi et al. 2004; Chiba, Kita et al. 2006). The subpopulation selected by this method is called side population (SP). The aldehyde dehydrogenase (ALDH) activity is another functional property of stem cells, used to isolate enriched CSC population (Ginestier, Hur et al. 2007; Charafe-Jauffret, Ginestier et al. 2009). An *in vitro* approach based on non-adherent serum-free culture has been shown to enrich CSCs population from different type of tumors (Lee, Kotliarova et al. 2006; Ricci-Vitiani, Lombardi et al. 2007). Using this non-adherent culture method, the cells from tumors (including brain, breast and colon), which have the self-renewal capacity and maintain stem-cell proprieties, can form spheroid colonies named spheres (Dontu, Abdallah et al. 2003).

Since its discovery, BORIS is described as a DNA-binding protein which shares with its paralog CTCF, 11 zinc-finger domains and for this also called CTCFL (Loukinov, Pugacheva et al. 2002). BORIS protein is involved in epigenetic reprogramming and it belongs to cancer testis antigen family, as it is expressed in normal germinal cells and reactivated in tumors. Recent reports demonstrate that BORIS expression is associated with poor prognosis in different cancers. In ovarian cancers, BORIS expression was correlated with advanced stage and decreased survival (Woloszynska-Read, James et al. 2010). It has been demonstrated that BORIS was involved in proliferation and invasion of esophageal squamous cell cancer

and BORIS-positive tumors had a poor overall survival (Okabayashi, Fujita et al. 2012). In hepatocellular carcinoma, a correlation between BORIS expression and poor overall survival, as well as, with the CSC marker CD90 have been observed (Chen, Huang et al. 2013). Our previous study has demonstrated the association of BORIS expression with stem cell and CSC marker genes in embryonic carcinoma cells (Alberti et al. submitted). All together these evidences prompted us to further investigate the presence and consequently also the molecular functions of BORIS in the CSCs-enriched populations from different type of tumor epithelial cells (cervical, colon and breast). Interestingly, we found that BORIS is highly expressed in CSC-enriched populations isolated from SP and spheres. Additional functional studies revealed that BORIS plays an important role in the self-renewal of tumors and/or acquisition of epithelial mesenchymal transition (EMT) signature in base of the different origin of tumor cells.

3.3. Materials and methods

3.3.1. Cells and spheres preparation

The human cell lines (HeLa, cervical adenocarcinoma; HT29, colon adenocarcinoma; NCCIT, embryonic carcinoma) were purchased from the American Type Culture Collection (ATCC) and the human breast cell lines (MCF7 and MDA-MB-231) were kindly provided by Dr Stéphanie Renaud (Biotechnology Institute, University of Lausanne). The cells were cultured at 37°C with 5% CO_2 either in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) for HeLa and HT29 cells, or in RPMI-1640 medium (Gibco, Invitrogen) for NCCIT, MCF7 and MDA-MB-231 cells, supplemented with 10% of heat inactivated fetal bovine serum (FBS; Invitrogen) and 1% of Penicillin-Streptomycin (Gibco, Invitrogen).

For sphere culture, cells (HT29, MCF7 and MDA-MB-231) were first detached with 0.25% trypsin solution (Invitrogen) and washed twice in PBS (Invitrogen). Then, cells were filtrated twice using a cell-strainer of 40 µm mesh size (Falcon) and cultured in serum-free medium containing DMEM/F-12 medium (Invitrogen) supplemented with B27 (Invitrogen), 5 µg/ml heparin (Sigma), 20 ng/ml EGF (Epidermal Growth Factor, BD Biosciences), 20 ng/ml FGF (Fibroblast Growth Factor, BD Biosciences) and 5 µg/ml insulin (Sigma). Cells were plated into ultra-low attachment 6 well/plates (Corning) at the density of 1,000 cells/ml for 10-15 days. Spheres were counted and collected for RNA extraction, an aliquot of spheres was seeded in normal medium with serum to allow the differentiation.

3.3.2. Fluorescence analysis of Side Population (SP) and BORIS expression using BORIS MB

Cells were prepared as previously described (Alberti et al. submitted). Briefly, cells in suspension (1 x 10^6 cells/ml) were incubated at 37°C for 1.5 hour in serum-free DMEM

medium with Cy3-BORIS MB (200 nM) and Hoechst 33342 (5 µg/mL) in presence of a Lipofectamine RNAiMAX siRNA transfection reagent (Invitrogen). The cells were washed, resuspended in PBS-5 mM EDTA and cytocentrifugated onto glass slide using a cytospin centrifuge and then examined under a fluorescent (Axioplan2 Imaging, Zeiss) or a confocal (LSM 710 Quasar, Zeiss) microscope.

3.3.3. ABCG2 immunofluorescence staining

Cells were prepared as described above. After cytospin, cells were fixed with ice-cold acetone for 8 min and stained at 4°C overnight with rabbit anti-human ABCG2 antibody (Sigma) used at 1:20 dilution in PBS. Slides were washed with PBS and incubated for 1 hour at room temperature with donkey anti-rabbit secondary antibody labelled with Alexa Fluor 488 (Sigma) used at 1:500 dilution in PBS. The slides were then examined under fluorescent microscope.

3.3.4. FACS analysis and sorting of SP

HeLa cells (1 x 10^6 cells/ml) were incubated in serum-free medium at 37°C for 1.5 hour with Hoechst 33342 (Invitrogen) at a final concentration of 12.5 µg/ml either alone or in combination with 50 µM verapamil (Sigma) as a control. The cell suspensions were periodically mixed during the incubation. After incubation, cells were washed with PBS and resuspended in PBS-5 mM EDTA. Before the analysis, the cells were incubated with propidium iodide (2 µg/ml) and filtrated using a cell-strainer of 40 µm mesh size (Falcon). SP analyses were performed using LSRII (Becton Dickinson) and the sorting of SP and NSP (non-SP) using FACS Aria (Becton Dickinson) at the facility of EPFL (Ecole Polytechnique Fédérale

of Lausanne). Hoechst 33342 dye was excited at 355 nm and its fluorescence was analyzed using dual-wavelength of emission, 445 nm for Hoechst blue and 650 nm for Hoechst red.

3.3.5. Ectopic BORIS transfection

Briefly, Hela cells were transfected with pCMV-BORIS plasmid using Lipofectamine 2000 transfection reagent (Invitrogen) as previously described (Alberti et al. submitted).

3.3.6. BORIS knockdown by inducible shRNA lentiviral system

Stables cell lines (HeLa, HT29, MCF7 and MDA-MB-231) with inducible expressing shRNAs targeting human BORIS mRNA were prepared as previously described (Alberti et al. submitted).

3.3.7. BORIS cDNA expression by inducible lentiviral system

Stable cell lines with inducible expressing human BORIS cDNA were generated using the doxycycline-inducible lentiviral system, (Meerbrey, Hu et al. 2011). BORIS cDNA from pCMV-BORIS plasmid was cloned into pINDUCER20 by Gateway Cloning system (Invitrogen). The lentiviral vector pINDUCER20 harbours the antibiotic selection marker of G418 (Geneticin), which enables to select only the transduced cells. This vector also contains a cassette with a doxycycline-inducible promoter that controls the transcription of the cloned cDNA. For the generation of lentivirus, we followed the previously described procedure (Alberti et al. submitted). The viral suspension combined with 8 µg/ml polybrene (Sigma) was used to infect target cells (HeLa, HT29, MCF7 and MDA-MB-231). Twenty-four hours post infection the medium was replaced with medium supplemented with 500 µg/ml G418 (Roche). After 2 weeks of antibiotic selection, 2 µg/ml of doxycycline (Sigma) was added to the medium to

allow induction of BORIS cDNA expression and doxycycline-containing medium was refreshed every 3 days.

3.3.8. Quantitative RT-PCR analysis

qRT-PCR was performed as previously described (Alberti et al. submitted). The sequences of primer used in addition are shown in Table 1.

Table 1 Primer sequences for qRT-PCR analysis

Gene	Forward primer	Reverse primer
ECADH	5' TGAAATTGGAAATTTTATTGATGA 3'	5' ATCATAAGGCGGGGCTGT 3'
CK19	5’ CTGCAGATGACTTCCGAACC 3’	5’ TTGATGTCGGCCTCCAC 3’
EpCAM	5’ GCAGCTCAGGAAGAATGTGTC 3’	5’ GACGATTATTATTCACAAAGCAGTTT 3’
NCADH	5’ CCTGAAGCCAACCTTAACTGA 3’	5’ TCTTGGGAACACTATTTCTTCAA 3’
VIMENTIN	5’ CAAAGTGGAATCTTTGCAAGAAG 3’	5’ GCAGCTCCTGGATTTCCTCT 3’
FIBRONECTIN	5’ TAAGCTGTACCATCGCAAACC 3’	5’ CCTCCAGGTGTCACCAATCT 3’
SNAIL	5’ CCCAATCGGAAGCCTAACTA 3’	5’ TAGGGCTGCTGGAAGGTAAA 3’
SLUG	5’ CAGACCCTGGTTGCTTCAA 3’	5’ GCAGTGAGGGCAAGAAAAAG 3’
TWIST	5’ CAGCAGGGCCGGAGAC 3’	5’ CCAGAGTCTCTAGACTGTCCATTTT 3’

3.3.9. CD44 and CD24 analysis by FACS

CD44 and CD24 expressions were analyzed in cells engineered to stably exhibit knockdown *BORIS* mRNA or BORIS cDNA. Cells were trypsinized and 10^6 cells were resuspended in 100 µL PBS-1% FBS. Monoclonal mouse anti-human CD44–APC-H7 antibody (BD Pharmingen) and a monoclonal mouse anti-human CD24–Alexa Fluor 647 antibody (BD Pharmingen) were added at dilutions of 1:20 and 1:5 respectively, as suggested by the manufacturer, and incubated for 40 min at 4 °C. DAPI was added at concentration of 1 µg/ml during the last 10 min of incubation. After washing with PBS-1% FBS, flow cytometry analysis were performed using Gallios flow cytometer (Beckman Coulter). At least 5 x 10^4 events were counted for all samples. The analysis of percentage of $CD44^+CD24^-$ cells was estimated after excluding dead

cells (DAPI positive cells) and gating on eGFP and tRFP positive cells. The results were analysed using FlowJo software. Three independent experiments were performed.

3.3.10. Colony forming assay

Cells were trypsinized and about three hundred cells were seeded in 6 well/plates. Each group of cells was measured in triplicates. Cells were cultured for 2 weeks and then fixed with 1 ml of 4% formaldehyde for 10 min at room temperature and stained with 1 ml of 0.1% crystal violet for 10 min. After washing with PBS, each well was photographed.

3.3.11. Migration assay

Cell migration was determined using cell culture inserts (BD Falcon) with 8 µm pore size. Briefly, the cells were harvested and resuspended in serum-free medium, 5 x 10^4 cells were plated into the top of inserts placed in 24 well/plates. At the bottom well of the inserts were added 500 µL medium supplemented with 10% FBS. After 48 hours of incubation, the non-migrating cells were removed with a cotton swab and the migrating cells (on the lower surface of the insert) were fixed with 1 ml of 4% formaldehyde and then stained with 1 ml of 0.1% crystal violet for 10 min. After washed 3 times with PBS, the migrating cells were counted under ten random high-power microscopic fields per insert and the mean number of migrating cells was calculated for each group of cells.

3.3.12. Chemo-sensitivity and cell proliferation assays

In vitro growth inhibition effect of 5-Fluorouracil (5-FU) on cells after BORIS silencing was determined by MTT assay. Briefly, each group of cells was seeded in triplicate at the density of 1 x 10^4 cells/well in 96 well/plates in doxycycline-containing medium. The day after, 5-FU

(Sigma) was added at different concentrations: 0.5, 5, 50 and 500 μg/ml. Cells were incubated for 2 days and then cell viability was measured by MTT assay. Growth inhibition or surviving fraction was expressed as a percentage of the untreated controls that were measured at once, using the equation: (absorbance of treated sample/absorbance of untreated sample) x 100.

Cell proliferation analysis after BORIS silencing and BORIS induction were assessed by MTT assay as previously described (Alberti et al. submitted).

3.3.13. Statistical analysis

Statistical significance was evaluated using two-tailed student t-test analysis. P-value <0.05 was considered statistically significant.

3.4. Results

3.4.1. Co-localization of BORIS mRNA with side population

Hoechst side population (SP) analysis is demonstrated to be a proven technique to enrich stem and early progenitor cells in different cell lines (Hirschmann-Jax, Foster et al. 2004; Kondo, Setoguchi et al. 2004; Chiba, Kita et al. 2006). Fluorescence imaging analysis was performed using Hoechst 33342 in combination with *in vivo BORIS* mRNA detection. Expression of *BORIS* was observed using BORIS-MB (BORIS-Molecular Beacon), as previously described (Alberti et al, submitted). The human tumor cell lines, HeLa (cervical), HT29 (colon), MCF7 (non-invasive breast) and MDA-MB-231 (invasive breast) were investigated. All these cells are classified as BORIS-low expressing cells. qRT-PCR confirmed low level of *BORIS* mRNA in HeLa and HT29, while in MCF7 and MDA-MBA-231 cells *BORIS* mRNA was almost undetectable (Figure 1A).

Fluorescence imaging confirmed *BORIS* qRT-PCR results and more specifically showed that all analysed cell lines constituted mixed population with only a very few cells expressing high levels of *BORIS*, and all resting cells lacking *BORIS* expression (Figures 1B). The estimated frequency of BORIS positive cells is approximately 0.02% for MCF7 and MDA-MBA-231, and 0.1%-0.5% for HeLa and HT29 cells. These observations were consistent with the results already obtained in embryonic (NCCIT) and ovarian (OVCAR3) tumor cell lines, in which *BORIS* mRNA was not present at the same level in all cells but rather occurs at higher level only in subset of cells. Interestingly, fluorescence imaging also showed that mostly all the BORIS positive cells were even Hoechst negative (white arrows, Figures 1A and S1). Therefore, *BORIS* expression was associated with SP phenotype (Hoechst negative cells) in

cervical, colon and breast tumor cells. This observation suggests that BORIS could be classified as a CSC marker.

3.4.2. Expression of BORIS in side population of HeLa cells

The presence of BORIS in CSC-enriched populations was firstly investigated in detail in HeLa cells. ABCG2 is described as the major responsible for Hoechst negative phenotype in HeLa SP cells (Katayama, Koike et al. 2009). Therefore, a possible co-expression of BORIS with the chemoresistance ABCG2 transporter protein was first investigated. HeLa cells were incubated with BORIS-MB and then with ABCG2 antibody. Figure 2A shows that mostly all the BORIS positive cells are both negative for Hoechst (white arrows) and positive for ABCG2 protein. This result indicates that *BORIS* is mainly expressed in the CSC-enriched population of HeLa cells.

To confirm this observation, the SP and NSP (non-SP) cell fractions were sorted from HeLa cells. Consistent with previous reports (Kondo, Setoguchi et al. 2004; Katayama, Koike et al. 2009), Hela contained from 0.5% to 1.5% SP cells (Figure 2B). The SP fraction was completely reduced by adding verapamil, an inhibitor of the ABC-transporters, indicating that the populations was bona fide SP. The qRT-PCR showed that *ABCG2* expression in SP cells was higher (about 1.5 fold) than that in NSP and parental HeLa cells (Figure 2C). Notable, *BORIS* expression analysis showed that *BORIS* mRNA level was significantly higher (about 12 fold) in SP sorted cells compared to that from NSP and parental cells (Figure 2D).

To further confirm the presence of *BORIS* in the CSCs enriched population, the frequency of SP was analyzed in pCMVBORIS-transfected HeLa cells (Figure 2E). As expected, the overexpressing BORIS cells were significantly more (2 fold, p=0.01) enriched in the SP cells than the HeLa parental cells. All these results strongly suggested that the isolation of

BORIS-positive cells could lead to a significant enrichment of the CSC populations in HeLa tumor cells.

3.4.3. Colon-sphere and mammo-sphere express high levels of BORIS mRNA

The ability of cells to grow in suspension in a serum-free medium is a common approach to CSC-enrichment (Singh, Hawkins et al. 2004; Ricci-Vitiani, Lombardi et al. 2007) and this property was reported to be restricted to stem/progenitor cells (Dontu, Abdallah et al. 2003). Therefore, *BORIS* expression was also investigated in forming-spheres of colon (HT29) and breast (MCF7) tumor cells. Cells were seeded at low density (1,000 cells/ml) in sphere culture medium in low attachment plates. After 10 days, the formed spheres were collected and an aliquot of spheres were seeded in serum-medium to allow the differentiation. Interestingly, *BORIS* expression analysis revealed a significant higher expression of *BORIS* in colon-spheres (from 5 to 45 fold, n=4) as well as in mammo-spheres (from 15 to 67 fold, n=4) compared to parental cells and to differentiated-spheres (Figure 3). These results indicated that *BORIS* is mainly expressed in CSC-enriched populations of colon and breast tumor cells.

3.4.4. Knockdown of BORIS reduces expression of stem cell and CSC marker genes, and affects hTERT expression and $CD44^{+}CD24^{-}$ phenotype in MCF7 cells

To establish a possible role of BORIS in CSCs, we selected a knockdown strategy using lentiviral system with inducible expressing shRNAs targeting human *BORIS* mRNA (Alberti et al. submitted). BORIS sh-3, BORIS sh-4 and scrambled-shRNA (CTRL sh) lentivirus were used to infect HeLa, HT29, MCF7 and MBA-MD-231 cells. According to the figure 1A all these cells are low-expressing BORIS. We has previously proven a direct role of BORIS in regulating *hTERT* and stem cell genes in NCCIT, a high-expressing BORIS cell line, and a similar relation

could be expected also in BORIS low-expressing cells. Interestingly, after 2 weeks of BORIS knockdown, *hTERT* expression was significantly down-regulated in all cells at the exception of MCF7, in which a strong up-regulation (13 fold) was observed (Figure 4A). *CTCF* expression was moderately decreased in all cells (from 40% to 20% compared to control). Notably, absence of BORIS trigged dramatic decrease of the chemoresistance ABCG2 transporter (25% for MDA-MB-231, 60% for HeLa, 80% for HT29 and 93% for MCF7). Concerning the other CSC marker, *CD44* was down-regulated in all but one cell line, MCF7 in which a 2.5 fold increase was observed compared to control. *ALDH1* (aldehyde dehydrogenase isoform 1) was decreased in all cells. Analysis of expression of stem cell genes (*NANOG, OCT4, SOX2* and *BMI1*) showed generally decrease for all cells when BORIS was depleted. Graph in figure 4A shows for all analyzed genes, the mean of the fold induction of both BORIS shRNA for each cell line. All together, these results suggested that BORIS could affect the regulation of the *hTERT* telomerase gene and the stem cell and CSC marker genes in BORIS low-expressing epithelial tumor cell lines.

$CD44^{+}CD24^{-}$ subpopulation has been found to be enriched with tumor-initiating features, especially in breast cancer cells (Al-Hajj, Wicha et al. 2003; Sheridan, Kishimoto et al. 2006). Therefore, we were interested to analyze by flow cytometry this CSC subpopulation in BORIS-knockdown tumor cells. A different behavior of MCF7 compared to the other cells was observed (Figure 4B). Of note, these analysis showed a remarkable acquisition of $CD44^{+}CD24^{-}$ phenotype (red rings) in BORIS-knockdown MCF7-derived cells. Indeed, from none $CD44^{+}CD24^{-}$ in the control cells to about 70% of the total cells in BORIS-knockdown cells. A decrease of $CD44^{+}CD24^{-}$ subpopulation was observed in BORIS-shRNA MDA-MB-231-derived cells, even if it was not significant. No change of expression was

noticed for HT29 and HeLa which displayed a typical $CD44^{+}CD24^{+}$ epithelial phenotype (Figure 4B).

3.4.5. Knockdown of BORIS affects cell proliferation in MCF7 breast cells

In NCCIT embryonic tumor cells, we have shown that BORIS silencing had an impact on cell survival and, in these BORIS high-expressing cells, the cell proliferation was inhibited through cellular senescence (Alberti et al. submitted). The impact of BORIS knockdown on cell survival was also analyzed in BORIS low-expressing tumor cell lines. Cell proliferation and the capacity to form colonies were measured each week during one month, after doxycycline-induced BORIS knockdown. For all, but one of cell lines, no significant differences on cell proliferation were observed (Figure 5A). The exception was MCF7 cells, in which the cell proliferation was dramatically increased 3-4 fold compared to control, already after 1 week of BORIS silencing. Colony formation assay confirmed these results (Figure 5B). After one month of BORIS-knockdown, the numbers of colonies were similar or slightly lower for HeLa, HT29 and MDA-MB-231, compared to controls. In contrast, the number of colonies was higher in MCF7. These results indicated that BORIS silencing in epithelial BORIS low-expressing tumor cells has not a significant impact on cell survival, at the exception of MCF7 breast cancer cells.

3.4.6. Knockdown of BORIS impairs the sphere formation capacity of colon and breast tumor cells

The effect of BORIS knockdown on self-renewal capacity of tumor spheres was investigated after BORIS silencing. Notable, the total number of tumor spheres was significantly decreased in all BORIS-shRNA derived cells (Figure 6). The number of spheres formed from

BORIS-shRNA engineered derived cells were decreased 90-95% for MCF7, 30-40% for MDA-MB-231 and 60% for HT29 compared to control. Representative images of spheres show that the size was similar between BORIS-sh and CTR sh derived spheres of MDA-MB-231 and HT29 (Figure 6B and C, right). However, the average size of spheres from BORIS-shRNA engineered MCF7-derived cells were found larger compared to the spheres from control-derived cells (Figure 6A, right). Indeed, the diameter of BORIS-depleted spheres was in average ≥ 400 µm and that of control spheres was ≤ 200 µm. Interestingly, it emerged that BORIS depletion had a negative effect on tumor sphere formation capacity in colon and in breast cancer cells in which the number of spheres were dramatically reduced. This result indicated that BORIS could have a role on the tumor self-renewal capacity of colon and breast cancers.

Analysis of expression profile was also investigated in formed spheres after BORIS silencing. The spheres formed by BORIS-depleted in HT29 and MDA-MB-231 cells showed a moderate down-regulation of *hTERT* expression (15-20% less expression compared to control) (Figure 6D). In contrast, BORIS depleted-MCF7 spheres displayed a strongly increase of *hTERT* expression (11 fold). No significant difference was observed for *CTCF* in the different cancer cell lines. These observations were consistent with the findings observed already in BORIS knockdown cells (Figure 4A).

The expression profile of stem cell and CSC markers genes of BORIS-depleted spheres showed that *NANOG, OCT4* and *SOX2* genes were dramatically down-regulated in both breast and colon tumor cells. However, *BMI1* was down-regulated in breast cells but moderately up-regulated in colon derived spheres. Expression of the chemoresistance transporter *ABCG2* was decreased for all BORIS-silenced spheres. For *CD44* and *ALDH1*, an up-regulation was observed only in the BORIS-depleted breast spheres (3 and 4.4 for MCF7

and 1.8 and 1.2 fold for MDA-MB-231 spheres, respectively compared to control spheres). In contrast, *CD44* and *ALDH1* expression were down-regulated in BORIS-depleted HT29 spheres. All these results demonstrated a different phenotype of formed spheres in which BORIS was knockdown. All the most important stem cell genes were down-regulated, which could explain the significant decrease of sphere formation capacity. Furthermore, a decrease of ABCG2, one of the most representative marker of CSCs, was observed.

3.4.7. Knockdown of BORIS up-regulates the epithelial-mesenchymal-transition (EMT)-related genes in MCF7, a luminal-like breast non-invasive tumor cell line

Analysis of BORIS-shRNA engineered MCF7-derived cells showed a remarkable difference of phenotype compared to the other breast cancer cell type as well to the other BORIS low-expressing cells. In BORIS-depleted MCF7 cells, we observed an increase of *hTERT* transcription, an acquisition of the CSC phenotype ($CD44^{+}CD24^{-}$) and an increase of cell survival. Interestingly, a change in morphology was also observed in BORIS-depleted MCF7 cells (Figure 7A). These cells formed structures irregular in shape with a unique spindle morphology. Considering that this particular morphology is characteristic of the mesenchymal cells, as MDA-MB-231 cells (Figure 7A), we further investigated to determine whether a modification of genes, controlling epithelial–mesenchymal transition (EMT) cellular process, occurred. EMT is a morphogenic cellular program in which epithelial cells acquire a mesenchymal phenotype characterized by dramatically alteration of their shape and increase of motility (Thiery 2002).

Expression of a panel of representative genes considered important during EMT program were analyzed by qRT-PCR after BORIS silencing in breast cancer cells (Figure 7B). Interestingly, it emerged that BORIS-depleted MCF7 cells acquired EMT gene signature. As

expected, the mesenchymal MDA-MB-231 cells neither changed of morphology (Figure 7A) nor modified significantly EMT gene profile (Figure 7B). In contrast, MCF7 cells after BORIS silencing, lost expression of epithelial markers, such as cytokeratin-19 (CK19), epithelial cellular adhesion molecule (EpCAM) and especially E-cadherin (ECADH). Simultaneously, we observed a remarkable up-regulation of several mesenchymal markers, including SNAI1 (SNAIL), Twist-related protein 1 (TWIST) and vimentin (43, 238 and 3567 fold increase, respectively). No significant change in expression was noticed in the other EMT-related genes, SNAI2 (SLUG), N-cadherin and fibronectin. All these results suggested that BORIS could affect the EMT process in breast cancer cells. Indeed, some of the most important EMT-related genes were transcriptionally up-regulated after BORIS silencing. However, our data indicated that BORIS may regulate some of the EMT-related genes, such as CDH1 (E-cadherin), SNAIL, TWIST and VIMENTIN, but apparently, only in luminal-like non-invasive breast cancer cells.

To confirm the acquisition of EMT phenotype of MCF7 cells after BORIS knockdown, we analyzed the migration capacity of these cells. As expected, the non-invasive breast MCF7 cells, which normally do not migrate, acquired the capacity to migrate after BORIS silencing (Figure 7C). This last finding confirmed the results obtained with expression analysis of epithelial and mesenchymal marker genes and the acquisition of EMT gene signature of MCF7 cells after BORIS silencing.

3.4.8. Evaluation of cell proliferation in BORIS knockdown-derived cells after 5-FU treatment

The impact of BORIS silencing on cell survival was also analyzed after treatment with a chemotherapeutic drug. We decided to use a drug among the most used in chemotherapy,

the 5-Fluorouracil (5-FU) (Sargent, Sobrero et al. 2009). This drug was applied for decades for the treatment of high risk stage colon cancer and also in breast, ovarian, head and neck and liver cancers at different stage.

After 2 weeks of BORIS silencing, the derived cells were treated with different concentration of 5-FU (0.5, 5, 50 and 500 μg/ml). The cell viability was determined by MTT assay and was expressed as the percentage of surviving 5-FU-treated cells compared with that of non-treated cells (Figure 8). As expected, BORIS-depleted MCF7 cells were significant ($p<0.01$) more resistant to 5-FU compared to the control cells at all concentrations, at exception of the highest concentration, where they were not significant different. BORIS-depleted MDA-MB-231 cells acquired chemoresistance only at low concentration of 5-FU. For HT29 and HeLa cells the treatment with 5-FU did not change the cell viability of the BORIS-depleted cells compared to control cells.

The increasing of chemoresistance of MCF7-BORIS depleted cells could be explained by the increasing of CSC-enriched population, $CD44^{+}CD24^{-}$ (Figure 4B). In contrast, the absence of 5-FU effect for HT29 and HeLa-BORIS depleted cells, was consistent with the none variation of the $CD44^{+}CD24^{-}$ profile.

3.4.9. At the exception of MCF7, the induction of BORIS expression inhibits cell growth of cancer cells and increases hTERT expression

In order to further investigate the biological effects of BORIS in the tumor cells, we generated stable cells in which BORIS cDNA was inducible expressed. Interestingly, the analysis of cell proliferation showed a significant decreasing of cell growth after BORIS-induction, at the exception of HT29 where no change was noticed (Figure 9A). The inhibition of cell proliferation was dramatically affected in MCF7 cells, already after 5 days of BORIS

induction. These results were in agreement with the analysis of colony formation (Figure 9B), which demonstrated a decreasing of clonogenic cell potential for all cells, at the exception of HT29. These data suggest a role of BORIS in tumor cell proliferation as a tumor suppressor gene.

The cell morphology after BORIS induction of all analyzed cells was not modified (data not shown). The analysis of expression profile after 10 days of BORIS induction showed that *hTERT* expression was significantly increased in MDA-MB-231, HT29 and HeLa cells (8.7, 3.3 and 4.8 fold, respectively) (Figure 9C). In contrast, a decrease of *hTERT* expression was observed in MCF7 cells compared to control cells. These findings are in correlation with the data found in BORIS-depleted cells in which *hTERT* expression was changed in an opposite manner. No significant change expression was found for *CTCF, ABCG2* and *CD44*. For *ALDH1* and stem cell genes, a cell-dependent behavior was observed with an up- or a down-regulation. However, a significant up-regulation was seen for *SOX2* and *NANOG* expression but not for all cell lines.

The analysis of CSCs-enriched population ($CD44^+CD24^-$ expression profile), showed that BORIS-induced cells did not have a different phenotype compared to control cells (data not shown).

All these data further confirm that BORIS plays an important role in the regulation of hTERT expression and stem cell genes, and in a cell type dependent manner.

3.4.10. BORIS induction can affect sphere formation capacity and sphere expression profile

The effect of BORIS induction was investigated on tumor sphere formation capacity. As expected, the spheres derived from BORIS-induced MCF7 cells formed 37% fewer spheres. Surprisingly, the number of formed spheres were significantly higher (2.6 fold, $p<0.01$) in

BORIS-induced HT29 cells compared to control (Figure 10A). MDA-MB-231 cells formed 44% fewer spheres. The analysis of expression prolife of formed spheres showed an up-regulation of *hTERT* expression, at the exception of MCF7 (Figure 10B). Interestingly, HT29-derived spheres showed a significant up-regulation of all analyzed genes. In contrast, for MCF7 and MDA-MB-231-derived spheres, a down-regulation was observed for all the investigated genes, at the exception of *CD44* and *ABCG2*.

The morphology of all spheres after BORIS induction was not modified compared to that of control spheres (data not shown). HeLa cells, that normally are not able to form spheres, were essayed to form spheres after BORIS induction, but were still not able to form spheres (data not shown).

All these data indicate that BORIS could increase the self-renewal of tumor cells, especially in colon tumor cells.

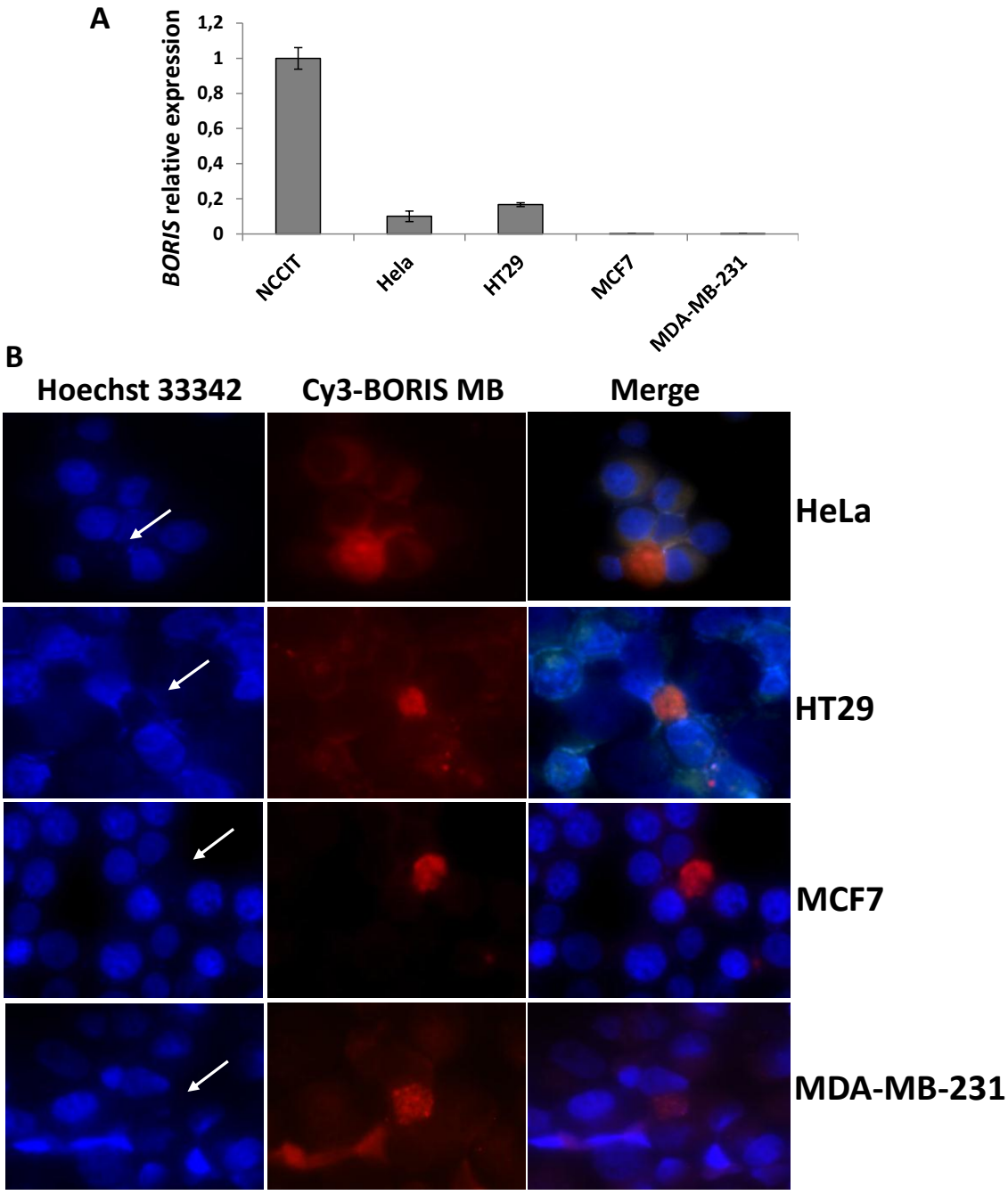

Figure 1. Analysis of SP and *BORIS* expression in human tumor cell lines using BORIS-MB. (A) *BORIS* expression in human tumor cell lines. Total RNA from NCCIT (embryonic), HeLa (cervical), HT29 (colon), MCF7 (non-invasive breast) and MDA-MB-231 (invasive breast) tumor cells were extracted and *BORIS* expression was analyzed by qRT-PCR. The results were normalized to *GAPDH* and were related to NCCIT cells. Error bars represent the mean ± SD (n=3). (B) Representative images of HeLa, HT29, MCF7 and MB-MDA 231 cells, 20X magnification. Cells were incubated with Cy3-BORIS MB (200 nM) and Hoechst 33342 (5 µg/mL) at 37°C for 1.5 hour in serum-free medium and then examined under fluorescent microscopy. In the merged images, we observed the localisation of *BORIS* mRNA in the Hoechst negative cells (indicated with white arrows in the Hoechst images).

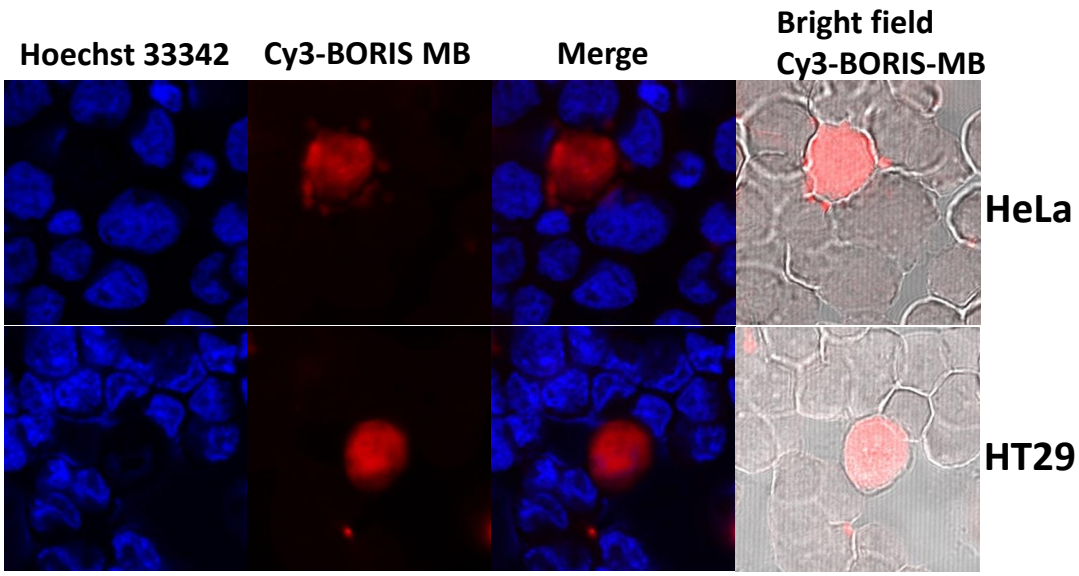

Figure S1. SP and *BORIS* expression analysis in HeLa and HT29 tumor cell lines. Representative images of HeLa and HT29 cells after incubation of BORIS-MB and Hoechst 33342. Cells were examined under confocal microscopy, 63X magnification.

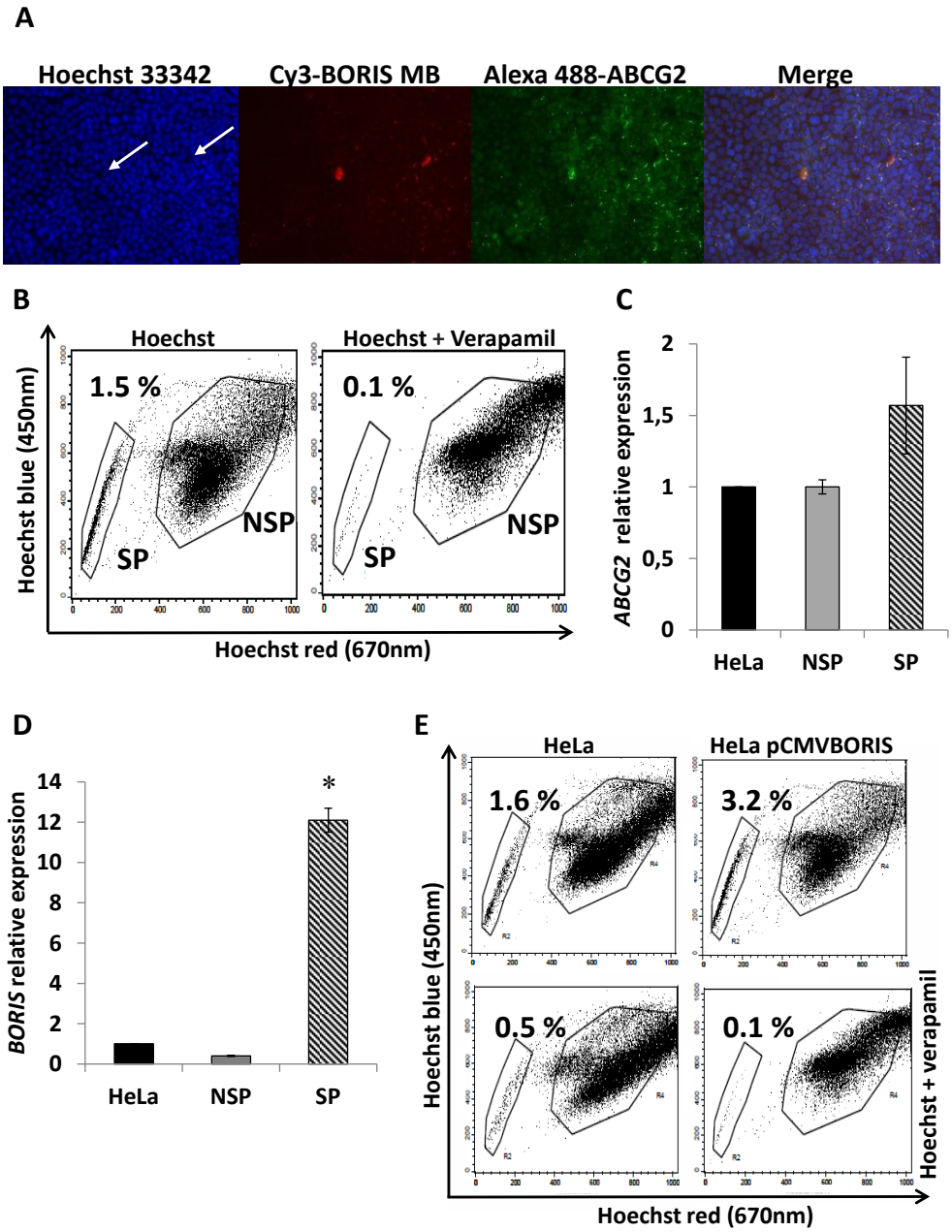

Figure 2. Expression of *BORIS* mRNA in isolated SP HeLa cells. (A) Immunolocalization of ABCG2 protein and *BORIS* mRNA in SP HeLa cells. The cells were incubated with BORIS-MB and Hoechst 33342 at 37°C for 1.5 h in serum-free medium. After cytocentrifugation the slides were fixed with cold acetone and then incubated with rabbit polyclonal ABCG2 antibody. The BORIS positive (red)-ABCG2 positive (green) - Hoechst negative cells are indicated with white arrows. 10X magnification. (B) Representative dot plot of flow cytometry analysis of SP. HeLa cells were staining with Hoechst 33342 (12.5 µg/mL) either alone or in combination with verapamil (50 µM). The analysis was performed using LSRII and the sorting using FACS Aria. The gates in each panel indicate the SP and NSP cells that were sorted. (C) *ABCG2* and (D) *BORIS* expression in SP and NSP isolated from HeLa. RNA was extracted from sorted SP and NSP HeLa cells and analysed by qRT-PCR. Graphics indicate the mRNA expression levels of the genes normalized with *GAPDH* and related to the parental HeLa cells. Data are represented as mean ± SD from 3 experiments. Asterisk indicates $p<0.05$. (E) SP analysis in BORIS overexpressed cells. HeLa cells were transiently transfected with a BORIS expression vector (HeLa pCMV BORIS). After 2 days, 1 x 10^6 cells were staining with Hoechst 33342 (12.5 µg/mL) either alone (top) or in combination (bottom) with 50 µM verapamil. The analysis was performed using LSRII flow cytometry and graphics shown one representative experiment of 3 independent experiments.

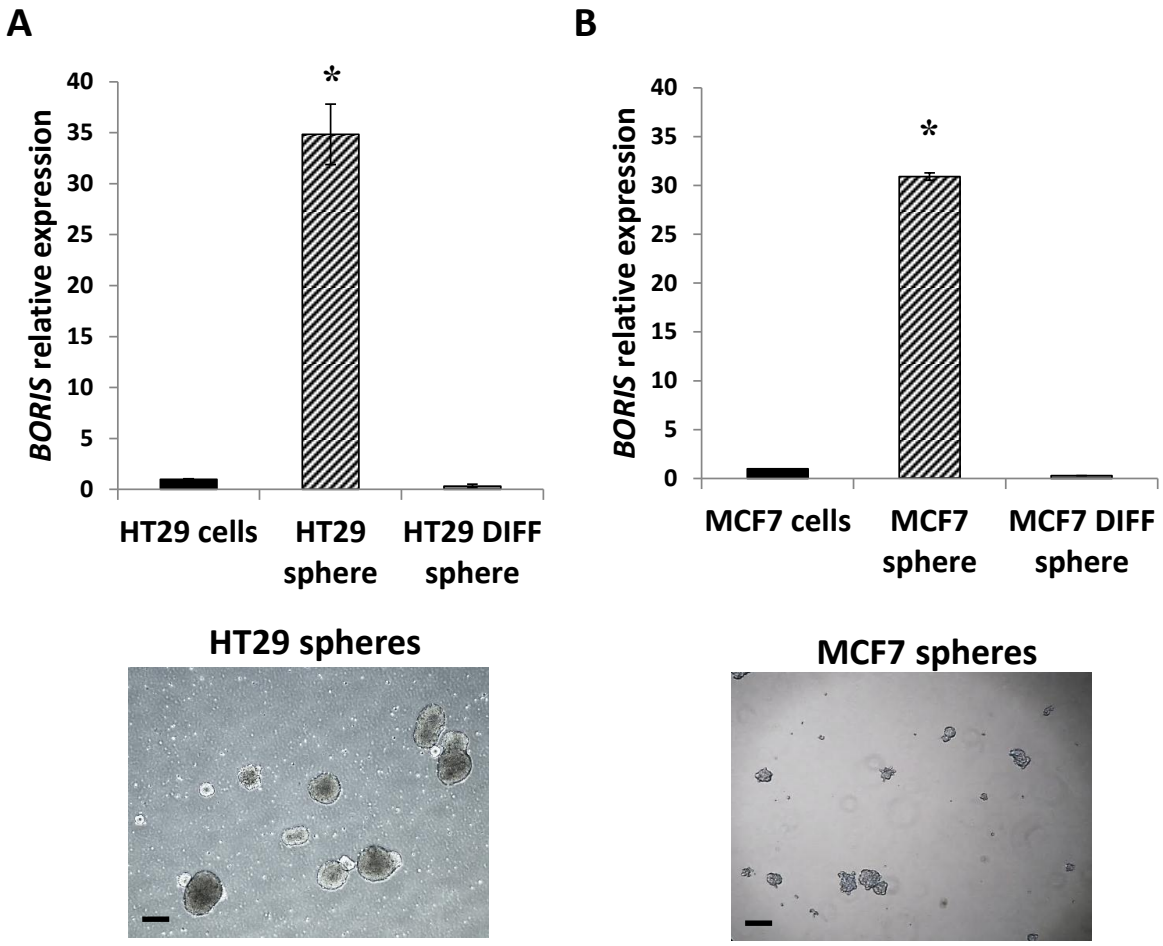

Figure 3. *BORIS* expression in colon-spheres and mammo-spheres. (A) *BORIS* expression was analyzed by qRT PCR in colon-spheres (HT29 sphere) and differentiated-spheres (HT29 DIFF sphere). (B) *BORIS* expression in mammo-sphere (MCF7 sphere) and differentiated-spheres (MCF7 DIFF sphere). Data were normalized to *GAPDH* and related to parental cells (cells). Graphics show one representative experiment of 4 independent experiments. Asterisks indicate statistically significant difference ($p<0.05$) between spheres and cells. Below the graphics are shown representative images of colon-spheres from HT29 (left) and mammo-spheres from MCF7 (right). 4X magnification. Black scale bars indicate 250 µm.

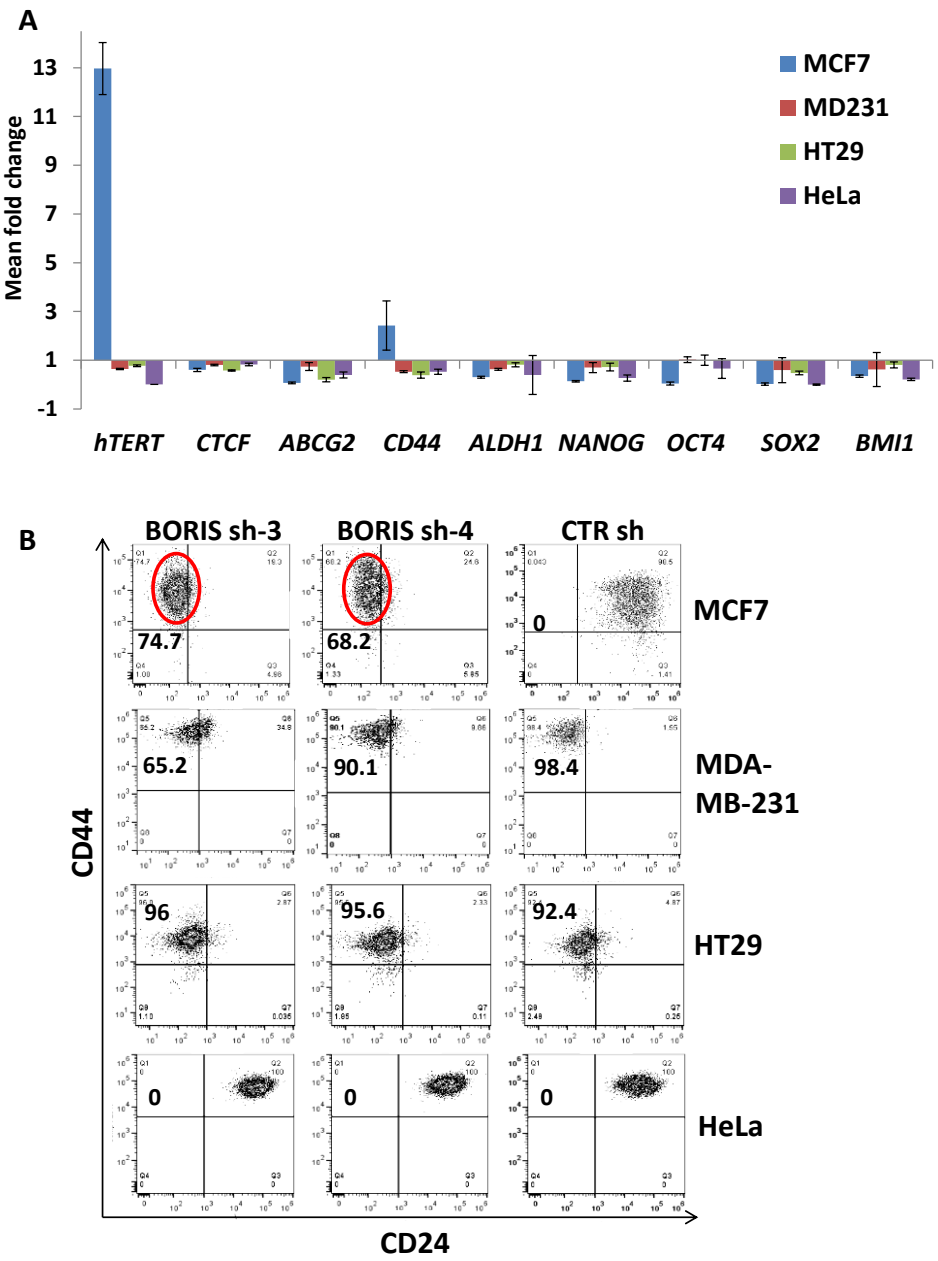

Figure 4. Impact of BORIS-knockdown on gene expression and CSCs profile. (A) MCF7, MDA-MB-231, HT29 and HeLa cells were engineered to stably exhibit knocked-down *BORIS* mRNA. BORIS sh-3, sh-4 and CTR sh (control with scrambled sequence) lentivirus were used to infect these cells. Each transduced cells were cultured with doxycycline to induce BORIS shRNA expression. Medium containing doxycycline was replaced every 3 days. After 2 weeks RNA was isolated from BORIS sh-3, sh-4 and CTR sh of each transduced cell line. mRNA levels of the indicated genes were analysed by qRT-PCR. Graphs represent for each gene the means of fold induction of both BORIS shRNA (BORIS sh-3 and sh-4) related to that of control of any cells. Standard errors were calculated considering error propagation of both BORIS shRNA analysis. Graphs show one representative experiment of 2 independent experiments. (B) Provided are representative flow cytometry dot plots of CD44 and CD24 expression of MCF7, MDA-MB-231, HT29 and HeLa cells engineered to stably exhibit knocked-down *BORIS* mRNA. CD44 and CD24 expression patterns of the two BORIS shRNA (BORIS sh-3 and sh-4) and the control (CTR sh) are shown. Anti-CD24 antibody labeled with AlexaFluor 647 and anti-CD44 antibody labeled with APC-H7 were used. The percentage of $CD44^{+}CD24^{-}$ population was estimated after gating on eGFP and tRFP positive cells (transduced and dox-induced shRNA, respectively) and the final gates are based on the isotype control corresponding to each cell line. All experiments were conducted independently 3 times and one representative experiment is shown for each group of cells.

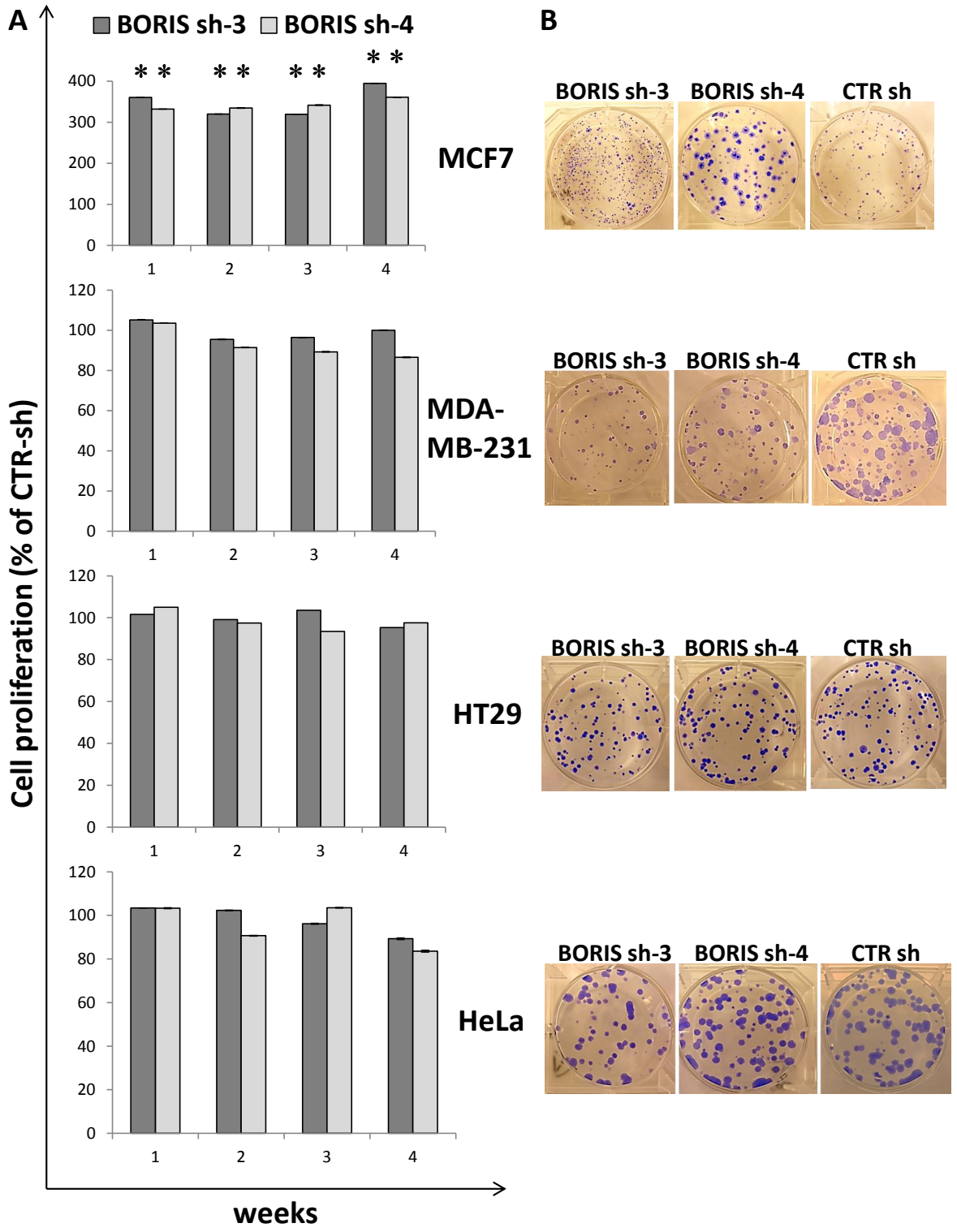

Figure 5. Impact of BORIS knockdown on cell survival in BORIS low-expressing cells. (A) Cell proliferation, over 1 month of dox-induced BORIS- and CTR- shRNA cells, were analysed by MTT assay at each week. Results of the two specific BORIS-shRNA (BORIS sh-3 and sh-4) are indicated as a percentage compare to the cell proliferation of control cells (scrambled shRNA, CTR sh). Error bars represent the mean ± SD (n=3). Asterisks indicate statistically significant difference ($p<0.05$) between BORIS sh and CTR sh. (B) Representative images of colony formation assay after 1 month of doxycycline induction. Three hundred cells were seeded in each well of 6 well/plates with medium containing doxycycline, each group were prepared in triplicate. Cells were cultured for 2 weeks, then were fixed and stained with crystal violet.

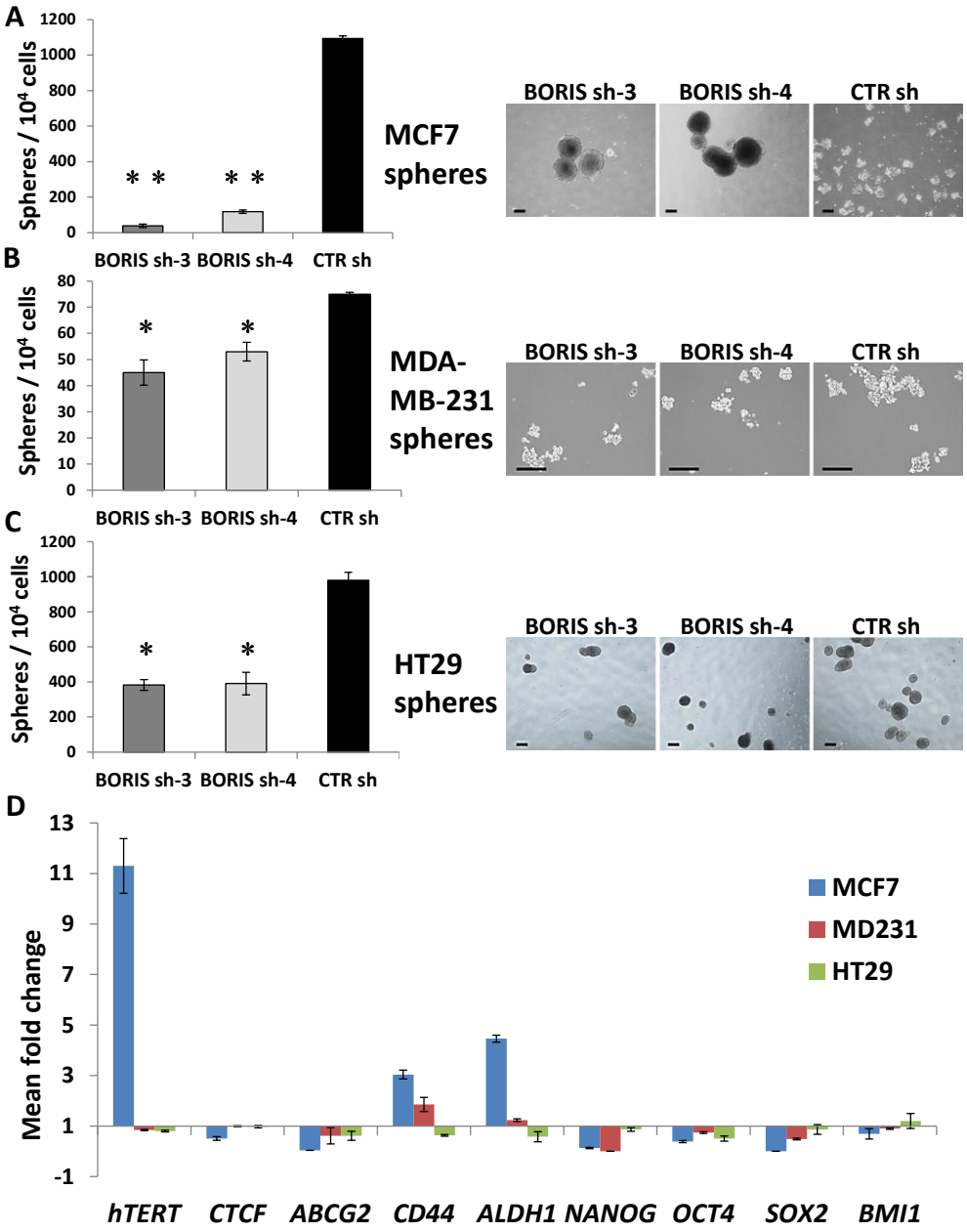

Figure 6. Knockdown of BORIS reduces the ability to form spheres of breast and colon tumor cells and decrease expression of stem cell genes. (A) MCF7, (B) MDA-MB-231 and (C) HT29 cells were engineered to stably exhibit knocked-down *BORIS* mRNA. After 2 weeks of dox-induction of BORIS- and CTR- shRNA cells were seeded at low density (1000 cells/ml) in sphere serum-free medium into ultra-low attachment 6 well/plates in triplicates. Doxycycline was added every 3 days to maintain the shRNA induction. After 10 days the total number of formed spheres were counted. Error bars represent the mean ± SD (n=3). One asterisk ($p<0.05$) or two asterisks ($p<0.001$) indicate statistically significant difference between BORIS sh and CTR sh spheres. On the right are shown representative images of spheres. MCF7-spheres and HT29-spheres, 4X magnification. MDA-MB-231-spheres, 10X magnification. Black scale bars indicate 250 µm. (D) Expression analysis of BORIS-depleted spheres. RNA was isolated from BORIS sh-3, sh-4 and CTR sh formed spheres of each cells. mRNA levels of the indicated genes were analysed by qRT-PCR. Graphs represent for each gene the means of fold induction of both BORIS shRNA (BORIS sh-3 and sh-4) related to that of control. Standard errors were calculated considering error propagation of both BORIS shRNA analysis. Graphs show one representative experiment of 2 independent experiments.

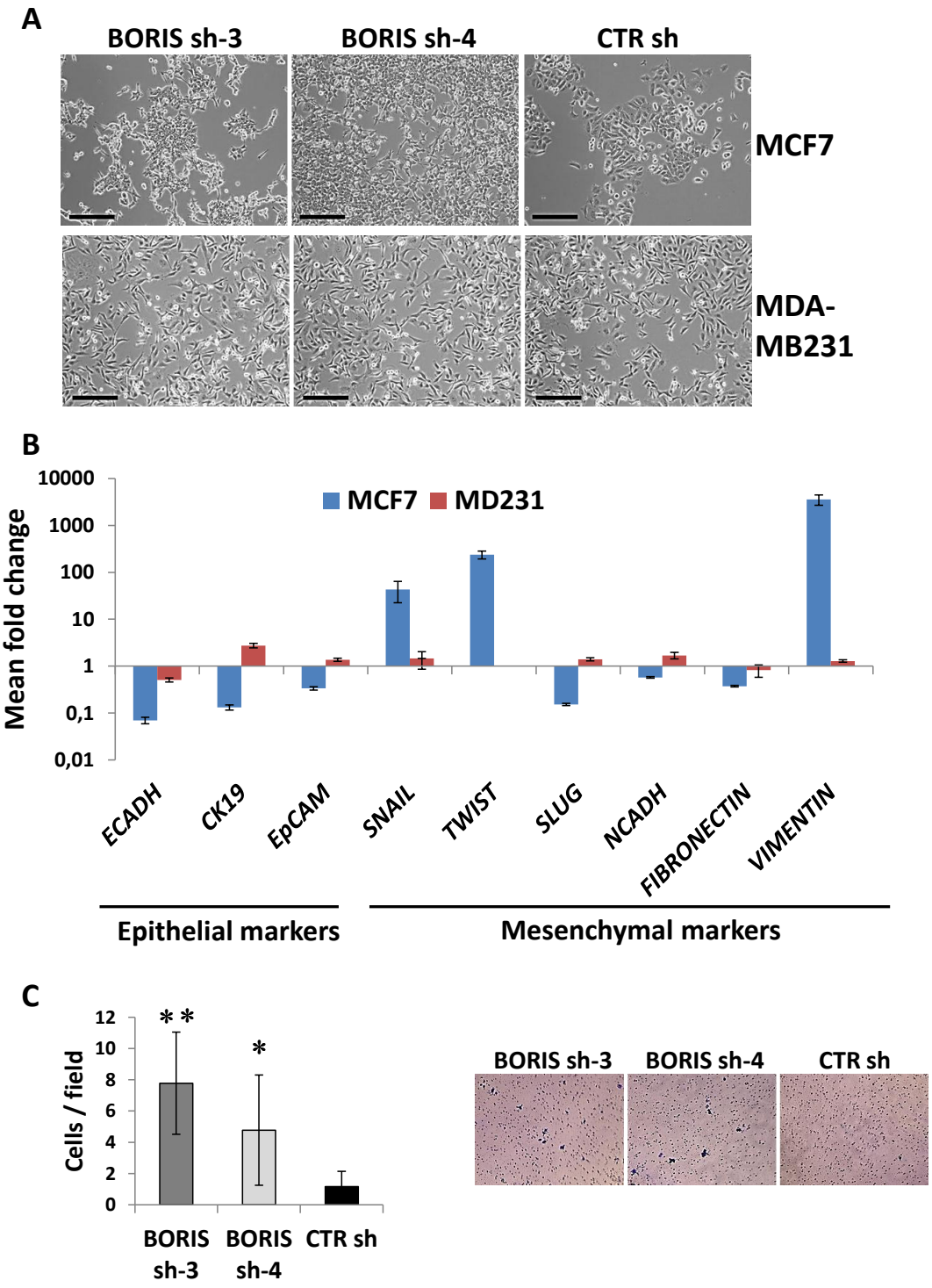

Figure 7. Acquisition of EMT phenotype and gene signature of MCF7 cells after BORIS silencing. (A) Representative images of MCF7- and MDA-MB-231- derived cells after dox-induction of BORIS- and CTR- shRNA. 10X magnification. Black scale bars indicate 250 µm. (B) After 2 weeks of dox-induction of BORIS- and CTR- shRNA, mRNA levels of the indicated genes were analysed by qRT-PCR. Graphs represent for each gene the means of fold induction of both BORIS shRNA (BORIS sh-3 and sh-4) related to that of control. Standard errors were calculated considering error propagation of both BORIS shRNA analysis. Graphs show one representative experiment out of 2 independent experiments. Results are shown in logarithmic scale. (C) Cell migration assay of MCF7 cells after BORIS knockdown. Graph shows the mean of cell number visualized in 10 different fields ± SD (n=3). One asterisk ($p<0.05$) or two asterisks ($p<0.001$) indicate statistically significant difference between BORIS sh and CTR sh. On the right, representative images. 10X magnification.

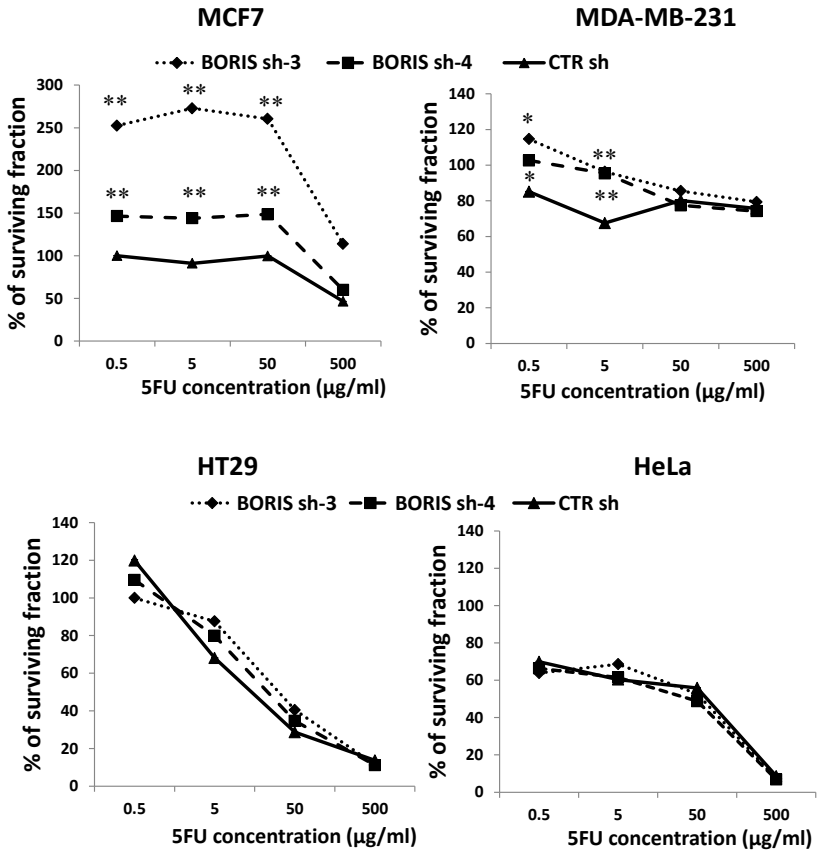

Figure 8. Effect of 5-FU on cell proliferation of BORIS-depleted tumor cell lines. MCF7, MDA-MB-231, HT29 and HeLa cells were engineered to stably exhibit knocked-down *BORIS* mRNA. After 2 weeks of dox-induction of BORIS- and CTR- shRNA cells were seeded (1 x 10^4 cells/well) in 96 well/plates in doxycycline-containing medium. The day after was added 5-FU at different concentrations: 0.5, 5, 50 and 500 µg/ml. Cells were incubated for 2 days and then cell viability was measured by MTT assay. The percentage of viable cells (% of surviving fraction) at each different concentration, is shown relative to that of the untreated control. Error bars represent the mean ± SD (n=3). One asterisk ($p<0.05$) or two asterisks ($p<0.01$) indicate statistically significant difference between BORIS sh and CTR sh cells.

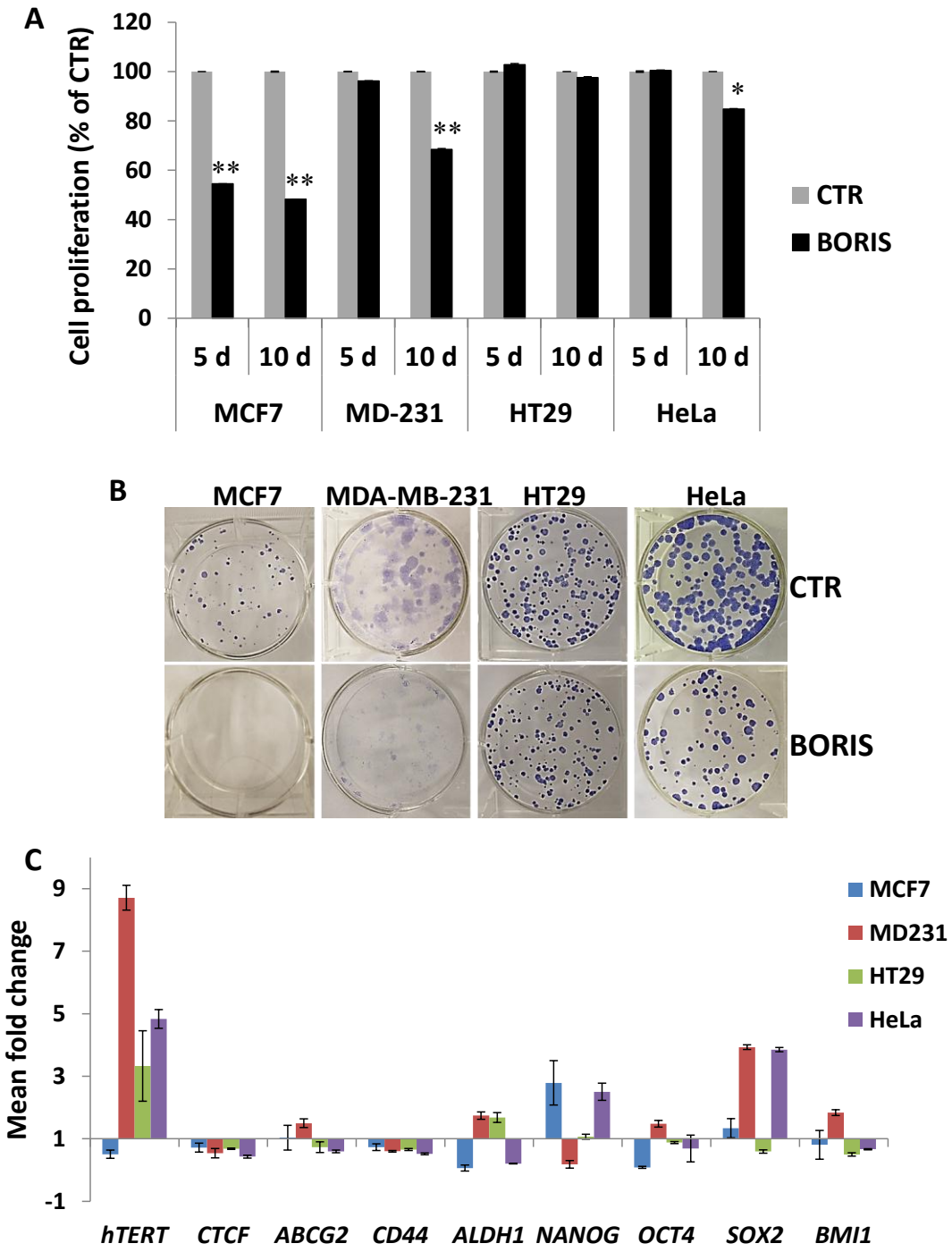

Figure 9. Inhibition of cell growth and increases of expression of *hTERT* and some stem cell genes, after BORIS-induction in cancer cells. MCF7, MDA-MB-231, HT29 and HeLa cells were engineered to stably express BORIS cDNA. After transduction with lentivirus harbour BORIS cDNA (BORIS) and control lentivirus (CTR), cells were selected by incubation with G418 for at least 2 weeks. (A) Cell proliferation analysis after 5 and 10 days of dox-induction of BORIS expression. MTT assay results of the BORIS cells are indicated as a percentage compare to the control cells (CTR). Error bars represent the mean ± SD (n=3). One asterisk ($p<0.05$) or two asterisks ($p<0.01$) indicate statistically significant difference between BORIS and CTR cells. (B) Representative images of colony formation assay. Three hundred cells were seeded in each well of 6 well/plates with medium containing doxycycline, each group were prepared in triplicate. Cells were cultured for 2 weeks, then were fixed and stained with crystal violet. (C) After 2 weeks of dox-induction of BORIS and CTR cells, mRNA levels of the indicated genes were analysed by qRT-PCR. Graph represents for each gene the fold induction of BORIS cells related to control cells. Error bars represent the mean ± SD (n=3).

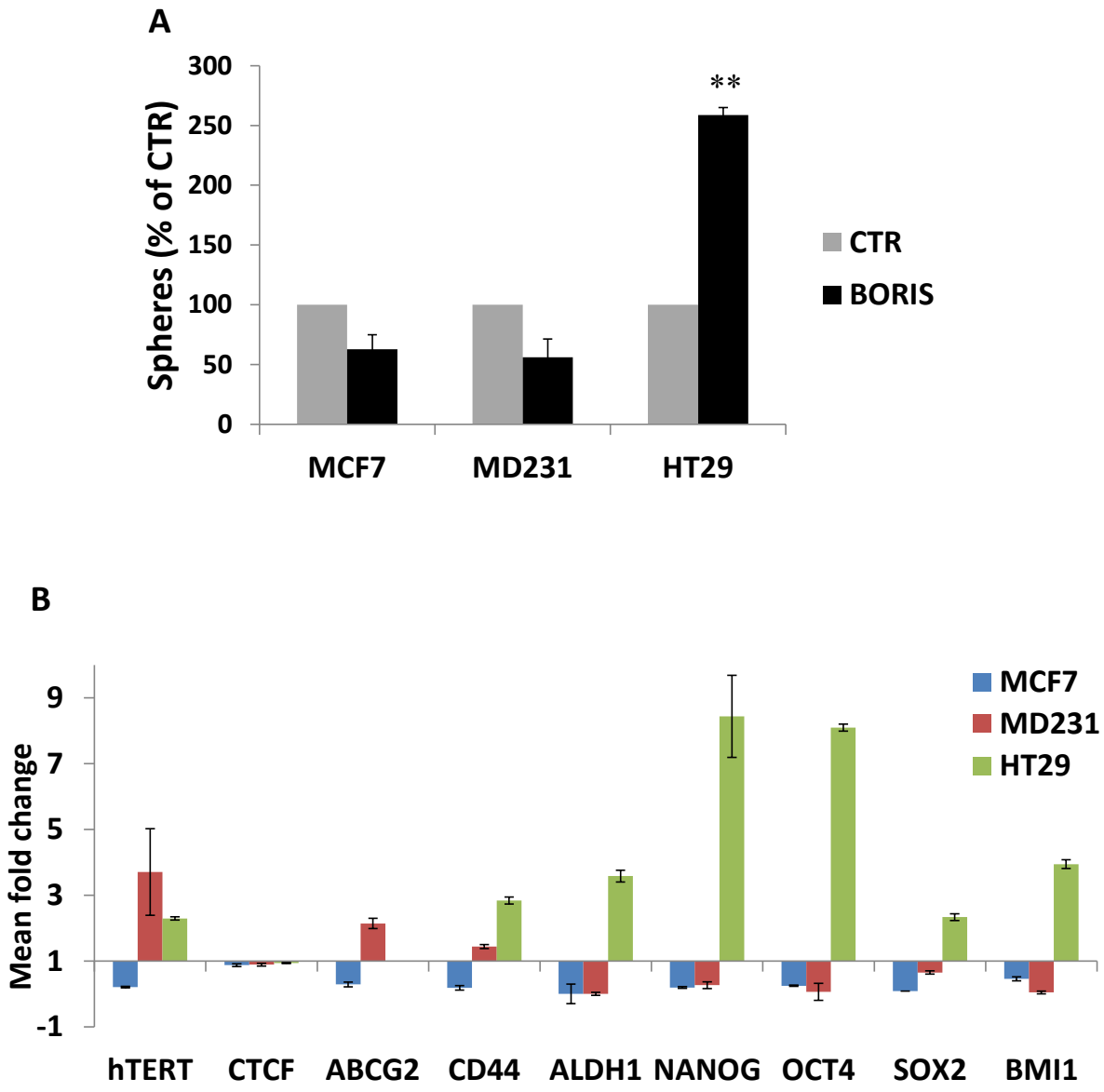

Figure 10. After BORIS induction, formed spheres and expression of stem cell genes were decreased in breast cancer cells and increases in colon cancer cells. (A) MCF7, MDA-MB-231 and HT29 cells that stably express BORIS cDNA were seeded at low density (1,000 cells/ml) in sphere serum-free medium into ultra-low attachment 6 well/plates. Doxycycline was added every 3 days to maintain the BORIS cDNA induction. After 10 days the total number of formed spheres were counted. Error bars represent the mean ± SD (n=3). Two asterisks ($p<0.01$) indicate statistically significant difference between BORIS and CTR spheres. (B) Expression analysis of spheres. RNA was isolated from BORIS-induced and CTR formed spheres of each cells. mRNA levels of the indicated genes were analysed by qRT-PCR. Graph represents for each gene the fold induction of BORIS cells related to control cells. Error bars represent the mean ± SD (n=3).

3.5. Discussion

Our study supports the evidence that BORIS could be classified as a CSC marker in epithelial tumors, including cervical, breast and colon cancers. Firstly, we analyzed *BORIS* mRNA expression by using BORIS-MB (Molecular beacon) in SP of different tumor cell lines, classified as BORIS-low expressing cells. We found a strong association between BORIS-positive cells and SP phenotype (Hoechst negative). In the same imaging analysis, we further demonstrated the presence of a small subset of BORIS-positive cells in the entire bulk tumor cells. Indeed, we found a significant high *BORIS* expression in the CSC-enriched populations isolated from HeLa SP cells and colon-spheres and mammo-spheres. Our data clearly demonstrated that BORIS-positive cells are only a rare subpopulation (0.02-0.5%) and this small cell fraction is CSC-enriched, supporting the hypothesis that BORIS could emerge as a novel CSC marker. These observations are consistent with recent reports showing a correlation of BORIS expression with poor outcome of survival in cancer patients (Woloszynska-Read, James et al. 2010; Okabayashi, Fujita et al. 2012; Chen, Huang et al. 2013). Interestingly, a correlation between BORIS expression and the CSC marker CD90 was recently found in hepatocellular carcinoma tissues (Chen, Huang et al. 2013).

BORIS knockdown significantly impairs the capacity to form spheres in colon and breast tumor cells, thus demonstrating a critical role of BORIS in self-renewal of tumor cells. These results could be a consequence of the down-regulation of *hTERT*, stem cell and CSC marker genes, that we observed after BORIS silencing, in the analyzed cells and derived spheres. Furthermore, these results confirm that BORIS could play an important role on the regulation of *hTERT* and stem cell genes, as already showed in embryonic tumor cells (Alberti et al, submitted).

Nevertheless, BORIS silencing did not lead a significant inhibition of cell proliferation, even after a relatively long time of BORIS silencing (2-3 months, data not shown). This is inconsistent with a previous work (Dougherty, Ichim et al. 2008) which has shown an increasing of apoptosis after BORIS-knockdown in MDA-MB-231 cells. This difference could be due to the different experimental conditions and settings. In our study, the experiments have been designed to minimize the risk of false or ambiguous results. For each cell line, we generated two different stable BORIS-depleted cells, and the analysis were done after 2 weeks of BORIS silencing. Notably, all the assays were performed with FACS-isolated transduced cells. Furthermore, the selected shRNA BORIS do not overlap with the homolog sequences of CTCF and target the exon 9, which is present in almost all the BORIS mRNA isoforms (Pugacheva, Suzuki et al. 2010). Although, we detected a moderate decreasing of clonogenic potential, we did not observed a significant inhibition of cell growth in HT29, HeLa and MDA-MB-231 cells. This result could be explained by the fact that these cell lines are BORIS-low expressing; hence, an inhibition of the very low subpopulation of BORIS positive cells would take long time to have a significant impact on cell proliferation of the entire bulk of tumor cells.

Conversely, a completely different phenotype after BORIS knockdown was observed in the non-invasive breast cell line (MCF7). First of all, we observed a remarkable different morphology in both of the two stable BORIS-shRNA engineered MCF7-derived cells compared to control cells and a significant enhancement of cell proliferation. The expression profile revealed a notable up-regulation of *hTERT* and also an up-regulation of *CD44* and *ALDH1* genes in formed spheres. Importantly, an acquisition of the CSC phenotype ($CD44^{+}CD24^{-}$) and an increasing of chemoresistance after 5-FU treatment, were observed. Although, we observed a decrease of sphere formation capacity, the size of spheres was

larger compared to those of control. This is correlated with the morphologic modification of the MCF7 cells after BORIS silencing and likely, these spheres are more tumorigenic than those of the control. Since a morphologic change to a mesenchymal phenotype was detected in MCF7 cells, we further explored the expression of the genes involved in EMT program. Interestingly, but also expected, we observed a down-regulation of epithelial genes (*E-cadherin, CYTOKERATIN-19* and *EpCAM*) and an up-regulation of the most important mesenchymal marker genes (*SNAIL, TWIST* and *VIMENTIN*). The increasing of migration capacity confirmed the acquisition of EMT signature observed in expression analysis.

A remarkable increase of *hTERT* expression was also observed. A putative explanation for all these observations, is that this phenotype modification in MCF7 cells is mediated by hTERT regulation. Indeed, it has been demonstrated that hTERT/telomerase has also telomere-independent functions (Stewart, Hahn et al. 2002) and some of these functions could explained our data. Overexpression of *hTERT* led cells more resistant to several insults, such as treatment with chemotherapeutic (Luiten, Pene et al. 2003; Dudognon, Pendino et al. 2004), and this observation is consistent with our data obtained with MCF7 cells. We and others have demonstrated an enhancement of cell proliferation and also a resistance to apoptosis after ectopic *hTERT* expression (Kang, Choi et al. 2004; Bollmann 2008; Renaud, Loukinov et al. 2011). Recent studies have shown that hTERT can also act as transcriptional modulator of Wnt/β-catenin signaling pathway (Park, Venteicher et al. 2009; Liu, Li et al. 2013). The Wnt pathway regulates EMT program that is involved in normal development during the embryogenesis process (Kim, Lu et al. 2002; Thiery, Acloque et al. 2009). As well, Wnt signaling aberrantly drives EMT genes to tumor formation in experimental models (Muller, Bain et al. 2002). EMT is a morphogenic cellular program, in which epithelial cells acquire a mesenchymal phenotype, characterized by dramatically alteration of their shape

and increase of motility (Thiery 2002). EMT is considered the first step in the metastasis process. Cells that undergo to EMT process acquire $CD44^{+}CD24^{-}$ expression profile pattern and the ability to form spheres (Mani, Guo et al. 2008). We found all these phenotypes in BORIS-depleted MCF7 derived cells and in addition, we observed a significant down-regulation of *CDH1* (E-cadherin) gene. The adhesive glycoprotein E-cadherin is the master-regulator of the epithelial phenotype and its loss is considered a hallmark of EMT. *CDH1* has been shown transcriptionally silenced by its transcriptional repressors, including SNAI1 (SNAIL), SNAI2 (SLUG) and TWIST (Peinado, Olmeda et al. 2007). Another important marker of EMT, which was up-regulated in BORIS-depleted MCF7 cells, is *VIMENTIN*, a major constituent of the intermediate filament family of proteins. VIMENTIN is ubiquitously expressed in normal mesenchymal cells and its overexpression is frequently associated with increased migratory and invasive capacity of cancer cells (Satelli and Li 2011). This is consistent with our finding that BORIS knockdown in MCF7 cells resulted in an increase of migration capacity.

Another putative explanation for this different phenotype, is that BORIS could regulate epigenetically other target genes. It has been shown that BORIS is involved in the epigenetic reprogramming in normal development in spermatocytes, during male germ line development (Klenova, Morse et al. 2002; Loukinov, Pugacheva et al. 2002; Jelinic, Stehle et al. 2006; Suzuki, Kosaka-Suzuki et al. 2010). Additionally, BORIS has been found to involve in the activation of several Cancer Testis genes (CTA) in cancers. CTA is a class of genes normally expressed in germinal tissues and up-regulated in many tumors by promoter hypomethylation (Caballero and Chen 2009). Several studies have demonstrated a direct role of BORIS in the regulation of the expression of some CTA genes. Indeed, overexpression of BORIS in normal cells allows *MAGEA1* expression by its promoter demethylation (Vatolin,

Abdullaev et al. 2005). It has been shown a correlation between BORIS and expression of CTA genes in non-small cell lung cancer and head and neck squamous cell carcinoma (Glazer, Smith et al. 2009; Smith, Glazer et al. 2009) and specifically, BORIS was implicated in the coordinated promoter demethylation and transcriptional activation of putative oncogenes. Consistent with all these evidences, BORIS could play a role in epigenetic regulation of some important genes implicated in the modification of phenotype.

In summary, all these data suggest that BORIS could have a critical role on the regulation of EMT process either by hTERT-mediated or by epigenetically regulation of other key-target genes.

Clearly more studies are required to connect BORIS to EMT process but another aspect to highlight is that, we did not observe the same phenotype in both of the two analyzed breast cell lines and this is due to a different origin of cancer. Indeed, MCF7 cells are luminal-like breast cancer cells, weakly proliferative and non-invasive, display epithelial phenotypic markers and express the nuclear hormone receptor, ERα. Instead, MDA-MB-231 cells are basal-like breast cancer cells, invasive with ability to migrate, display a mesenchymal phenotype and are ERα-negative. These cell lines are phenotypically and genetically different, therefore BORIS could affect target genes that are not present in both cell lines and this could explain the different phenotypes observed after BORIS silencing. Furthermore, MCF7 cells after BORIS-silencing were analysed for 2-3 weeks without induction of BORIS-shRNA (thus, without doxycycline in the culture medium) and surprisingly, cells did not revert the phenotype and remained with the mesenchymal characteristics (data not shown). This result was unexpected as EMT is reversible process. Supplementary studies are needed to further understand the mechanism by which BORIS regulated this process.

To go further in the biological functions of BORIS in tumorigenesis, we induced BORIS expression in BORIS-low expressing cells. Another function in tumorigenesis was highlighted for BORIS. Indeed, cell proliferation analysis indicated that BORIS could act as a tumor suppressor gene, since we observed an inhibition of cell growth. These results were confirmed with the colony formation assay. Our data with MCF7, MDA-MB-231 and HeLa were consistent with those of recent reports in which BORIS overexpression inhibits cell growth (Gaykalova, Vatapalli et al. 2012; Rosa-Garrido, Ceballos et al. 2012; Tiffen, Bailey et al. 2013). Contradictory results have been shown in relation to the cell cycle progression in BORIS-overexpressing cells. One study demonstrated that BORIS induction did not alter the cell cycle profile of both normal and cancer cell lines (Tiffen, Bailey et al. 2013). Another study reported that BORIS overexpression in normal cells (HEK293 cells) led to an accumulation of cells in S phase, increase of cell size and a decrease of cell cycle markers PCNA and Cyclin A (Rosa-Garrido, Ceballos et al. 2012). In our experiments, no change in cell size was observed by flow cytometry analysis after BORIS-induction.

The expression profile analysis shows that BORIS induction promotes *hTERT* expression in MDA-MB-231, HT29 and HeLa cells. These results are consistent with the data already observed (Renaud, Loukinov et al. 2011; Alberti et al. submitted) and with our last BORIS-silencing experiments in which *hTERT* was down-regulated. All together these data support again the evidence that BORIS has a role on transcriptional regulation of *hTERT* gene.

In addition, it has been shown that BORIS can be expressed in 23 different isoforms and translated in 17 different proteins, each isoprotein has a unique combination of zinc-finger domains and N- and C- termini (Pugacheva, Suzuki et al. 2010). Furthermore, BORIS is transcriptionally controlled by three alternative promoters (Renaud, Pugacheva et al. 2007). MCF7 cells have a different promoter usage compared the other analyzed cells. The

promoters A and C are equally active in MCF7, whereas in the other cell lines, BORIS is transcriptionally regulated mainly by promoter C. Taken together, all these evidences could suggest another explanation for the different data obtained with the all analyzed cells. Different BORIS protein expressed in the diverse cells could lead to diverse biological functions.

In the experiments of BORIS induction, we observed a significant increasing of sphere formation capacity in colon tumor cells (HT29) and also an up-regulation of stem cell genes in the formed spheres. On the contrary, in both breast tumor cells BORIS induction led to decreasing of formed spheres and down-regulation of stem cell genes expression. This observation demonstrates again a different role of BORIS in breast and colon tumor cells.

In summary, our results support the evidence that BORIS can be classified as a CSC marker and reveal a novel mechanism in which BORIS plays a critical role in tumorigenesis by transcriptional regulation of *hTERT,* stem cell genes and genes involved in the EMT process. This study opens new findings to better understand of tumor development and provides the opportunity for a potential new anti-tumor therapy against BORIS that can simultaneously target multiple tumorigenic pathways.

4. CONCLUSIONS AND PROSPECTIVES

Recent evidences support the view that cancers are complex tissues where aberrant cell growth is driven by a small population defined as cancer stem cells (CSCs) (Reya, Morrison et al. 2001; Jordan, Guzman et al. 2006; Dalerba, Cho et al. 2007). CSCs are characterized by stem cell proprieties and allow tumor metastasis and drug resistance. Their presence could explain the recurrent tumor relapses and the failures of cancer treatments (chemotherapy, radiotherapy). Numerous markers have been proposed as possible new CSCs-targeted therapies. Despite the enormous efforts in research, almost of these CSCs markers are expressed also in normal cells.

BORIS (Brother of Regulator of Imprinted Sites) or CTCFL (CTCF-like) is a gene expressed in normal tissues only in germinal cells and it is re-expressed in a wide variety of tumors. Furthermore, recent studies have showed an association of *BORIS* expression with a poor prognosis in different type of cancer patients (Woloszynska-Read, James et al. 2010; Okabayashi, Fujita et al. 2012; Chen, Huang et al. 2013).

Although the expression of BORIS in tumors is well documented, there were still several open questions. Firstly, it was not known the frequency of BORIS-expressing cells within the tumors. Secondly, it was not known the roles of the population of BORIS-expressing cells in tumor development.

To address the first question, we developed a new technology to target specifically *BORIS* mRNA expressing cells. Using the BORIS-MB (Molecular Beacon), we demonstrated that the BORIS expressing cells represent only a subset of tumor cell population. The estimated frequency of BORIS expressing cells depends on the origin of the tumor cells. In embryonic and ovarian tumor cells is about 3-5%, in HeLa and HT29 cells is 0.1-0.5% and in breast tumor cells is about 0.02%. This different number of BORIS expressing cells could explain the different phenotype that we observed in the functional studies.

The first indication that BORIS could be considered as a CSC marker was the finding we observed in the isolated BORIS-high expressing cells in embryonic tumor cells. Indeed, this cell fraction expressed high level of *hTERT* and stem cell genes (*NANOG, SOX2* and *OCT4*). The embryonic tumor cells was a good model to study the eventual association of BORIS with CSC, as they are BORIS-high expressing cells and they show gene expression profiles close to those of human embryonic stem cells (Sperger, Chen et al. 2003). By using BORIS-MB, we demonstrated that BORIS expressing cells co-localize with the side population (Hoechst 33342 negative cells) in different human tumor cells. Furthermore, we found a high expression of BORIS in the SP population of the isolated HeLa cells, as well an increase of SP in BORIS-overexpressing HeLa cells. We further investigated the presence of BORIS expression in other CSC-enriched populations. A significant high BORIS expression was observed in colon- and mammo-spheres. Taken together, all these evidences support the hypothesis that BORIS could become a CSC marker, as it is expressed only in a small fraction of tumor cells and this fraction is CSC-enriched. Obviously, further *in vivo* studies are needed to demonstrate the tumorigenic capacity of these BORIS-positive tumor cells.

The functional studies have showed that BORIS could play different roles in tumorigenesis according to tumor cell type. In one hand, BORIS gene has a behavior of oncogene, such as in embryonic tumor cells, where its inhibition allows a decreasing of cell growth. In another hand, BORIS acts like a tumor suppressor gene, such as in breast cells and HeLa cells, where BORIS overexpression inhibits cell proliferation. Different expression profiles were also observed in the tumor cells analyzed after knockdown or induction of BORIS. From our experimental data emerges the fact that BORIS is an important factor that regulates the expression of key-target genes for tumor development and progression. One crucial gene is *hTERT*, which was down- and up-regulation after knockdown and induction of

BORIS, respectively. The only exception was observed with MCF7 cells, in which an opposite behavior was noticed concerning the *hTERT* regulation. Interestingly, after BORIS silencing we observed an acquisition of mesenchymal phenotype in MCF7 cells. Likely, BORIS has an important function on the genes involved in EMT process. This phenotype of the non-invasive epithelial breast cancer cells was unique and we did not observed an opposite phenotype in the invasive mesenchymal breast cells (MDA-MB-231). Understanding the different mechanisms through BORIS acts in the EMT process would be interesting. The stem cell genes are another group of genes that we detected to be transcriptionally modified. We observed that their transcriptional modifications were generally comparable with those of *hTERT* gene. A significant down-regulation of stem cell genes was noticed after BORIS silencing in embryonic cancer cells, this reveals an evident association of BORIS on the transcriptional regulation of these genes. Consistent with this, an important up-regulation was observed after BORIS induction in colon tumor cells and furthermore, as mention above, we observed a high expression of stem cell genes in the BORIS-high fraction. To better understand which other genes and pathways could be affected by BORIS, it is required a gene expression analysis by microarrays that could provide a snapshot of all the transcriptional activities. Using BORIS-MB it could be possible to isolate BORIS-positive cells from different tumor cell types and by microarray analysis, we could analyze their differences in gene expression. To complete the study, it will be necessary to investigate by microarray analysis also the diverse gene expression profiles after BORIS silencing and BORIS induction, especially in MCF7 cells which displayed a particular phenotype.

In human malignancies the difference of gene expression of cancer specific genes has been frequently associated with epigenetic alterations (promoter methylation, histone acetylation). The main mechanism of the inactivation of tumor suppressor genes is the DNA

hypermethylation, nevertheless, in almost all tumors a global hypomethylation has been reported (Ehrlich 2002; Dunn 2003; Das and Singal 2004). Recent reports provide evidences that BORIS has an important role on controlling the epigenetic modification of cancer target genes (cancer testis antigens, hTERT) and commonly these alterations are associated with promoter demethylation and gene reactivation. This BORIS function, as demethylation promoting factor, could explain all the gene expression modifications that we observed after BORIS silencing or induction. We speculate that BORIS could contribute to tumorigenesis through epigenetic modification, such as hypomethylation of cancer target genes. The methylation profile of the cancer target genes need to be investigated in the future, to highlight the correlation with methylation and gene expression profiles. Another epigenetic mechanism by which BORIS could regulate gene expression is by histone modification. Indeed, it has been shown that the binding of BORIS to cancer testis antigen genes leads to an enrichment of modification of two histones, H3K9 and H3K4 (Bhan, Negi et al. 2011). Another study showed that the binding of BORIS causes changes in the local chromatin organization allowing altered *Rb2/p130* expression (Fiorentino, Macaluso et al. 2011). Likely, the BORIS binding leads to modify the conformation of chromatin from a close state to open state. It has been reported that during development and differentiation, numerous events could be regulated by a complex system of transcription factors, chromatin regulators and coordinated chromatin states (Suva, Riggi et al. 2013). In pluripotent cells the chromatin is more open, generally accessible and hyper-dynamic, instead, after differentiation, histone modifications and transcriptional activities lead to inactive genomic regions and change of chromatin configuration to a more close state. It could be interesting to identify the downstream transcription factors for which BORIS cooperates in the change of chromatin configuration state. This may help to further define the pathways in which BORIS is involved

during differentiation and the pluripotent cells. A difference of pathways regulated by BORIS among differentiated and stem cells could explain the different phenotypes observed between the two breast cancer cells. Indeed, the two analyzed breast cancer cell lines have different origin, MCF7 cells are luminal tumor cells and more differentiated, compared to the other breast cancer cell line (MDA-MB-231), which is derived from luminal progenitor cells (Perou 2010).

In summary, the experiments performed during this PhD research project validate the classification of BORIS as a new CSC marker and support the hypothesis that BORIS plays a critical role in tumorigenesis. The molecular mechanisms that cause modifications in phenotype and gene expression should provide a resource for future studies addressing to better understand the specific pathways regulated by BORIS. Our results can also open the way to the development of new anti-cancer therapies.

5. APPENDIX

Renaud S, Loukinov D, Alberti L, Vostrov A, Kwon YW, Bosman FT, Lobanenkov V, Benhattar J. BORIS/CTCFL-mediated transcriptional regulation of the hTERT telomerase gene in testicular and ovarian tumor cells. Nucleic Acids Res. 2011 Feb;39(3):862-73.

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BORIS/CTCFL-mediated transcriptional regulation of the *hTERT* telomerase gene in testicular and ovarian tumor cells

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ABSTRACT

Telomerase activity, not detectable in somatic cells but frequently activated during carcinogenesis, confers immortality to tumors. Mechanisms governing expression of the catalytic subunit *hTERT*, the limiting factor for telomerase activity, still remain unclear. We previously proposed a model in which the binding of the transcription factor CTCF to the two first exons of *hTERT* results in transcriptional inhibition in normal cells. This inhibition is abrogated, however, by methylation of CTCF binding sites in 85% of tumors. Here, we showed that *hTERT* was unmethylated in testicular and ovarian tumors and in derivative cell lines. We demonstrated that CTCF and its paralogue, BORIS/CTCFL, were both present in the nucleus of the same cancer cells and bound to the first exon of *hTERT in vivo*. Moreover, exogenous BORIS expression in normal BORIS-negative cells was sufficient to activate *hTERT* transcription with an increasing number of cell passages. Thus, expression of BORIS was sufficient to allow *hTERT* transcription in normal cells and to counteract the inhibitory effect of CTCF in testicular and ovarian tumor cells. These results define an important contribution of BORIS to immortalization during tumorigenesis.

INTRODUCTION

Telomerase activity is one of the most important factors that has been linked to multiple developmental processes including cell proliferation, differentiation, aging and senescence (1,2). This complex enzyme stabilized telomeres length by adding hexameric repeats (TTAGGG) to telomeric ends of linear chromosomes, thus compensating for the continued erosion of telomeres (3). Maintenance of telomeres is required for cells to escape from replicative senescence and proliferate indefinitely. In adult humans, telomerase activity is not detectable in most somatic cells (4). In contrast, highly proliferative cells, such as germ cells and stem cells, and 85–95% of cancers express telomerase (5). Among the various components of human telomerase, only the telomerase RNA component, *hTERC*, and the human telomerase reverse transcriptase, *hTERT*, are essential for the reconstitution of telomerase activity *in vitro* (6–8). Moreover, it has been shown that ectopic expression of *hTERT* is sufficient to restore telomerase activity in telomerase-negative cells (8–10). Therefore, *hTERT* expression is defined as the rate-limiting factor in regulating telomerase activity (11), and many studies suggest that the regulation of *hTERT* occurs primarily at the transcriptional level.

Following the characterization of the *hTERT* genomic sequence and gene organization, a minimal promoter encompassing a 283 bp region upstream of the initiation ATG codon and numerous binding sites for transcription factors have been described (12–15).

The fact that the *hTERT* promoter lies within a CpG island has led to studies of transcriptional regulation through DNA methylation. Contradictory results have been published (16–21), but apparently hypermethylation of *hTERT* is required for its expression in the majority of telomerase-positive cells (18,22). We previously showed that the proximal exonic region of *hTERT* is involved in transcriptional inhibition of the gene (23) caused by binding of CTCF to the first two exons (24). In fact, hypermethylation of the *hTERT* exon 1 region in cancer cell lines and tumors prevents the binding and the repressive effects of CTCF. In addition, a specific 110 bp region

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within the core promoter was found to be hypomethylated in *hTERT*-expressing cells thereby allowing the transcriptional effect of the minimal promoter (25). A recent study also demonstrated that this region of the *hTERT* promoter upstream of the transcriptional start site is unmethylated and linked with active chromatin in cancer cells, thus explaining *hTERT* activity in the face of hypermethylation of the 5′ regulatory region (26). Moreover, Meeran *et al.* have reported that down-regulation of DNMTs in response to HDAC inhibition by treatment with potential cancer-prevention drug Sulforaphane (SFN) generates site-specific CpG demethylation primarily in the first exon of the *hTERT* gene, which, in turn, leads to the repressive recruitment of CTCF to the same sites we mapped earlier (23,24,27).

Although methylation of its promoter appears to be the most frequently observed mechanism of the *hTERT* gene regulation in tumor tissues and tumor cell lines (18,20,28), it is obvious that one or more other methylation-independent mechanisms exist for certain types of telomerase-positive cells (16,17,19,28–30) including testicular and ovarian cancers (19,28). Therefore, a mechanism that does not require *hTERT* methylation must be active in these telomerase-positive and *hTERT* umethylated tumors to prevent the repressive effects of CTCF.

Previous studies shown that BORIS (Brother of the Regulator of Imprinted Sites), also termed CTCFL (CTCF-like), is the mammalian paralogue of a highly conserved (31,32), multi-functional chromatin factor encoded by the candidate tumor suppressor gene, *CTCF* (33–36). BORIS was found to be present mainly in testicular and has first been found to be express in a mutually exclusive manner with CTCF during male germ cell development (37). It has recently been shown to be critical to normal spermatogenesis in mice, by regulation of the CTS (Cerebroside Sulfotranscferase) gene (38). In addition to its normal expression in testis, studies revealed that various tumors and cancer cell lines, also expressed BORIS (39–43). *BORIS* is also involved in epigenetic reprogramming, both in normal development and in tumorigenesis (34,37,44). In addition, it has been shown that conditional expression of BORIS induced expression of a series of Cancer Testis Antigens (CTA) genes, as MAGE-A1, NY-ESO-1 (40,42) and SPANX (45). Althought BORIS and CTCF were shown to bind to the same recognition sequences, data provided clear demonstration that BORIS is functionally different from CTCF (38). Therefore, BORIS could also bind the CTCF target site within *hTERT* exon1.

In this study, we examined the potential contribution of BORIS to *hTERT* transcriptional regulation in testicular and ovarian cancer cells. We first analyzed the methylation pattern of *hTERT* in primary tumors of these types, in the NCCIT and OVCAR-3 cell lines, and investigated the role of BORIS in the *hTERT* gene regulation. Our data indicate that expression of BORIS in tumor cells is permissive for the transcription of *hTERT* in spite of the presence of the CTCF repressor. We concluded that BORIS and CTCF have opposite effects on *hTERT* transcription. Moreover, we showed that expression of BORIS in normal cells is sufficient to allow *hTERT* transcription and to extend their lifespan *in vitro*, revealing an important role for BORIS in immortalization.

MATERIALS AND METHODS

Tissue samples and cell lines

Ten testicular and five ovarian tumors were analyzed. The use of human tissue samples for this study was according to the guidelines of the ethical committee of the Medical Faculty of Lausanne. The human tumor cell lines (HeLa, cervical adenocarcinoma; NCCIT, testis teratocarcinoma, OVCAR-3, ovary carcinoma) and normal BJ fibroblast cells were obtained from the ATCC and were grown in the medium recommended by the ATCC. HeLa, NCCIT and OVCAR-3 cell lines are telomerase-positive, whereas BJ cells are telomerase-negative. The Normal Human Bronchial Epithelial cell line (NHBE) was obtained from Lonza (Basel, Switzerland) and maintained in medium without Retinoic Acid as recommended by Lonza. The mouse normal fibroblast NIH3T3 cell line was used in transient transfection experiments and was maintained in DMEM supplemented with 10% FBS.

DNA methylation analysis

DNA was extracted from cultured cells using the DNeasy tissue kit (Qiagen, Hilden, Germany). After bisulfite modification (EpiTect Bisulfite, Qiagen, Hilden, Germany), methylation analysis of the *hTERT* promoter was done by amplification of a 224 bp fragment (−443 to −274 from the ATG translational start site) and analyzed by a methylation-sensitive single strand conformation assay (MS-SSCA) as previously described (46). For cell lines, methylation analysis of the promoter and first exon of *hTERT* (from −200 to +100) was also performed after amplification of bisulfite modified DNA with the primers 5′-CTACCCCTTCACCTTCCAA-3′ and 5′-GTTAGTTT TGGGGTTTTAGG-3′. Triplicate PCR products were cloned into the TOPO TA cloning kit (Invitrogen). Plasmid DNAs were extracted from bacterial clones with the QIAprep Spin Miniprep Kit (Qiagen). Each clone was analyzed by sequencing, with the M13 forward primer (5′-GTAAAACGACGGCCAG-3′) by the NIAID sequencing facility.

Plasmid construction and siRNA sequences

hTERT reporters. pTERT–297 contains the *hTERT* minimal promoter (23). The pTERT–297/ex1 vector contains the *hTERT* minimal promoter and 80 bp of the first exon. To generate this vector, an *hTERT* fragment was generated by PCR and cloned into the pGL3 basic vector (Promega). The pTERT-297/ex1mut contains a mutated version of the CTCF binding site located in exon1 (24). All constructs were used in transient transfection experiments.

BORIS and CTCF expression vectors. In the pCMV-BORIS and pCMV-CTCF vectors, BORIS cDNA and CTCF cDNA were cloned in pCMV6-XL4 by

ORIGENE Technologies (Rockville, MD, USA). A pBIG-BORIS vector was created on a template containing the tetracycline-responsive, autoregulated, bidirectional expression vector pBIG2i (42). The original plasmid was used as a control. A pBORIS-puro vector contained the cDNA of BORIS under control of the CMV promoter and contains the puromycin gene as a selective marker. The empty vector was used as a negative control in transfection experiments.

siRNA BORIS and CTCF sequences. siRNAs were ordered from Sigma Life Science. An siRNA sequence targeting BORIS was: 5′-CGAGUUGAUGCCGGAAA AA[dT]-3′ and a siRNA sequence targeting CTCF was: 5′-UUGGUUCGGCAUCGUCGUU[dT]-3′.

Transient transfection and luciferase assays

Cells were seeded at a concentration of 2×10^5/3.8 cm^2 for NIH3T3, HeLa, NCCIT and OVCAR-3, and 5×10^4 cells/3.8 cm^2 for BJ and cultured overnight. Transient transfections of reporter plasmids (0.75 µg/well) and expression vector (1 µg/well) were carried out in triplicate using JetPEI Cationic Polymer Transfection reagent (8 µl/well) (Polyplus-transfection, Illkirch, France). All experiments were performed at least twice. The pRL-tk vector (0.25 µg/well) (Promega, Madison, WI) was co-transfected as an internal control for transfection efficiency in transient transfection experiments. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

NHBE cells were seeded at 3.5×10^2 cells/cm^2 in 60 mm dishes. The day after, cells were transfected using FUGENE6® 3 µl for 2 µg of DNA (Roche, Rotkreuz, Switzerland) In co-transfection experiments, a reporter: expression vector ratio of 1:1 was used. Expression of pBIG-BORIS was induced by two different concentrations of doxycycline: a low concentration of 0.0625 µg/ml and a high concentration of 1 µg/ml.

Co-transfection of expression plasmids and siRNA was performed in triplicate in six-well-plates for OVCAR-3 and NCCIT cell lines using the jetPRIME™ transfection reagent (Polyplus-transfection, Illkirch, France) following the manufacturer's instructions.

Immunfluorescence

HeLa, NCCIT and OVCAR-3 cells were grown for one day on glass slides in a four-well tissue culture chamber. Cells were washed in 1× PBS and fixed 10 min in 4% paraformaldehyde/1× PBS solution, freshly prepared. After two washes with 1× PBS, cells were permeabilized for 15 min in freshly prepared 1× PBS/0.2% Triton solution. Slides were washed in 1× PBS and then cells were blocked in a 1× PBS/10% goat serum for 90 min. Rabbit anti-BORIS and mouse anti-CTCF antibodies (produced by D.L. *et al.*) were diluted at 1/200 and 1/400, respectively, in antibody diluent solution (DakoCytomation, Carpinteria, CA) with 0.5 M NaCl. Slides were incubated at 4°C overnight. The next day, cells were washed three times in 1× PBS/1% milk/0.5% TritonX-100. Secondary antibodies including FITC-anti-rabbit and Cy3-anti-mouse (Sigma-Aldrich, St. Louis, MO) were added at a 1/100 diloution in 1× PBS and incubated for 1 h at RT in the dark. Cells were then washed three times in 1× PBS/1% milk/0.5% TritonX-100 followed by three washes in 1× PBS. Slides were mounted in 1× PBS/20% glycerol. Images were analyzed on a confocal microscope (Nikon, Chiyoda-ku, Tokyo).

RNA extraction and RT–PCR

RNA was extracted from cells using the TRIzol Reagent (Invitrogen, Basel, Switzerland). Platinum quantitative RT–PCR Thermoscript one-step system (Invitrogen) was used to amplify *hTERT* mRNA as previously described (24). RT–PCR products were analyzed on 2% agarose gels.

BORIS expression was screened with the primers RT-BORIS-FW 5′-AAGCCGCGAACGGAGACG AAG-3′ and RT-BORIS-REV 5′-ACGCCTTCATCCAC TTCCTCTTT-3′. CTCF expression was screened with the primers RT-CTCF-FW 5′-GTGGCGCGGAGAATGAT TAC-3′ and RT-CTCF-REV 5′-TCCACAATGGCTTCG ACTGC-3′. RT–PCR products were analyzed on 2% agarose gels.

For quantitative RT–PCR, RNA was converted to cDNA using random primers and superscript III reverse transcriptase (Invitrogen). Quantitative real-time RT–PCR (qPCR) analysis was performed using the TaqMan Universal PCR master Mix (Applied Biosystems, Foster city, CA) and using the Applied Biosystem 7900HT Real-Time PCR system. CTCF expression was determined using the following primers and probe: 5′-TGACACAGT CATAGCCCGAAAA-3′ (FW), 5′-TGCCTTGCTCAAT ATAGGAATGC-3′ (REV) and 6FAM-TGATTTGGGT GTCCACTTGCGAAAGC-MGB (probe). Human Glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), human BORIS (hBORIS) and human Telomerase Reverse transcriptase (hTERT) primers/probe mixtures were purchased as pre-developed assays (Applied Biosystems, Foster city, CA).

Chromatin immunoprecipitation assay

NCCIT, OVCAR-3 and HeLa cells were used for chromatin immunoprecipitation (ChIP) assays to show the *in vivo* binding of CTCF and BORIS on *hTERT* exon1. We used a ChIP Assay kit (Upstate, Charlotteville, VA, USA) and followed the manufacturers' recommendations. One ChIP reaction used 10 µg anti-CTCF monoclonal antibodies previously described (32) or 10 µg of anti-BORIS polyclonal antibody (Abcam, Cambridge, UK). Immunopurified DNAs were used in qPCR using SYBR green mix and the following specific primers for *hTERT* exon1: FW 5′-GCGGCGCGAGTTTCAG-3′ and REV 5′-GCAGCACCTCGCGGTAGT-3′. The human Nmyc CTCF binding site was used as positive control for CTCF binding using the following primers: FW 5′-GGC TCTGTGAGGAGGCAAGGTG-3′REV and 5′-GCTCT CTATTTGGAGTGGCGGG-3′. Primers used to co-amplified immunoprecipitated DNA of hTERT exon1 and H19 after ChIP were previously described (25).

RESULTS

Testicular and ovarian tumor cells exhibited the same methylation profile as normal cells

Using the MS-SSCA approach, we analyzed methylation of 20 CpGs within the *hTERT* 5′ regulatory region (between −443 and −274) in primary tumor samples obtained from five testis and five ovaries (Figure 1A). Analyses of the *hTERT* promoter showed an unmethylated pattern except for one ovarian tumor that had a hypermethylated *hTERT* gene (Figure 1A). Methylation analysis of the testicular (NCCIT) and the ovarian (OVCAR-3) tumor cell lines identified an unmethylated *hTERT* 5′ regulatory region in contrast with the hypermethylation observed in the telomerase-positive HeLa cervical tumor cell line (Figure 1A). RT–PCR analyses showed that *hTERT* was expressed in all of the primary tumors and cell lines regardless of the methylation profile (Figure 1B). The methylation status of the *hTERT* CpG island in NCCIT and OVCAR-3 cell lines was examined in greater detail around the ATG within sequences previously shown to be involved in *hTERT* regulation (25). Figure 1C presents data from representative clones of the HeLa and BJ cell lines as reference methylation profiles from a cancer cell line and a normal cell line, respectively (24,46). Sequencing of bisulfite-modified DNA from NCCIT and OVCAR-3 revealed hypomethylation of these regions at levels close to that observed in the normal cell line BJ, with, respectively, 17% and 0% of methylation within the region B containing the CTCF binding site. This result contrasted with data we reported previously for the HeLa cell line. In NCCIT and OVCAR-3, the mechanism preventing CTCF binding to the first exon of *hTERT* is thus independent of DNA methylation and had to involve another mechanism.

The repressor effect of the proximal exonic region of the *hTERT* gene is less efficient in testicular and ovarian tumor cell lines than in HeLa cells

The inhibitory effect of the first exon of *hTERT* on its minimal promoter was tested in NCCIT and OVCAR-3 and compared to HeLa and BJ fibroblasts. These four cell lines were transiently transfected with the pTERT-297 and the pTERT-297/ex1 luciferase reporter vectors containing, respectively, the *hTERT* minimal promoter alone or with *hTERT* exon 1 (Figure 2A). The transcriptional activity of the *hTERT* minimal promoter was different in these four cell lines, ranging from 16.5 to 88% of relative luciferase activity in BJ and HeLa cells respectively. Transfection of the pTERT-297/ex1 construct resulted in a 6-fold diminution of luciferase activity in HeLa cells. Interestingly, this reduction was only 3-fold and 2.3-fold in NCCIT and OVCAR-3, respectively (Figure 2B). It is interesting to note that in BJ, the low activity of the reporter vector containing the *hTERT* minimal promoter was completely inhibited by the addition of the first exon containing the CTCF binding site. Therefore, in NCCIT and OVCAR-3 cell lines, the inhibitory effect of CTCF on *hTERT* minimal promoter transcription was less efficient than in HeLa cells, showing that cellular representation of transcription factors were distinct and might differentially influence *hTERT* expression.

CTCF and BORIS were co-expressed in the testicular and ovarian tumor cell lines

As BORIS, a paralogue of CTCF, is expressed in male germ cells, we investigated the expression of these two factors in NCCIT and OVCAR-3. RT–PCR analyses of CTCF and BORIS transcripts showed that these two factors are expressed in both lines (Figure 3A). In normal cells, BORIS and CTCF were found to be expressed in a mutually exclusive manner (37). This prompted us to investigate the expression of these two proteins by immunofluorescence in NCCIT, OVCAR-3 and HeLa. These studies showed that BORIS and CTCF were coexpressed in the nuclei of NCCIT and OVCAR-3 (Figure 3B, a–f). In HeLa cells, CTCF was localized in the nucleus, whereas BORIS could be detected only at background levels (Figure 3B, g–i). In NCCIT and OVCAR-3, the merger of CTCF and BORIS signals showed that BORIS is likely localized in the nucleus where it could compete with CTCF.

BORIS and CTCF bound *in vivo* to the same region of the *hTERT* gene

As the BORIS protein has the same 11 zinc finger domain as CTCF (34,37), we investigated the hypothesis that BORIS can replace CTCF on *hTERT* repressive site(s), thus allowing *hTERT* expression. Consequently, we studied *in vivo* binding of CTCF and BORIS to *hTERT* exon 1. Quantitative ChIP assays confirmed that CTCF did not bind the exon 1 of *hTERT* in HeLa cells *in vivo*, whereas it did bind exon 1 of *hTERT* in OVCAR-3 and NCCIT cells with a 3.6- and 2.2-fold change respectively (Figure 4A). Similarly, BORIS bound to the first exon of *hTERT* in OVCAR-3 and NCCIT cells but not in HeLa cells (Figure 4A). These results indicated that BORIS and CTCF can bind the same region of *hTERT* in the telomerase-positive cell lines OVCAR-3 and NCCIT. These results were confirmed by ChIP analyzed on agarose gels (Figure 4B and C) as described previously (24,25).

BORIS and CTCF expression levels modulated *hTERT* transcription

The finding that CTCF and BORIS bound *hTERT* exon1 *in vivo*, prompted us to examine the effect of BORIS downregulation on *hTERT* transcription. First we co-transfected NCCIT and OVCAR3 cells with reporter plasmids containing the *hTERT* minimal promoter and the first exon with siRNA against BORIS. The data in Figure 5A showed that a decrease in BORIS expression induced a decrease of reporter activities in both cell lines. These results demonstrated that BORIS acted as an inhibitor of the *hTERT* minimal promoter in transient transfection experiments. Because the use of reporter vectors did not fully reflect *hTERT* transcription *in vivo*, we performed additional transfection experiments in OVCAR-3. Then we modulated the balance between BORIS and CTCF expression levels using specific

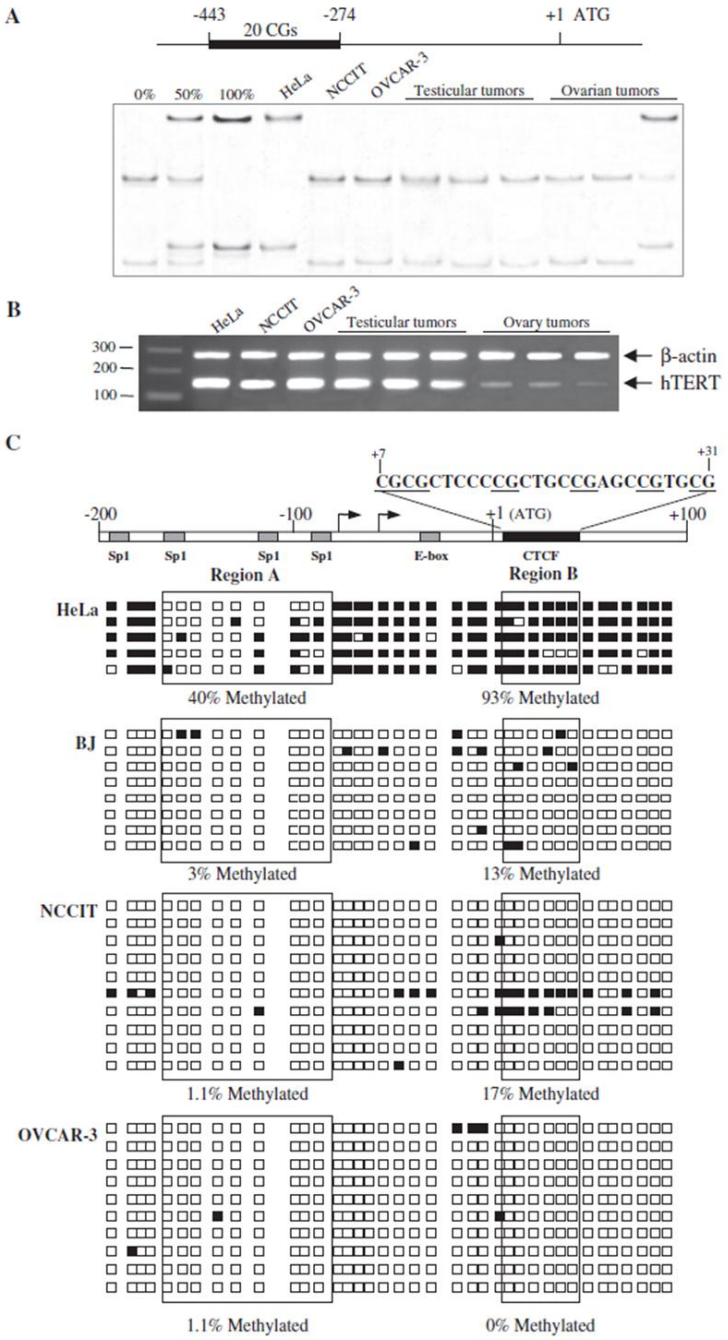

Figure 1. Expression and methylation patterns of *hTERT* in human tumors and cell lines. (**A**) Schematic representation of *hTERT* promoter sequence: +1 represents the translational start site, the black bar represents the region of promoter analysed by MS-SSCA. Controls obtained from plasmids containing *hTERT* sequences that are either unmethylated (0%), or 1:1 mixture of unmethylated and fully methylated (50%), or fully methylated (100%). The cell lines are: HeLa, cervical adenocarcinoma; NCCIT, testicular teratocarcinoma and OVCAR-3, ovarian carcinoma. (**B**) *hTERT* mRNA was detected by RT–PCR, with β-actin as internal control. (**C**) Genomic bisulfite sequencing of *hTERT* promoter and proximal exonic region (–200 to +100 nt bases around the ATG transcriptional start site). After PCR amplification and cloning, 10 clones of NCCIT and OVCAR-3 were analyzed. As a comparison, five clones representing the methylation in HeLa cell line and 8 clones representing the methylation in BJ normal cell line are shown (25). Each square represents one CpG site. Methylated CpG sites are indicated in black and unmethylated in white. On top of the bar is displaying the entire sequence of the region B, in which CGs are underlined.

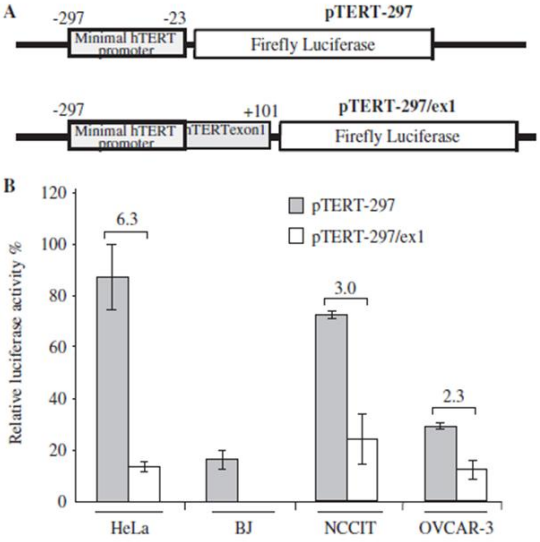

Figure 2. Transcriptional activity of the *hTERT* minimal promoter with or without the proximal exonic region. (**A**) Schematic representation of the luciferase reporter plasmids pTERT-297 and pTERT-297/ex1. (**B**) Luciferase reporter plasmids containing the *hTERT* minimal promoter, without (pTERT-297) or with (pTERT-297/ex1) the proximal exonic region, are transfected into HeLa, BJ, NCCIT and OVCAR-3 cell lines. For each cell line, the fold differences between the activity of pTERT-297 and pTERT-297/ex1 were calculated and added on top of the graphic bars.

siRNAs against BORIS and CTCF. The endogenous levels of *hTERT*, BORIS and CTCF were then quantified by qPCR. Transcript levels in siRNA-treated cells were normalized to the levels detected after transfection with a scrambled siRNA. A 2-fold decrease in *BORIS* transcripts was observed in cells transfected with siRNA against BORIS and levels of *CTCF* transcripts were decreased by 30%. In contrast, levels of *hTERT* transcripts were essentially unchanged. (Figure 5B). A 2.5-fold decrease in *CTCF* transcripts was observed in cells transfected with siRNA against CTCF while levels of *BORIS* and *hTERT* transcripts were unaffected. These results demonstrated that control of *BORIS* and *CTCF* transcription were closely linked such that modulation of one could affect the expression of the other.

In a second experiment, we overexpressed CTCF in OVCAR-3 cells and measured the transcript level of endogenous *hTERT* and *BORIS* using levels in unmanipulated cells referenced as 100%. Overexpression of CTCF resulted in increased *BORIS* transcription, but had no effect on the transcriptional levels of *hTERT* (Figure 5C). This suggests that the predicted repressive effect on *hTERT* of increased CTCF expression was countered by the increase in BORIS expression, thereby indicating that transcription of *hTERT* cannot be repressed by CTCF in cells that also express BORIS. Taken together, these results demonstrated that the equilibrium between the levels of CTCF and BORIS expression have a great importance for regulating *hTERT* transcription.

In studies of HeLa cells that do not express BORIS, a reporter vector with a mutated CTCF binding site in exon1 exhibited increased *hTERT* promoter activity. Parallel studies of BORIS-positive OVCAR-3 cells revealed that the mutation had no effect on *hTERT* promoter activity (Figure 5D). This experiment showed that in BORIS-negative cells, mutation of the CTCF binding site diminished the inhibitory effect of CTCF on *hTERT* promoter, and that in BORIS-positive cells, the presence of BORIS maintained *hTERT* promoter activity in the presence of CTCF.

Expression of BORIS induced *hTERT* transcription in telomerase-negative cells

To further investigate the contributions of BORIS to *hTERT* transcriptional activation, NIH3T3 cells were co-transfected with a BORIS expression vector (pBIG-BORIS) and *hTERT* reporter constructs. Expression of BORIS was induced with doxycycline and luciferase activities were measured 24 h after transfection. The level of reporter activity of pTERT-297, which contains only the *hTERT* minimal promoter, was set as the reference point of 100%. The activity of the pTERT-297/ex1 vector was 2.5-fold lower in NIH3T3 cells co-transfected with the empty vector. In the co-transfection experiments with pBIG-BORIS, the reporter activity of pTERT-297/ex1 reporter activity was only 1.5-fold lower (Figure 6A), a level similar to that observed in OVCAR-3 cells transfected with pTERT-297/ex1 (Figure 2B). To confirm the stimulating effect of BORIS on *hTERT* transcription, the pCMV-BORIS expression vector was transfected in the BJ normal human fibroblast cells that do not express either *BORIS* or *hTERT*. After transfection with the pCMV-BORIS vector, *BORIS* and *hTERT* transcripts were easily detected by RT–PCR (Figure 6B). We also performed RT–PCR assays for the full-length and the α-spliced variants of *hTERT* (24). The amplified BORIS fragment obtained from BJ cells transfected with pCMV-BORIS was purified and directly sequenced, revealing the presence of only the full-length *hTERT* transcript (data not shown). In addition, qPCR analysis showed that high levels of BORIS transcripts were associated with substantial expression of *hTERT* while expression of CTCF was unchanged (Figure 6C).

Expression of BORIS in NHBE cells induced expression of *hTERT* and promoted cell viability

The lifespan of Normal Human Bronchial Epithelial cells (NHBE) in culture is very limited such that after 5 passages, virtually all cells detach and die. NHBE cells were transiently transfected with either a BORIS expression vector or an empty vector and BORIS expression was induced with doxycycline. After days 4, 7 and 10, the cultures were split. Half the cells were returned to culture and the other half was used for RNA extraction and qPCR to quantify *BORIS* and *hTERT* transcripts (Figure 7). Between Days 7 and 10, cells transfected with

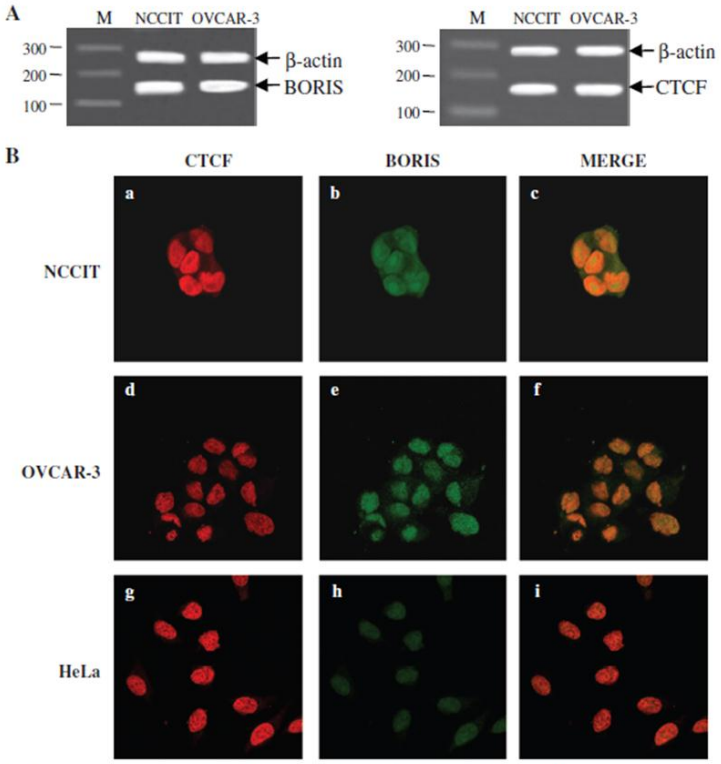

Figure 3. Expression and localization of CTCF and BORIS in NCCIT, OVCAR-3 and HeLa cells. (**A**) Transcriptional expression of CTCF and BORIS were performed by RT–PCR from extracted RNA of NCCIT and OVCAR-3. β-actin was co-amplified as internal control (**B**) Immunofluorescence staining of CTCF and BORIS in NCCIT (a, b, c), OVCAR-3 (d, e, f) and HeLa (g, h, i) cells. Images a, d and g: CTCF immunostaining with a mouse monoclonal CTCF antibody, followed by incubation with an anti-mouse-Cy3 antibody. Images b, e and h: BORIS immunostaining with a rabbit polyclonal BORIS antibody, followed by incubation with an anti-rabbit-FITC antibody. Images c, f and i: merge of BORIS and CTCF staining revealed a coexpression within the nucleus.

empty vector detached and died. At Day 10, cells transfected with the BORIS expression vector exhibited high levels of *BORIS* and *hTERT* transcripts. Lower levels of both *BORIS* and *hTERT* transcripts observed at Day 7 could be explained by plasmid dilution occurring with cell growth. The difference between the levels of *BORIS* transcripts at days 4 and 10 was ~6-fold with 0.625 μg/ml of doxycycline and 55-fold with 1 μg/ml doxycycline. It is interesting to note that a low or a high concentration of doxycycline did not significantly affect the levels of *BORIS* transcripts at Day 4 while the levels of *hTERT* transcripts were almost 2-fold lower with the higher concentration of the drug. These observations lead us to suspect that a high level of *BORIS* transcripts were not necessarily associated with high levels of active BORIS protein. At Day 10, as we considered that plasmids were integrated, the levels of *BORIS* transcripts were 10-fold higher with a high concentration of doxycyline than with a low concentration (Figure 7). The levels of *hTERT* transcripts detected at low concentration of doxycycline did not differ at Days 4 or 10. With a high concentration of doxycycline, however, *hTERT* transcripts were 28-fold higher at Day 10 than at Day 4. Cells kept in continuous culture after day10 were viable for two more weeks, but then they stopped proliferating and finally detached. As we had found that low or high levels of BORIS transcripts affected the levels of *hTERT* transcripts after 10 days, the same experiments were performed using the non-inducible pBORIS-puro vector with similar results; the cells finally stopped proliferating and detached (data not shown).

DISCUSSION

Our studies of *hTERT* regulation in testicular and ovarian tumors showed that the presence of unmethylated CTCF repressor sites in the gene were not inhibitory to transcription raising the question as to how these cancers, despite a normal methylation profile, can express *hTERT*. Here we describe a novel mechanism that disrupts CTCF repression of *hTERT*—occupancy of the CTCF binding sites by BORIS. Since BORIS has the same DNA binding domain as CTCF and is known to be expressed at a high level in testis (37,38), in oocytes (47), as well as in various tumors

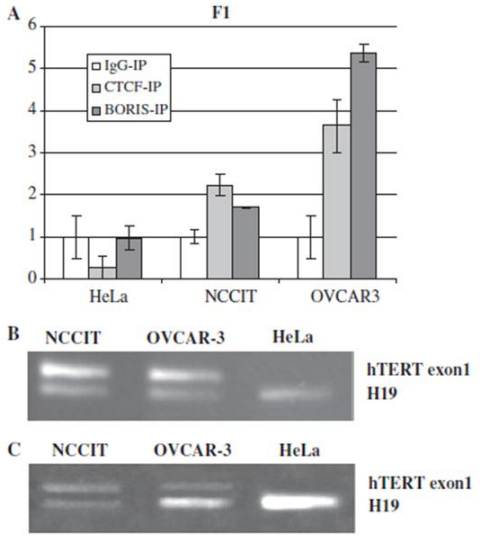

Figure 4. *In vivo* binding of CTCF and BORIS on *hTERT* exon1. Chromatin ImmunoPrecipitation (ChIP) Assays were performed in HeLa, NCCIT and OVCAR-3 cell lines. After immunoprecipitation with serum or specific antibodies against CTCF or BORIS, DNAs were purified and amplified by PCR and quantitative PCR. (**A**) Quantitative PCR were preformed to amplify the exon1 of *hTERT*. Bars represent the mean value of three independent experiments. (**B**) PCR coamplification of the *hTERT* exon1 and H19 from immunoprecipitated fractions bound by CTCF antibody in NCCIT, OVCAR-3 and HeLa cells. (**C**) PCR coamplification of the *hTERT* exon1 and H19 from immunoprecipitated fractions bound by BORIS antibody in NCCIT, OVCAR-3 and HeLa cells.

and cancer cell lines (39–43), we investigated its potential role in the regulation of *hTERT* in testicular and ovarian cancers. Analysis of testicular and ovarian cancer tissues showed, at the exception of one ovarian cancer, the *hTERT* promoter was hypomethylated, that is in correlation with what was observed in a previous study (28). Parallel studies of the same 5′ regulatory region of *hTERT* in OVCAR-3 and NCCIT cell lines showed they were also hypomethylated. A more detailed bisulfite analysis of the promoter and exon 1 regions of *hTERT* in NCCIT and OVCAR-3 cell lines showed they were unmethylated as in normal cells, findings that contrast with the hypermethylated state of these sequences in the HeLa carcinoma cell line, that reflected the *hTERT* methylation profile in most cancer cells (16–18,20).

In transient transfection experiment using HeLa cells, it was shown that the activity of a reporter containing only the minimal *hTERT* promoter was 6-fold higher than the activity of a reporter containing the minimal promoter together with the first exon. In BJ cells, the reporter containing the minimal promoter and the exon 1 of *hTERT* did not exhibit any activity, most likely due to the absence of factor(s) essential for activation of the *hTERT* minimal promoter. In the same experiment, we showed that the activities of reporters containing the minimal promoter and the exon 1 were 2- to 3-fold higher in NCCIT and OVCAR-3 than in HeLa cells. It is noteworthy that these two tumor telomerase-positive cell lines expressed relatively high level of BORIS transcripts. Therefore, the inhibitory effect of *hTERT* exon 1 in reporter assays was less pronounced in BORIS-positive cells than in HeLa or BJ cells. The levels of BORIS expression is correlated with reduced inhibitory effects of the first exon of the *hTERT* gene. Similarly, co-transfection of a BORIS expression and *hTERT* reporter vectors in NIH3T3 cells revealed a reduced inhibitory effect of the first exon on its promoter activity. Indeed, when BORIS was transiently expressed in NIH3T3 cells, the reduction in luciferase activity observed with the *hTERT* exon1 reporter was less pronounced compared to the activity resulting in co-transfection of *hTERT* exon1 reporter with the empty vector. Moreover, this activity was close to that observed in the both BORIS-positive cell lines NCCIT and OVCAR-3. We also showed that transfection of telomerase-negative normal fibroblast BJ cells with a BORIS expression vector was sufficient to induce transcription of the endogenous *hTERT* gene. These results demonstrated that BORIS has the opposite effect of CTCF on *hTERT* transcription. BORIS might counteract the repressive effect of CTCF by binding the target sequences in the *hTERT* gene thereby permitting transcription of the *hTERT* promoter. Moreover, NHBE cells transfected with BORIS exhibited active transcription of *hTERT* and the lifespan of transfected cells was prolonged.

BORIS and CTCF were found to be coexpressed in the nuclei of NCCIT and OVCAR-3 cells and both proteins bound to the first exon of *hTERT in vivo*. Thus in NCCIT and OVCAR-3 cells, CTCF and BORIS might compete for the same binding sites in the *hTERT* gene. We demonstrated *in vitro* that downregulation of BORIS in NCCIT or OVCAR-3 was associated with reduced *hTERT* minimal promoter activity. However, experiments measuring the endogenous levels of *hTERT* after downregulation of BORIS did not lead to the same conclusion. Indeed, the levels of *hTERT* transcripts were not significantly different. This could be explained by the facts that (i) BORIS expression was reduced by only 50% and (ii) downregulation of BORIS was also associated with downregulation of CTCF. Conversely, overexpression of CTCF was associated with increased levels of BORIS transcripts. Taken together, these results demonstrated that BORIS or CTCF expression could influence levels of each other in cancer cell lines. To further examine the effects of reduced BORIS expression on *hTERT* transcription, we performed serial siRNA transfections and observed massive cell death. Similar observations were made in recent studies showing that downregulation of BORIS by siRNA induced apoptosis in a breast cancer cell line (48). These results suggest that disturbances of the equilibrium between CTCF and BORIS in these cells might induce cell death. In order to avoid deregulation of the balance between CTCF and BORIS, we performed transient transfection of *hTERT* luciferase reporters containing or lacking exon1 of *hTERT* with or without mutation of the CTCF binding site. In HeLa cells, where BORIS levels are low, when we expressed *hTERT* with a mutant CTCF target site, we observed an increase

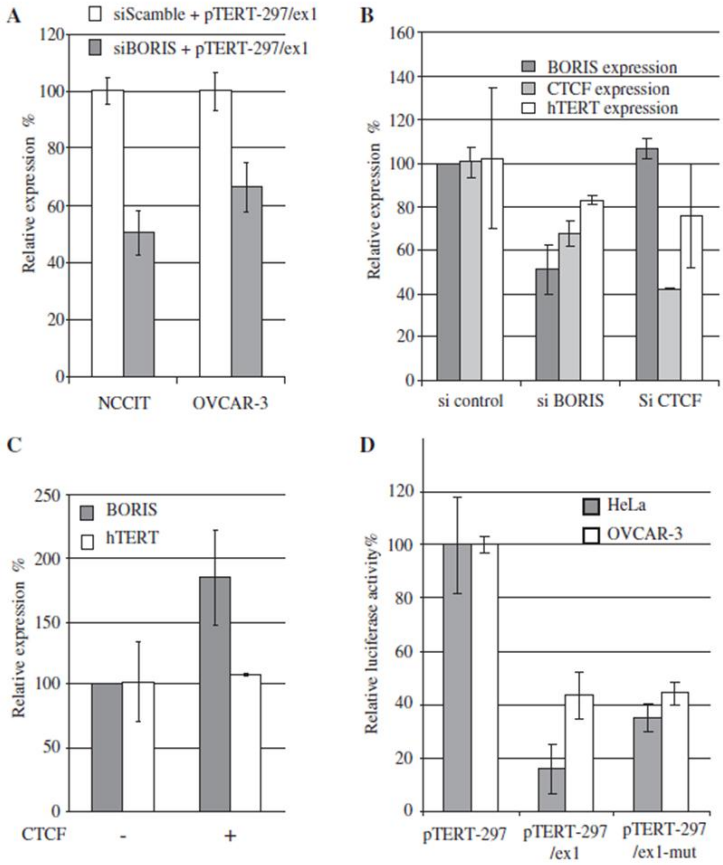

Figure 5. Modulation of BORIS and CTCF expression levels. (**A**) Co-transfection of siRNA scramble or against BORIS with luciferase reporters containing the *hTERT* minimal promoter or the *hTERT* minimal promoter + exon1, in NCCIT and OVCAR-3. Relative luciferase expression is expressed in percentage with the expression of the minimal *hTERT* promoter referred as 100%. (**B**) Transfection of siRNA scramble or against CTCF or BORIS in OVCAR-3. Endogenous transcriptional levels of *BORIS*, *CTCF* and *hTERT* are measured 24 h after transfection by quantitative PCR. Relative expressions were calculated with the expression of each gene after transfection with the scramble siRNA referred as 100% of transcription. (**C**) Transcriptional level of endogenous *BORIS* and *hTERT* in OVCAR-3 after transfection of CTCF expression vector. Relative levels were calculated taking the transcriptional levels of *BORIS* and *TERT* in OVCAR-3 without over-expression of CTCF as 100%. (**D**) Transfection of hTERT reporters in BORIS-negative and BORIS-positive cell lines. Luciferase reporter constructs containing either the minimal promoter of *hTERT*, or the minimal promoter of *hTERT* + exon1, or the minimal promoter of *hTERT* + exon1 with CTCF mutated binding site, were transfected into HeLa and OVCAR-3 cell lines. Relative luciferase activities were calculated taking the level of the minimal promoter as 100%.

in luciferase activity, indicating that CTCF cannot bind this site, its inhibitory effect is diminished. In contrast, parallel studies of the BORIS-positive cell line OVCAR-3 revealed that luciferase activity was the same in cells bearing the normal or mutant CTCF binding site. There are multiple explanations to the observation but most feasible that BORIS isoforms that have different Zinc Finger composition can bind to minimal promoter of *hTERT* (E.Pugacheva *et al.*, submitted elsewhere). The mechanism by which BORIS counteracted the inhibitory effect of CTCF on the *hTERT* transcription resulting in activation of *hTERT* transcription in testicular and ovarian cancers has to be elucidated. However, such mechanism involving CTCF and other proteins counteracting its effect on *hTERT* transcription has already been suggested recently. Indeed, in lymphoid cells, the PAX5 and CTCF factors bound simultaneously the *hTERT* exon 1. In this situation, PAX5 might antagonize the chromatin-mediated transcriptional repression by CTCF on *hTERT* promoter (30).

Since recent studies have shown that *hTERT* is an endogeneous inhibitor of the mitochondrial pathway of apoptosis (49), our data might explain how BORIS acts as inhibitor of apoptosis in cancer cells by activating *hTERT* transcription.

Taken together, these findings show the importance of BORIS for transcriptional activation of *hTERT* and, possibly, to the process of immortalization during

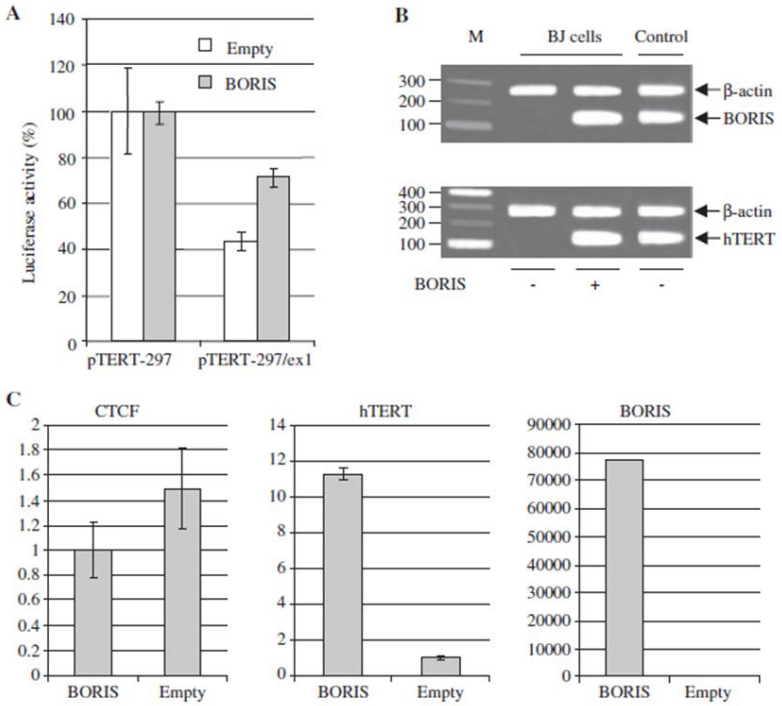

Figure 6. Transient transfection of a BORIS expression vector in normal cells. (**A**) Firefly Luciferase activities of pTERT-297 or pTERT-297/ex1 co-transfected with pBIG-BORIS or pBIG-empty in NIH3T3 mouse normal fibroblasts. The Renilla Luciferase was used to normalize the firefly luciferase. In each experiment, the activity was calculated by setting the activity of *hTERT* minimal promoter as 100%. pTERT-297: minimal *hTERT* promoter; pTERT-297/ex1: minimal promoter and first exon of the *hTERT* gene. (**B**) RT–PCR of *BORIS* and *hTERT* from total RNA of BJ cells 24 h after transfection with the pCMV-BORIS expression vector. (**C**) Real-time RT–PCR of CTCF, hTERT and BORIS from RNA of BJ cells after transfection of pCMV-BORIS or pCMV-empty. All qRT–PCR were normalized on GAPDH levels.

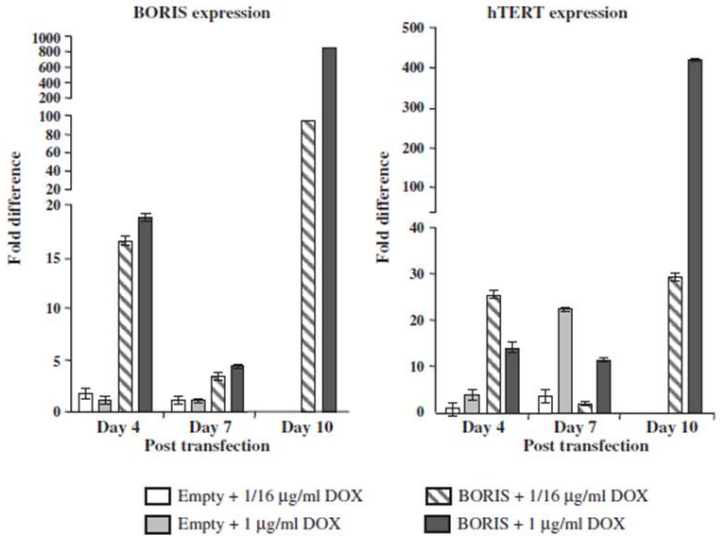

Figure 7. pBIG-BORIS inducible expression vector or the pBIG-empty vector were transfected into NHBE cell line (Normal Human Bronchial Epithelial). BORIS expression was induced with 1/16 µg/ml (0.0625 µg/ml) or 1 µg/ml of doxycycline and maintained in culture. RNAs from cells were extracted 4, 7 and 10 days after transfection. Parental cells and cells transfected with empty vector died after 7 days. The transcriptional expression of *BORIS* and *hTERT* were measured by quantitative RT–PCR and normalized on GAPDH levels.

carcinogenesis. As CTCF binds directly to SIN3A, which recruit histone deacetylase and thus prevents transcription (50), we hypothesize that BORIS might have the opposite effect and open chromatin around the transcriptional start site of *hTERT*. This opening would then allow other factors to activate the *hTERT* transcription. Moreover, recent studies showed that down-regulation of DNMT by anti-cancer drug or TSA, induced a demethylation of *hTERT* exon1 within the CTCF binding site, allowing binding of CTCF and inhibition of *hTERT* (27,51). In addition, downregulation of DNMT also induced an increase in CTCF binding on BAG-1 oncogene promoter, while an overexpression of DNMT led to increase in BORIS binding (52). This opposite behavior is further reflected by changes in H3K4Me2 and H3K9me2 ratio. Taking together these studies showed that promoters regulation by CTCF/BORIS involved epigenetic changes.

In tumors, co-expression of CTCF and BORIS could be directly responsible for epigenetic deregulation leading to cancer (34). The demonstrated utility of BORIS as a target for immunotherapy (53,54) is consistent with the importance of BORIS in carcinogenesis. The importance of BORIS for *hTERT* activation suggests that it might be an essential factor in the immortalization of testicular and ovarian cancer cells, possibly opening new ways to understanding of carcinogenesis and for potential anti-cancer therapies.

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