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Molecular insights for overcoming Hepatocellular Carcinoma

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SUMMARY

Introduction. Hepatocellular Carcinoma (HCC) ranks fifth in frequency of cancers in the world. Orthotopic Liver Transplantation (OLT) or liver resection represents the best treatments for HCC. However, most patients cannot be subjected to potential curative OLT or resection because of extensive tumor involvement of the liver, metastasis, invasion of the portal vein or advanced underlying hepatocellular disease at the time of diagnosis. Systemic chemotherapy or chemoembolization represent a good alternative for the treatment, however drug therapy of cancer in general is hampered by multidrug resistance (MDR) that is a phenomenon caused by the up-regulation of the ABC-transporters (ABC) leading to chemotherapy failure.

To overcome these problems new therapeutic approaches, such gene therapy, are needed. Selective down-regulation of an essential and specific cancer gene such as telomerase (hTERT) could represent an emerging strategy that could prevent cancer progression and diminish numerous side effects derived from drug usage.

The present study include two tasks whose aims are:

Task 1: a) Assess if the extent of tumoral differentiation results in a different ABCB1, ABCC1 and ABCG2 expression.

b) Assess whether the treatment with a chemotherapeutic drug(s) may affect the expression of the three ABC transporters under study.

Task 2: to overcome the obstacle of MDR-induced chemoresistance using new therapeutic approaches such as gene therapy, silencing a cancer essential and specific gene.

Results and discussion. Task 1: We assessed the ABCB1, ABCC1 and ABCG2 expression in three hepatic cell lines: IHH (non tumoral control), HuH7 (differentiated tumoral cells) and JHH6 (undifferentiated tumoral cells). Only ABCG2 expression correlates with the degree of tumoral differentiation.

Through confocal microscopy analysis we observed that the Doxorubicin (Dox) is able to reach the cell's nucleus within 10 min. After 24h and 48h Dox is completely concentrated into the nucleus where some nuclear damage occurs. The presence of damaged nuclei could explain the decreased mRNA in most of the ABCs under study. The treatment with Dox doses lower than the LC_{50} for 24h and 48h has different consequences for all the ABC considered in the three cell lines, with an mRNA expression pattern not in line with the protein one in most of the cases, suggesting that the possible mechanism that determines the ABCs protein

upregulation in the tumoral cell lines (Huh7 and JHH6) is not the *de-novo* transcription but probably something related to the protein turnover.

After the treatment ABCC1 protein expression increases in the tumoral cell lines but not in the non tumoral one (IHH). Regarding ABCB1 and ABCG2, these transporters seem to play a role only in Huh7 and JHH6 cells respectively. We were not able to correlate the tumorigenic potential of the two tumoral cell lines with the ABC expression since the different behaviour of ABCs and the different contribution to MDR. Thus in order to better clarify the contribution of each single ABC to MDR our future steps will consider the use specific inhibitors.

Task 2: From our *in vivo* data, among four cancer related genes we selected hTERT as the best candidate for silencing experiments due to its exclusive expression in tumoral samples. A functional non-inflammatory siRNA targeting hTERT was designed: SirTel 1.

Silencing experiments were conducted in JHH6 cell line. The hTERT silencing effect was dose dependent, at least at the three considered doses (25-50-100nM). For all the subsequent determinations the experimental concentration was 25nM. After 72h of silencing we observed a significant reduction in both hTERT mRNA expression and enzymatic activity ($p<0.001$).

The effects observed in the cells after silencing are:

- morphological changes, from a fibroblast-like to an hepatocyte-like shape;
- increased albumin expression. The expression of this hepatic hallmark increases after silencing in JHH6 cells that, due to their poor degree of differentiation, at basal conditions do not express quantifiable levels of albumin. The peak of the higher albumin expression corresponds to the maximum hTERT silencing effect.
- decreased cell viability ($p<0.01$). Interestingly, the siRNA induced a reduction in cell viability higher than Dox.
- cell cycle arrest in G1 phase ($p<0.01$)

All data were validated using a hTERT negative cell line (primary culture of human fibroblast).

After 72h silencing, we observed that hTERT expression reaches its minimum, and the expression is recovered after 264h although it does not reach the initial expression levels. Re-exposing the cells to additional 25nM of siRNA induces a reduction of mRNA levels by 76% compared to the amount already present after the first treatment.

Taken together all this results suggest the pivotal role of hTERT silencing in a HCC derived cell line. Therefore, hTERT represent a promising candidate for gene-therapy strategies in HCC.

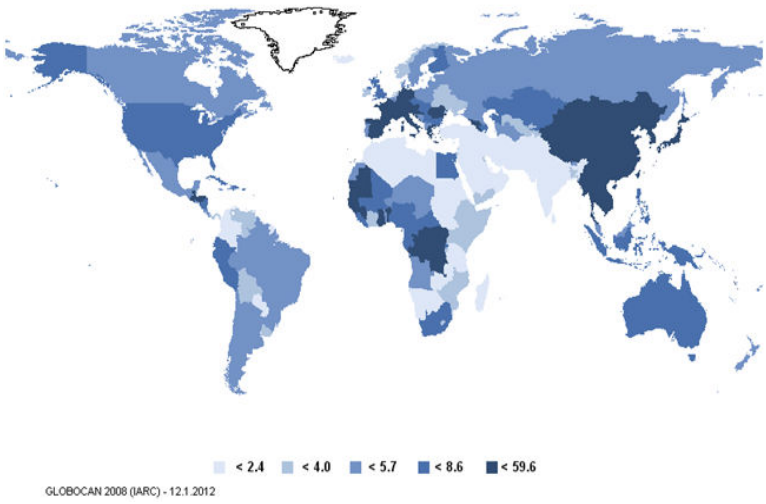
INTRODUCTION

TASK 1. MULTIDRUG RESISTANCE IN HEPATOCELLULAR CARCINOMA

Hepatocellular Carcinoma

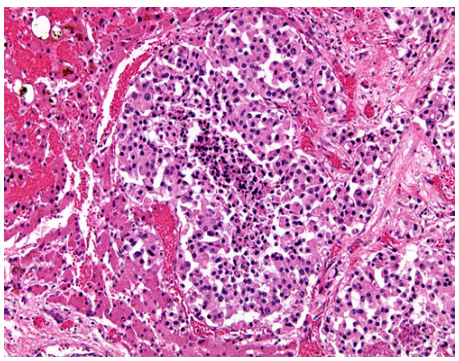
Hepatocellular Carcinoma (HCC) is the third most common cause of cancer death worldwide counting 700,000 death per year. The presence of several relevant risk factors such as HCV and HBV infections, alcoholic cirrhosis and non-alcoholic steato-hepatitis explains the geographic distribution of liver cancer with the majority of cases seen in the developing countries where the HCV and HBV infections are common [1] (Fig.1).

Figure 1. Estimated crude incidence rate per 100,000 habitants, both sexes, all ages.



The HCC presents as nodular, multinodular or with an infiltrative growth pattern. Tumor nodules are round to oval, grey or green (if the tumor produces bile), well circumscribed but not encapsulated. The diffuse type is poorly circumscribed and infiltrates the portal vein, or more rarely the hepatic veins [2] (Fig. 2).

Figure 2. HCC, 10x magnification, hematoxylin-eosin stain.



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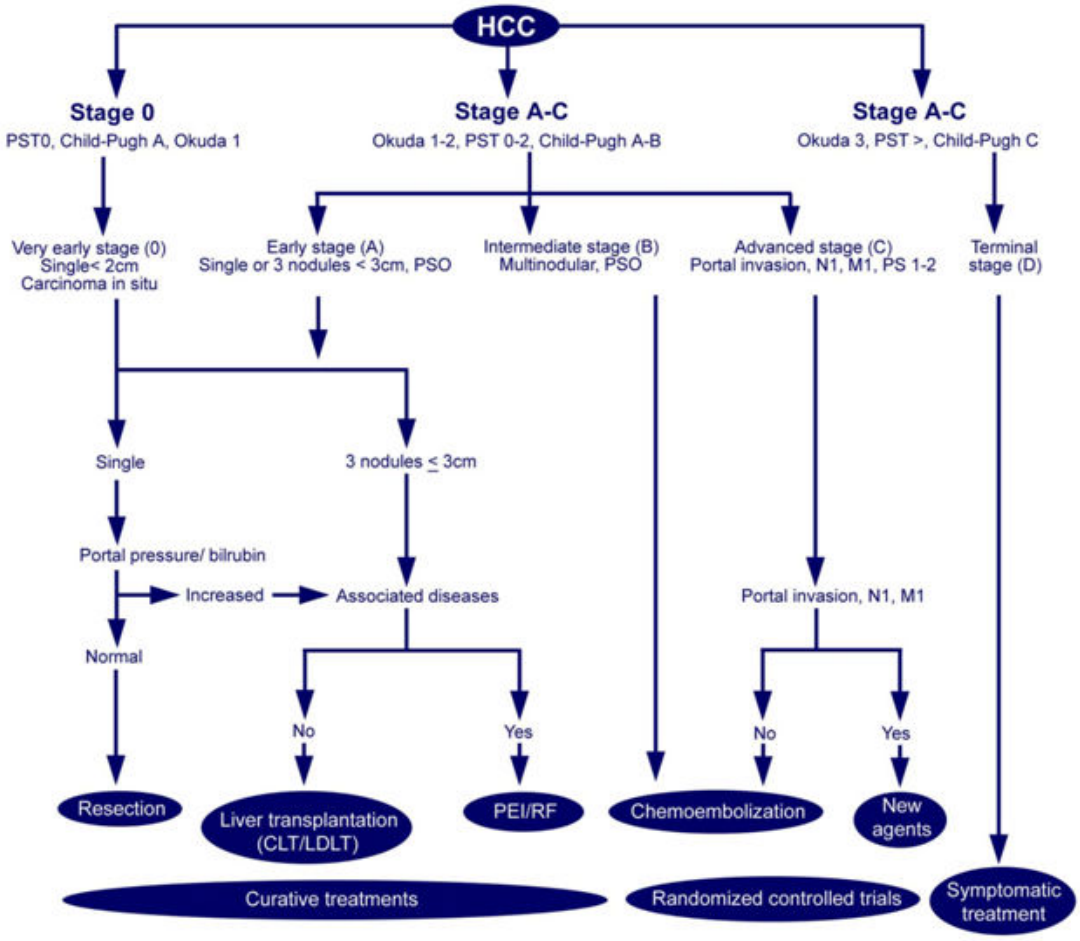
The carcinogenesis remains still unclear although it has been hypothesized that chronic diseases, continuous cell proliferation and direct oncogenic action of viruses/toxins lead genomic instability that enhances the rate of genomic alteration required for cellular transformation (loss of tumor suppressors, de-repression of oncogenes). Despite considerable progress in HCC treatment, the overall prognosis is still not good, since majority of the patients are identified with an advanced

disease, consequently that preventing potentially curative treatments [3]. Surveillance with abdominal ultrasound (US) of patients at risk, is an end-point that is achieved in a minority of patients, especially in the developed world [4]. American Association for the Study of the Liver Diseases (AASLD), European Association for the Study of the Liver (EASL) and Asia Pacific Association for the Study of the Liver (APASL) share common guidelines for semestral surveillance with abdominal US of all patients at risk [4-6], as the growth rate of the tumor takes 6 months to double its volume, on average [4]. The co-existence of multiple diseases in the HCC have substantial influence on the choice of therapy and survival. The guide lines for the treatment of HCC are provided by the Barcelona Clinic Liver Cancer (BCLC) staging system that suggests curative treatments such as resection or Orthopic Liver Transplantation (OLT) for the lower stages. Drug based palliative treatments are recommended for the intermediated stages while for the higher grade tumours a symptomatic treatment represent the only option available [7] (Fig. 3).

Figure 3. Barcelona Clinic Liver Cancer (BCLC) Staging and Treatment Strategy scheme.



Late diagnosis, stage, severity of the underlying liver disease and the lack of liver donors are responsible for the poor outcome of the HCC. Liver Resection (LR) is still the treatment of choice for early-stage HCC with well-preserved liver function; surgery provides good long-term survival but can be applied in only to 20–30% of patients with HCC on cirrhosis [8]. Several cohort studies comparing LR and loco-regional ablation treatment (LAT) for patients affected by HCC on cirrhosis have been published in literature, however the results of these studies are often conflicting and are affected by the heterogeneity of selection and patient management [9,10]. Moreover, two recent randomized trials failed to clarify the role of LAT and LR; the first [11] of the two studies showed that survival rates in patients with early HCC (single, ≤5 cm) were similar after LAT and LR, and the second [12] demonstrated the superiority of LR also in small HCC (single, ≤3 cm).

Same observational studies [12] have found that in small HCC (4 or 5 cm), survival and disease-free survival are comparable between surgery and LAT, other recently published RCT comparing 115 patients within Milan criteria showed the superiority of LR in both survival and disease-free survival; these results were confirmed also in single and small HCCs [11].

HCC and chemoresistance: The role of ABC transporters

Although LR or OLT represent the eligible choice for HCC treatment, most patients cannot be subjected to these potential curative therapies because of extensive tumor involvement of the liver, metastasis, invasion of the portal vein or advanced underlying hepatocellular disease at the time of diagnosis. Systemic chemotherapy or chemoembolization represent a valuable alternative for the treatment, however drug therapy of cancer in general is hampered by multidrug resistance (MDR) [13-15]. MDR is the phenomenon in which cancer cells exposed to one anticancer drug show resistance to various antitumoral agents that are structurally and functionally different from the initial one.

MDR is a multifactorial process since up to now no single mechanism has been identified accounting for resistance to the entire spectrum of anticancer drugs commonly used, however after the identification of the first ATP binding cassette (ABC) protein [16], was demonstrated that a single protein could confer resistance to a wide range of chemical compounds [17].

Mechanisms involved in MDR are activation of the drug efflux systems, phase I and II enzymes, alterations of the genes and the proteins involved into the control of apoptosis, absorption, metabolism and delivery, DNA methylation.

Soon after the introduction of chemotherapy in 1950s it was observed that cancer cells could became resistant to cytotoxic drugs [18]. During the next thirty years the primary role of ABC transporters in MDR was established [16,19,20] and during this period became evident the association between ABC overexpression and HCC resistance in animal models [21,22].

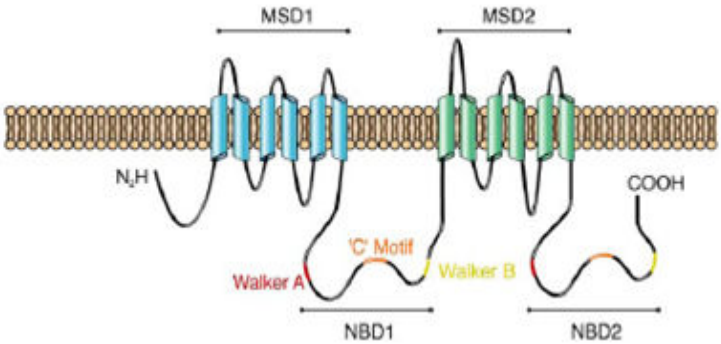
ABC transporters

ABC transporters are large membrane-bound proteins that use energy to drive the transport of various molecules across the plasma membrane as well as intracellular membranes of the ER, peroxisome and mitochondria [23,24]. They are present in practically all living organisms from prokaryotes to mammals [24]. ABC transporters are expressed basically in all tissues, with differential subcellular localization; in polarized cells they can be expressed in apical or basolateral membranes [25-27].

The ABC family comprehend 49 genes wich are widely dispersed in the genome. Based on similarity in gene structure in eukaryotes ABCs can be divided into seven subfamilies named from A to G in where every member is numbered consequently [25,28].

In humans, the three major types of multidrug resistance (MDR) proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MRP1, ABCC2/MRP2, probably also ABCC3-6, and ABCC10-11), and ABCG (ABCG2/MXR/BCRP) subfamily [27].

Figure 4. Example topology of an ABCC transporter.



The figure illustrates a probable topology of a single chain encoded ABC. In blue and green are evidenced the two transmembrane domains (MSD). In red and yellow the Walker A and B domains respectively. In orange the "C" motif.

Functional ABC transporters contain two membrane-spanning domains (MSDs) (from 5 to 10 elices, tipically 6) and two nucleotide binding domains (NBDs) [24]. They can be encoded in a single polypeptide in a order NH2-MSD-NBD-MSD-NBD-COOH (Fig.4) or can be homo- or heterodimer following the order NH2-MSD-NBD-COOH that sometimes could be reversed as in ABCG2 [29]. The ABC unit harbours several conserved sequence motifs: the Walker A (P-loop), a glycine-rich sequence; the Walker B motif; both

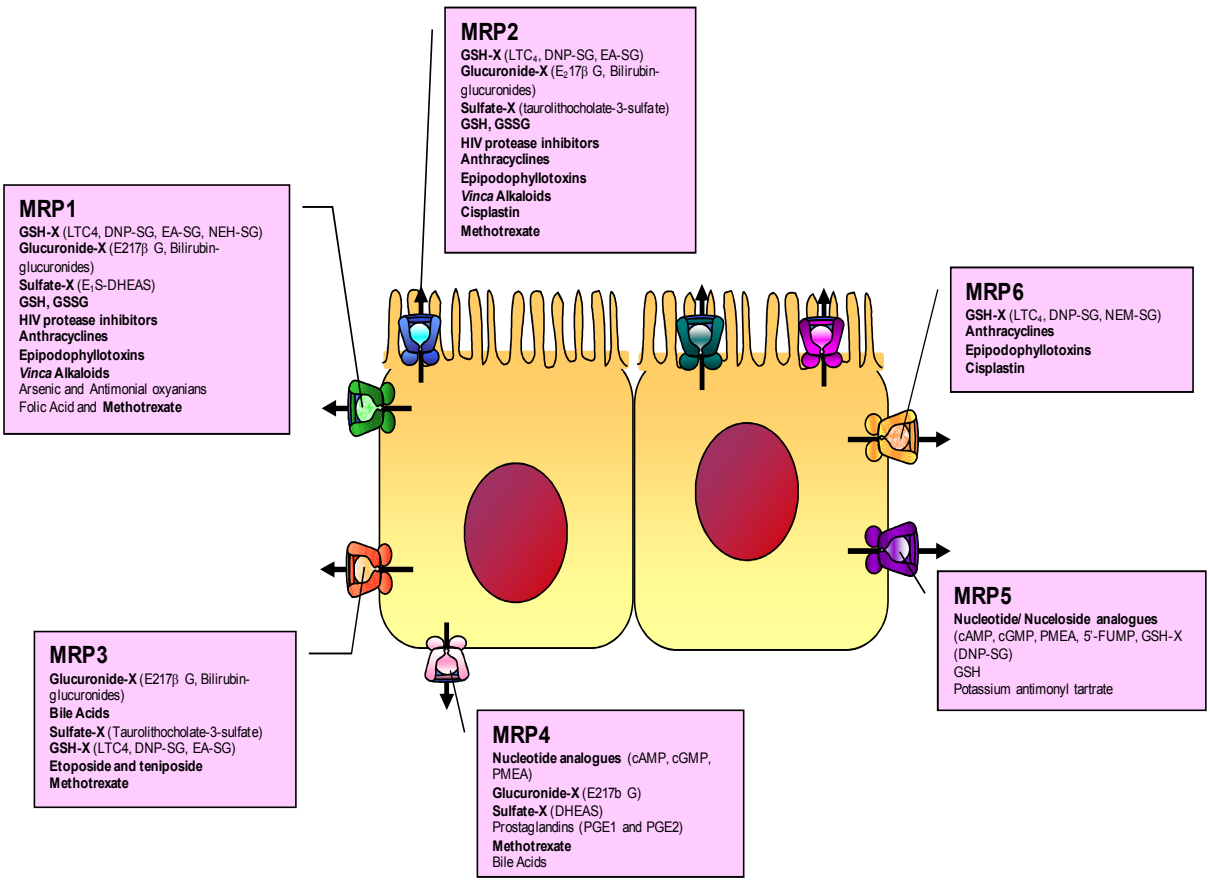
involved in ATP binding and hydrolysis [30]; and a conserved glutamine (Q-loop) also known as C signature or C motif, which is characteristic of ABC ATPases and the has the core motif LSGGQ [31].

ABC pumps are mostly unidirectional, in bacteria they principally import essential compounds into the cell, in eukaryotes they move compounds from cytoplasm to the extracellular compartment or into cellular organelles: ER, mitochondria, peroxisome.

This transporters use ATP hydrolysis derived energy to move the substrate.

The transport across the membrane involve a cyclic process which starts with the transporter in a "open" state with two ATP molecules loosely bound to the NBDs. The substrate binding to a high affinity site(s) induces conformational changes that enhance the ATP binding to NBD1. The initial binding of ATP by NBD1 stabilizes the interaction between NBDs by establishing contacts with the C signature of NBD2, facilitating the binding of a second

Figure 5: Subcellular localization and substrate specificity of some ABC transporters.



The cartoon shows two polarized cells. The subcellular location(s) of each protein on the apical (upper) or basolateral membranes is shown. Some of the major classes of substrates for each protein are indicated, as well as specific examples of substrates chosen to illustrate the overlap in substrate profiles among the ABCs.

molecule of ATP. The ATP binding induces a tighter interaction between the NBDs which transfer the movement to the MSDs resulting in a decrease in the substrate affinity [27,32]. At this stage only one ATP is tightly bound and hydrolyzed, Senior and Coll. [33] suggest that the binding of one ATP molecule at NBD1 promotes the hydrolysis of the ATP molecule at NBD2. The hydrolyzation is a multistep process which ends with a phosphate release [33]. This step can be blocked by phosphate-mimicking molecules, such as vanadate, that stabilize the complex ADP:V_i:protein [33]. After the ADP release the protein is ready for another cycle with the ATP hydrolysis occurring in the other NBD since the NBDs are functionally equivalent. The fact that the NBDs can be exchanged without loss of function provides strong support for this cycling model [34]. Although the ATPase activity is required for transport and substrate increase the rate of ATP hydrolysis, it is not know which steps are associated with binding, transport and release of substrate [35]. In the case ABCB1, considerable evidence exists to support a model in which hydrolysis of ATP at either NBS results in transport of one molecule of substrate [33]. A more recent variation of this model proposes that the binding and hydrolysis of one ATP molecule drives a “power stroke” in which the protein shifts from a high- to low-affinity substrate binding state with the concomitant transport and release of one molecule of substrate [35]. Hydrolysis of a second ATP is then required to reset the protein in a high-affinity state for the next transport cycle.

In contrast with these studies some researchers sustain that it is ATP binding rather than hydrolysis that converts the protein from a high- to low-affinity substrate binding state [36,37].

Mutational studies have also identified individual amino acids that are important for the transport of a range of diverse substrates [38,39]. Substrates establish multiple, often but not always, overlapping interactions with amino acid residues that collectively form a relatively large binding pocket, as a consequence a single amino acid mutation can alter transport of some substrates and not others [40-43]. In ABCCs TM11 and TM17 and in ABCB1 TM6 and TM12 play major roles in determining its substrate specificity. Moreover mutational studies of TM17 in ABCC1 ABCC2 and ABCC3 have revealed multiple polar and/or aromatic residues and basic residues that have pronounced effects on substrate specificity, with respect to various classes of natural product drugs and conjugated organic anions, such as E217_G and LTC4 (Leukotriene C4), as well as folic acid analogs such as methotrexate and leucovorin [44-48].

One of the most striking examples of a major alteration in substrate specificity resulting from single amino acid variation came from the functional characterization of mammalian ABCC1

orthologs. ABCC1 is relatively highly conserved among mammals, and the human protein exhibits 88, 86, 92, and 98% sequence identity with the mouse, rat, dog, and macaque proteins, respectively [49-52]. However, with the exception of macaque ABCC1, the other orthologs fail to confer resistance to anthracyclines and are poor transporters of E217_G [49-51,53]. The lack of anthracycline resistance has been traced to the presence of a Gln rather than Glu residue in TM14 (Glu1086 in human ABCC1), while the poor E217_G transport seems attributable in large part to the presence of Ala rather than Thr in TM17 (Thr1242 in human ABCC1) [54,55].

Finally should be noted that several residues have been identified that, rather than being important for the activity or substrate specificity of some ABC, such as ABCC1 for example, play a critical role in the stable expression of the transporter in mammalian cell plasma membranes [42,43,56].

ABC proteins have a relevant role in the transport of both endo- and xenobiotics [25].

Each ABC has a broad overlapping substrate spectrum which encompasses GST-, glucuronide- and sulphate- conjugates [57-59], nucleotide or nucleoside analogous such as cAMP, cGMP, 5'-fluorouracil [60], GSH, GSSG [61], bile salts [62], steroids, prostaglandins and drugs, such as cisplatin and anthracyclines [25] (Fig. 5).

In addition of the broad substrate specificity there are growing number of examples of compounds that bind to ABCs without being transported and many of these act as competitors. Of interest would be interesting could be the role of dietary flavonoids such as genistein or quercitin, as well as synthetic flavonoids, such as flavopiridol in the inhibition of ABCC1 and ABCC2; that could influence the drug ADME-tox (absorption, distribution, metabolism, excretion and toxicity) during therapies [63-65].

In addition there has been considerable interest in developing novel compounds that may prevent or reverse clinical MDR [66] such as the quinolone derivative MS-209, ABCB1 and ABCC1 inhibitor [67]; the pipecolinate derivative VX-710 (biricodar), ABCB1, ABCC1 and ABCG2 inhibitor [68]; and pyrrolpyrimidine analogs, ABCC1 specifics inhibitors [69].

Most of the trials of ABC reversing agents have had disappointing results [70-74], the explanation is that each ABC has a broad spectrum of substrate with an overlapping specificity as a consequence the role of a inhibited ABC can be supplied by an alternative transporter and this leads to a difficult interpretation of patients' outcome. Furthermore, earlier ABCB1 reversing agents were of relatively low specificity and affinity and in some cases were found to have significant pharmacokinetic effects that required reduction in dosing of the chemotherapeutic agent(s) used. The second generation of ABCB1 reversing agents,

such as PSC833 (a nonimmunosuppressive derivative of cyclosporine), showed a role also in hepatic ABCC2, ABCB11, a bile salt transporter, and CYP3A modulation that could influence pharmacokinetics [74,75]. More recently, high-affinity highly specific, ABCB1 specific reversing agents have been developed. One of these, zosuquidar (LY335979), has shown minimal pharmacokinetic effects, combined with confirmed inhibition of ABCB1 in recent phase I trials involving solid and hematological malignancies [76-78]. At present the outcomes of phase II trials of zosuquidar are not so promising. A randomized, placebo-controlled, double-blind phase II study on metastatic breast cancer patient revealed that there was no difference in progression-free survival, overall survival, or response in patients treated with anticancer drug plus placebo and anticancer drug plus zosuquidar 3HCl (DZ) [79].

Dozens trials have been performed in the last twenty years and among these very few showed an increased overall survival in patients [26] and this is the reason why ABCs still remain a open field of investigation.

Alternative approaches to target MDR come from peptides analogues, antibodies, efflux evading drugs, gene downregulation [26].

ABCB1 mediated drug resistance can be reversed by hydrophobic peptides that are high-affinity ABCB1 substrates. Such peptides, showing high specificity to ABCB1, could represent a new class of compounds for consideration as potential chemosensitizers [80]. Peptide analogues of TMDs are believed to interfere with the proper assembly or function of the target protein and they can be specific and potent ABCs inhibitors as demonstrated for ABCB1 [81]. Studies suggest that immunization could be an alternative supplement to chemotherapy. A mouse monoclonal antibody directed against extracellular epitopes of ABCB1 was shown to inhibit the *in vitro* efflux of drug substrates [82]. Similarly, immunization of mice with external sequences of the murine gene *abcb1* elicited antibodies capable of reverting the MDR phenotype *in vitro* and *in vivo*, without eliciting an autoimmune response [83].

The epothilones represent a novel class of anticancer therapy that stabilizes microtubules, causing cell death and tumor regression in preclinical models. They are not recognized by ABCs, providing proof of the concept that new classes of anticancer agents that do not interact with the multidrug transporters can be developed to improve response to therapy [84].

Selective downregulation of resistance genes in cancer cells is an emerging approach in therapeutics. Using peptide combinatorial libraries, Bartsevich and Coll. [85] designed transcriptional repressors that selectively bind to the ABCB1 resulting in a selective reduction in protein levels and a marked increase in chemosensitivity in highly drug-resistant cancer

[85,86]. Similarly, interference technologies could be a promising new strategy that is not only highly specific but also could prevent ABCs expression during disease progression. However, at present antisense oligonucleotides has produced mixed results; in certain cases sufficient downregulation of ABCs has proved difficult to attain and in others the safe delivery of constructs to cancer cells *in vivo* remains a challenge [87,88].

ABCs mainly involved in MDR: ABCB1, ABCC1 and ABCG2

Fulfilling their role in detoxification, several ABC transporters have been found to be overexpressed in cancer cell lines. In humans, the three major types of MDR proteins include members of the ABCB (ABCB1/MDR1/P-glycoprotein), the ABCC (ABCC1/MRP1, ABCC2/MRP2, probably also ABCC3–6, and ABCC10–11), and the ABCG (ABCG2/MXR/ BCRP) subfamily [27].

ABCB1, also known as MDR1 or P-gp (P-glycoprotein), was the first ABC transporter discovered, cloned and characterized through its ability to confer a multidrug resistance phenotype to cancer cells that had developed resistance to chemotherapy drugs [16,89-91].
ABCB1 has a four-domain structure, as is typical of most eukaryotic ABC transporters, with two NBDs each preceded by a MSD composed of six transmembrane helices (MSD-NBD-MSD- NBD) [65].
ABCB1 has been demonstrated to be a promiscuous transporter of hydrophobic substrates including drugs such vinca alkaloids, anthracyclines, epipodophyllotoxins and taxanes [92] as well as lipids, steroids, xenobiotics, and peptides [93]. ABCB1 is thought to play an important role in removing toxic metabolites from cells but is also expressed in cells at the blood–brain barrier, where presumably plays a role in transporting compounds into the brain that cannot be delivered by diffusion and in adrenal gland where it is involved in steroid hormones excretion [27].
ABCB1 is expressed in many cell types such as brain, including choroid plexus, astrocytes, microglia, and capillary endothelium where the protein prevents the passage of drugs and toxins into the brain [94,95]. It is also expressed in apical surface of proximal tubule cells of the kidney, in luminal membranes of cells of the gastrointestinal tract, in the canalicular membranes of hepatocytes liver. Lower levels are expressed in the placenta, the adrenal cortex, and CD34+ hematopoetic stem cells [96,97].

The expression in the apical membranes of the epithelial cells have an important role in regulating drug distribution since ABCB1 influences drug distribution in three ways: it limits drug absorption in the gastrointestinal tract; it promotes drug elimination in the liver, kidney, and intestine; and it regulates drug uptake. The orally administration of drugs in *abcb1* knockout mice lead to a 50-100 fold increase in drug accumulation in tissues especially in brain where ABCB1 plays a predominant role in toxins defence [98,99].

Since its role in detoxification, in tumours ABCB1 become overexpressed and there are evidences linking the protein expression with a poor clinical outcome with a reduction in response to chemotherapy in breast cancer, sarcoma and certain types of leukaemia [100,101]. In HCC ABCB1 overexpression has been reported to be associated with shorter overall survival [102,103], interestingly Ng and Coll. [104] found this association only in patients previously treated with chemotherapy. The high-level expression ABCB1 in tumours is either due to gene amplification or to elevated level of transcription [105].

Overexpression of this transporter raised the possibility that oncogenes or tumor suppressor genes may regulate constitutive *ABCB1* expression. The proteins p63 and/or p73 in certain types of tumors play a complex role in the regulation of *ABCB1*, which may depend on the cellular environment, the cytotoxic drug used during selection or treatment, and mutations in p53 [106-109].

Many ABCB1 inhibitors were discovered (Verapamil, Tariquidar, Disulfiram and others) and despite promising *in vitro* results, using several resistance cell models [26,110], successful modulation of clinical MDR through the chemical blockage of drug efflux from cancer cells remains elusive [26,65].

ABCC1 was first member of ABCCs subfamily being cloned 1992 from drug-selected human lung cancer cell line H69AR [111,112]. Initially it was identified as multidrug resistance-associated protein (MRP) and subsequently multidrug resistance protein 1 (MRP1). ABCC1 do not respect the typical structure of an ABC (Fig. 4) since it is composed of five domains with an extra NH2-proximal MSD which has five TM segments and an extracytosolic NH2-terminus (MSD–MSD–NBD–MSD–NBD) [111,113,114]. The ABCC1 protein is thought to play both a role in protecting cells from chemical toxicity and oxidative stress and to participate in inflammatory due its active role in the transport of leukotrienes such as leukotriene C4 (LTC4) [115]. Despite structural differences there is considerable overlap with others ABCs in the spectrum of drugs to which ABCC1 confer resistance.

The ABCC1 pump confers resistance to doxorubicin, daunorubicin, vincristine, colchicines, and several other compounds, very similar profile to that of ABCB1 [116]. However, unlike ABCB1, ABCC1 transports drugs that are conjugated to glutathione by the glutathione reductase pathway [57,115,117,118].

Several ABCC1 inhibitors or reversing agents were developed such as Verapamil, PSC-833, Laniquidar, Disulfiram, all of this agents failed the clinical trial test since they do not ameliorate patients' outcome or worse, they owed secondary toxicity [26].

ABCC1 is expressed in most tissues throughout the body with relatively high levels found in the lung, testis, kidneys, skeletal muscle and peripheral blood mononuclear cells, while less amount is found in liver [52,111,119]. In most tissues ABCC1 is localized to the basolateral cellular surface, which in certain tissues results in the efflux of its substrates into the blood.

High levels of ABCC1 expression has been found in non-small cell lung cancer (NSCLC) and have been correlated with a higher grade of differentiation of NSCLC, particularly in adenocarcinoma [120-122]. Despite the higher grade of differentiation that might be expected to have a better prognosis, the higher expression of ABCC1 is a negative indicator of response to chemotherapy and overall survival for these kind of tumours [123-125].

Several independent studies indicate that ABCC1 expression is a negative prognostic marker for some types of breast cancers associated with shorter times to relapse and reduced overall survival [120,126,127]. In prostate cancer ABCC1 expression levels have been reported to increase with cancer stage and invasiveness [128] and to be positively associated with mutant p53 status of the tumor which is reported to be a suppressor of *MRP1/Mrp1* transcription [128-130].

ABCC1 expression is reported to increase in severe human liver disease [131] and in hepatocellular carcinoma where it is associated with a more aggressive tumour phenotype [132,133]. Despite the increased ABCC1 expression in liver malignancies some studies reported no statistically significant difference in ABCC1 expression levels between the neoplastic and perineoplastic tissue [132]. Moreover Nies and Coll. evidenced no role for ABCC1 in MDR phenotype in HCC [134].

ABCG2 (MXR/BCRP/ABCP) is a so-called half-transporter consisting of a single hydrophobic MSD predicted to contain 6 TM helices preceded by a single NBD (NBD-MSD) [29], it is an atypical ABCG subgroup member since it has a large extracellular loop between TM5 and TM6. It was cloned independently from two drug selected cell lines and a human cDNA library and was given three different names. In the first study it was isolated from a

multidrug-resistant breast cancer cell line co-selected in doxorubicin and verapamil (a ABCB1 inhibitor) in an effort to elucidate non-ABCB1 mechanisms of drug resistance [29,135]. Although the first name suggested from this study was Brest Cancer Resistance Protein (BCRP), there is no evidence at present that this transporter is preferentially expressed in normal or malignant breast tissue and its clinical relevance is not yet well established.
Subsequently it was isolated from a mitoxandrone resistant cell lines and named Mitoxantrone Resistance Protein (MXR) [136] and last from a human cDNA library from placenta (ABCP) [137].
As the others ABC transporters ABCG2 is widely expressed around the body. In lung it appears low but detectable, and is found in the epithelial layer and seromucinous glands [138]. It is expressed at the apical surface of the epithelial cells throughout the small intestine and colon preventing and/or modulating the passage of certain xenobiotics or their metabolites from the gut into the circulation [139-141]. It is highly expressed at the luminal surface of brain capillaries [142,143] where it as a relevant role in the transport since both its mRNA and protein expression increases in ABCB1 knockout mice versus wild-type mice suggesting a compensatory up-regulation the absence of ABCB1 [142]. ABCG2 is highly expressed in the trophoblast cells of the placenta [144]. This suggests that the pump is responsible either for transporting compounds into the foetal blood supply or removing toxic metabolites [145]. It is also highly expressed in liver, where it localizes to the apical regions of canalicular cells and various stem cells.
The high ABCG2 expression levels in “barrier” tissues indicate a key role in the protection of the body from xenobiotics, especially in the gastrointestinal track. Indeed ABCG2 transports a wide variety of anticancer agents, their partially detoxified metabolites, toxins, and carcinogens found in food products, as well as endogenous compounds [27,146].
As for ABCB1 the importance in protecting tissues become evident from knockout experiments where a*bcg2* (-/-) mice have elevated plasma levels and decreased intestinal, fecal, and hepatobiliary excretion of the food carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) [147] and an increased intestinal absorption and decreased biliary secretion of pheophorbide, a toxic compound derived from ingested food, expecially plant-derived nutrients or food supplements [148].
Fulfilling its role in detoxification ABCG2 have been found overexpressed in many cancer cell lines and human tumours especially in adenocarcinomas of the digestive tract, lung, and endometrium [149]. In retrospective studies the chemotherapy response rate in patients was found to be correlated with ABCG2 expression [150,151]. Regarding HCC at present, no

clear results associate ABCG2 expression with clinical outcome although some studies evidenced the up-regulation of both ABCG2 mRNA and protein in HCC [152].

In tumours ABCG2 confers resistance to a narrower range of anticancer agents than ABCB1 and ABCC1. Nevertheless, the spectrum includes anthracyclines, mitoxantrone, and topoisomerase I inhibitors such as camptothecin. On the other hand, ABCG2 does not confer resistance to the vinca alkaloids, epipodophyllotoxins, paclitaxel, or cisplatin [153].

Recently was evidenced that ABCG2 is also able to alter absorption, metabolism and toxicity of Tyrosine Kinase Inhibitors (TKIs) such as Imatinib (STI-571) and Iressa (ZD 1839) [154].

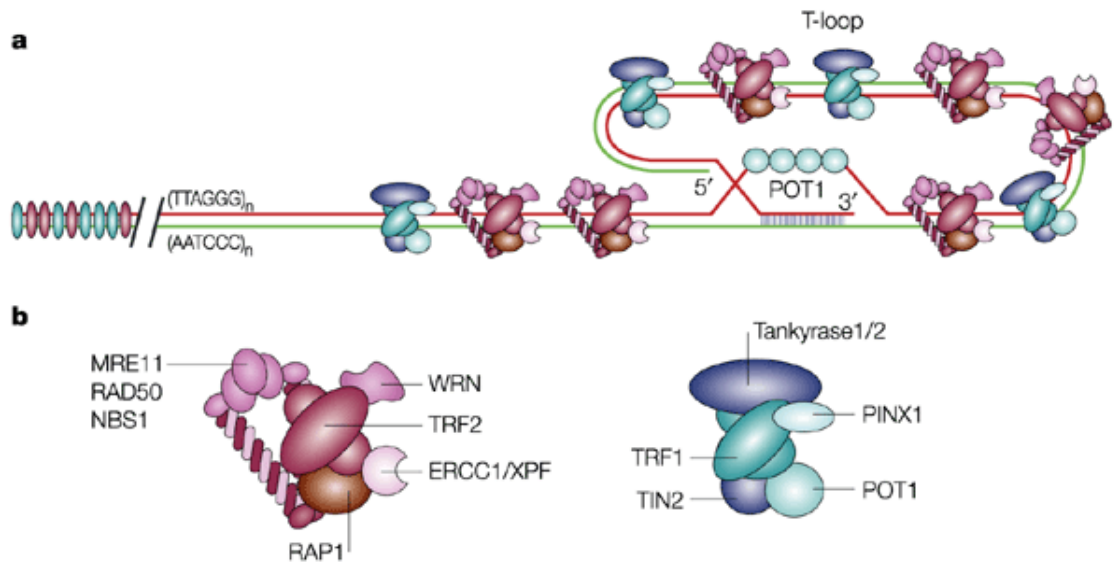
TASK 2. TELOMERASE SILENCING EFFECTS IN HCC

Telomeres and cellular senescence

The ends of linear eukaryotic chromosomes contain specialized structures called telomeres [155]. The telomeres consist of DNA-protein complexes, termed helterin complexes [156], that protect chromosome ends from end-to-end fusion and degradation. Telomeric DNA typically ends in a 3' single-strand G-rich overhang of 50-300 nucleotides, which has been proposed to fold back onto duplex telomeric DNA forming a "T-loop" structure [157,158] and avoiding the linear ends of chromosome from being recognised as single and/or double-strand DNA breaks (Fig. 6).

Telomere length varies among chromosomes and among species [159,160]. In human generally telomeric DNA consists of about 15–20kbp tandemly repeated G-rich sequences (TTAGGG) that form a molecular scaffold containing many binding sites for telomeric proteins, including TTAGGG repeat binding factor 1 (TRF1) and 2 (TRF2) (Fig. 6) [161]. TRF1 seems to regulate telomere length by inhibiting telomere elongation once telomeres reach a critical size [162]. TRF2, in contrast, suppresses end-to-end fusions chromosomes and serves to stabilize chromosome ends [163].

Figure 6. Telomeres and t-loop organization.



The figure represents human telomeres t-loop organization **(a)** with the main protein complexes involved in stabilization **(b)**. Taken from De Lange *et al*. (2004) [160].

The DNA-protein complexes are extremely dynamic especially during interphase when the telomere-bound proteins are rapidly exchanged on and off [164].

Telomeres are subjected to progressive ends shortening at every cell cycle due to the inability of DNA polymerase to replicate the chromosome ends during lagging strand synthesis ("end replication problem"), oxidative damage and other processing events [165-167]. Cells that lack a compensatory mechanism to counteract this gradual loss exhibit a growth arrest state, called replicative senescence [168], that is thought to occur when one or more critically short telomeres trigger a p53 (and perhaps RB) -regulated DNA damage response [169,170]. Human cells can temporarily bypass this growth arrest when RB and p53 are disabled [171,172], but ultimately so many telomeres become critically shortened that multiple chromosome end fusions occur, resulting in loss of cell viability in a process termed "crisis".

Cellular senescence was discovered in the early 1960s, Leonard Hayflick observed that human cells placed in tissue culture stop dividing after a limited number of cell divisions by a process now known as replicative senescence [173]. Actively growing cells, such as embryonic stem cells, stem cells, lymphocites, some epithelials, and cancer cells posses several mechanism counteracting progressive telomere shortening and senescence.

By virtue of its ability to repair telomeric DNA, the telomerase, a reverse transcriptase, plays a key role in preserving chromosomal stability and genetic integrity in eukaryotes leading to an anti-aging effect [174-178]. However telomerase dependent telomeres maintenance is not the only anti-senescence mechanism known.

Some eukaryotic species have apparently completely lost the telomerase-mediated mode of telomeric DNA maintenance during evolution. In these organisms, the telomeric DNA is composed of other types of sequences, which provide exceptions to the usual type of canonical telomeric repeats. For example in the fruit fly *Drosophila melanogaster*, telomeres are primarily composed of a complex mosaic of large, non-LTR-type retrotransposons called HeT-A and TART elements [179]. Sporadically, one of these retrotrasposons is added onto the termini of chromosomes by a variant retrotransposition mechanism, counteracting over time the gradual sequence loss from chromosome ends.

Yeasts can use a telomerase-independent Rad52-mediated DNA recombination mechanism to maintain telomeres stability [180-183], and a small percentage of tumours and immortalized human cell lines can utilize an apparently similar mechanism known as "alternative lengthening of telomeres" ALT [184], however these cells are less tumorigenic in mouse xenografts and they have weak metastatic potential [185].

The telomerase reverse transcriptase

Telomerase was discovered by Carol W. Greider and Elizabeth Blackburn in 1984 as novel telomere terminal transferase involved in the addition of telomeric repeats necessary for the replication of chromosome ends in the ciliate *Tetrahymena* [210].

Table 1. Protein interactors of the telomerase holoenzyme components.

hTR	hTERT	RNP
hTEP [186]	PKCα [196]	hEst1A [208]
Dyskerin [187]	p23 [197]	hnRNP A1 [209]
hStau [188]	Hsp90 [197]	
L22 [188]	p53 [198]	
hGar1 [189]	c-Abl [199]	
hNHP2 [190]	PinX1 [200]	
hNOP10 [190]	SMN [201]	
hnRNP C1 [191]	Ku70/80	
hnRNP C2 [191]	[193,202]	
La [192]	CRM1 [203]	
Ku70/80 [193]	Ran [203]	
SmB [194]	KIP [204]	
SmD3 [194]	Nucleolin [205]	
hNaf1 [195]	MKRN1 [206]	
	hPif1 [207]	

Subsequently telomerase was identified as a 650 to 670 kDa ribonucleoprotein (RNP) complex composed of hTERT (127kD), a catalytic subunit, dyskerin (57kD), a putative pseudouridine synthase [211], and a 451 nucleotide RNA (hTR or hTERC) (153kD). The ribonucleoprotein dyskerin, also known as NOLA4 (nucleolar protein family A, member 4), encoded by the *DKC1* gene on the X chromosome, is a putative pseudouridine synthase within the class of H/ACA (Hinge-hairpin-ACA) box ribonucleoproteins [212]. It is required for proper folding and stability of telomerase RNA [213].

The RNA molecule carries the template for the addition of 6 base repeats (TTAGGG)n to the 3'end of telomeres that became shorter due to incomplete extremities replication at every cell cycle or due to oxidative damage [214-217]. Other proteins are also associated with the complex such as GAR1, NHP2, NOP10 (also known as NOLA1, NOLA2 and NOLA3, respectively) and TEP1 which are proposed to aid the function and the location of the resulting telomerase complex [218].

Although telomerase is active as a monomer [219], from *in vitro* experiments there is evidence that telomerase in many others organism such as yeasts and human exists as a dimer [220-223] to which at least 32 distinct proteins have been proposed to associate (Table 1) [211]. Some of these components are necessary for telomerase attachment to the telomere at a certain cell cycle phase [224], while others are required for regulation of telomerase activity

[225]. Some proteins are necessary for maturation of the telomerase complex and degradation of its components [226] most of which dissociate during the activation process [226].

Normal human cells have hTERT distributed in the nucleolus and nucleoplasm, but in human cancer cells hTERT primarily locates in the nucleoplasm, and it is generally not detected in the nucleolus [227].

The human telomerase RNA subunit is expressed in both telomerase positive and telomerase-negative tissues [228] as a consequence the expression of the human catalytic subunit gene (*hTERT*) seems to be the rate-limiting process for telomerase activity.

In yeast, telomerase is not active at each telomere in every cell cycle [229]. Instead, individual telomeres might experience several rounds of shortening in successive cell cycles before a certain length is reached that will make telomerase more likely to act on them. Indeed telomerase preferentially associate with short telomeres, compared to unshortened ones and this association markedly increased in S phase or G2/M, apparently being coupled with DNA replication [230-233].

Without telomerase, the cycle of alternating lengthening and shortening of telomeres in dividing cells is broken. As a result, telomeres progressively shorten, and as the cells divide, a gradually increasing fraction of the cells exit the cell cycle until the cell population senesces [234]. In some mammalian cells, apoptosis is also provoked.

Human cells that overexpress both the RNA and protein components of telomerase experience continuous telomere elongation that is independent of telomere length [235]. This suggests that limiting amounts of telomerase might be an important factor in ensuring the preferential elongation of the shorter telomeres and might help to explain the role of negative regulators of overall telomerase activity such as PinX1 [236]. The protein PinX1 [200,237] is a negative regulator of telomerase that interacts with hTERT via the RNA-binding domain (TRBD). The role of the interaction of hTERT with PinX1 is still unknown. It is supposed that in this way hTERT not bound to hTR is “preserved” in an inactive state [236]. PinX1 it is often found to be diminished in amount in human cancers thus it could be considered as tumor suppressor [237].

Telomerase expression profile

During embryonic development, human telomerase activity is detectable at the blastocyst stage and in most embryonic tissues although before 20 weeks of gestation is subsequently lost [238]. Fetal tissues show temporally distinct patterns of regulation, with activity remaining longer in liver, lung, spleen and testes than in heart, brain and kidney [239]. In heart tissue, loss of activity occurs concomitant with loss of hTERT mRNA expression; loss of activity in kidney instead occurs concomitant with a change in the pattern of hTERT expression [240]. Although telomerase expression is restricted to embryonic stem cells, in adulthood a weak expression is detectable in activated stem cells [238,241,242], grow-stimulated lymphocytes, uroepithelial cells [243], intestinal epithelium [244], esophageal epithelium [245], cycling endometrium [246], basal keratinocytes [247], cervical epithelium [248], and hematopoietic stem cells [249]. All the other somatic cell types do not express telomerase.

These various telomerase-positive human somatic cell types produce different relative amounts of catalytic activity, this is because of cellular telomerase activation does not necessarily act to maintain a constant telomere length. In some cases, telomeres erode with cell proliferation despite telomerase activation [250,251], in other cases, telomeres make dramatic gains in net length despite cell proliferation [252]. Moreover the telomerase activation in human somatic cells is transient, not within a given cell cycle out over the course of multiple cell divisions. A stem or progenitor cell with weak telomerase activity can generate strongly telomerase-positive lineage-committed descendants, which will subsequently lose telomerase activity with additional differentiation [252-254]. This transient telomerase activation in normal human somatic cells contrasts sharply with the constitutive activation of telomerase in most cancers.

In contrast to its physiological expression pattern, telomerase become up-regulated in many cancers since the maintenance of a correct replicative *status* is an essential step in tumorigenesis. Telomerase is overexpressed in 85–90% of human cancers and over 70% of immortalized human cell lines [241,255], particularly in cancers telomerase activity is highly increased by up to 100-fold of expression in tumoral portions in respect of the adjacent normal cells [253]. Telomerase expression in cancers and immortalized cells is usually associated with a short and stable telomere length [184,256-258].

Telomerase expression in HCC

Telomerase is overexpressed in 80-100% of human HCCs and its expression is positively correlated with its activity [259-261]. The tumor-surrounding affected tissue presents a weaker telomerase activity, generally this observation is more frequent in cirrhosis than in hepatitis [260], however there is not a general agreement with this statement among the several studies in this field [262,263].

Some reports did not observe any correlation between telomerase expression with tumoral progression [262-264] whereas other studies reported that the telomerase activity progressively increases during the dedifferentiation process of HCC from well-differentiated to poorly differentiated HCC [265-267]. These observations support strongly that the high enhancement of telomerase expression is an essential event for malignant transformation during hepatocarcinogenesis like other malignant cancers and for the immortality of the transformed cells.

It worth to be noticed is that in all studies no telomerase expression was detected in non diseased liver [259,260] reinforcing the relationship between cancer and telomerase expression.

Telomerase and cellular immortalization

Due to its pivotal role in stabilizing telomeric DNA and in preventing telomere shortening-induced cell proliferative senescence [268-271], telomerase is required for immortalization of primary cells.

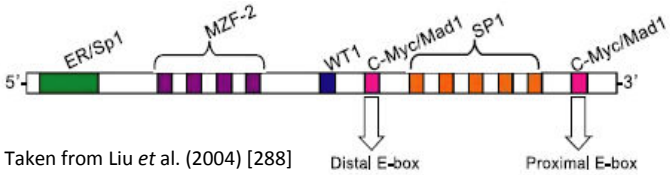
When placed into culture, most normal human somatic cells have a limited lifespan. Human fibroblasts, for example, can divide an average of 40 to 50 generations before they stop dividing [272]. Transformation with viral and/or cellular oncogenes extends the lifespan of human cells beyond the first growth arrest point, known as senescence, but these transformed cells eventually enter a phase known as crisis, where cells suffer chromosome aberrations and massive cell death [273,274]. Rare immortal cell clones escape from crisis and survive by telomerase activation. From this observation became evident the role of telomerase for cell immortalization. In some reports the ectopically induced overexpression of the reverse transcriptase subunit of telomerase (hTERT) activates the telomerase activity and indefinitely extended the proliferative lifespan of fibroblasts [268]. Although telomerase expression is

sufficient to immortalize some cell types, such as fibroblasts, other cell types require the cotransfection of an oncogene for the inactivation of other growth suppressing pathways and thereby for direct immortalization [275]. For example the cotransfection of SV40 large antigen, mutant H-*ras*, and the *hTERT* gene has been shown to be capable of transforming both human fibroblasts and human epithelial cells into tumor cells [276]. In these cells telomerase is not only necessary for maintaining the immortality of the cells [277] but, in certain cases it also increases cell proliferation and invasion ability [278]. This definitively shows that *hTERT* expression, and presumably the resultant telomerase activation represents an important step in tumor development.

Genetic modulation of telomerase activity

Telomerase levels are regulated at multiple levels including transcription, alternative splicing, assembly, subcellular localization, and post-translational modifications of various components and of the enzyme complex itself.

Figure 7. Core promoter region of hTERT.



Taken from Liu *et* al. (2004) [288]

Telomerase activity mainly depends on hTERT availability since the RNA component is ubiquitously expressed in somatic cells. The transcriptional regulation of hTERT is determined by the binding of either repressors or activators to the core promoter which is essential for transcriptional activation in cancer cells and immortalized cells. However, the exact molecular mechanism underlying the tumor-specific expression of telomerase remains unclear.

Transcriptional activators include c-Myc [279], Sp1 [280,281], estrogen [282] and USF1 and 2 (upstream stimulatory factor) [283]. Transcriptional repressors include the tumor suppressor protein p53 [284,285], Mad1, myeloid-specific zinc finger protein 2 (MZF-2) [286], Wilms' Tumor 1 (WT1) [287], TGF-h and Menin [288] (Fig. 7). Overexpression of p53 can trigger a rapid downregulation of hTERT mRNA expression [284,285]. However, the inhibition of p53 activity failed to reactivate hTERT expression [289] suggesting the involvement of others regulators in hTERT expression.

p53 inhibits Sp1 binding to the hTERT promoter by forming a p53-Sp1 complex [285]. Indeed the mutations in all five Sp1 binding sites abolished the p53-mediated hTERT promoter repression. Menin can bind directly to the hTERT promoter, whereas TGF-h acts through Smad-interacting protein-1 (SIP1) [289]. The presence of MZF-2 significantly represses hTERT transcription [286], but it is assumed to play a minor role in the regulation of hTERT.

Mad1 and c-Myc play antagonistic roles in the regulation of hTERT, they both bind to the consensus sequence 5V-CACGTG-3V, called an ‘‘E-box’’ [280,290]. High levels of c-Myc often correlate with high levels of hTERT, and high levels of Mad1 are observed in cells with repressed hTERT [291]. c-Myc is an oncogene and its product complexes with Max protein as a heterodimer to activate gene transcription [292]. c-Myc/Max heterodimer binds at the E-boxes after induction of cellular transformation [293] whereas there is a preferential binding of the Mad1/Max heterodimer at the E-boxes of the hTERT promoter in untransformed cells [293]. The hTERT regulatory region contains two estrogen response element (ERE) and an increased transcription of hTERT follows the binding of the hormone estrogen and its receptor to ERE [282].

Located within the hTERT promoter there are clusters of CpG dinucleotides [294] that are targets for DNA methylation generally leading to gene silencing. The methylation state seems not to clearly correlate with hTERT expression since contrasting data are available in literature [295-297]. This could be due to the involvement of a large variety of transcription factors interacting with the hTERT promoter.

Telomerase post-transcriptional regulation

To date, seven alternatively spliced sites (ASPSs) in the *hTERT* mRNA have been described [298-300]. Two ASPSs, α-deletion and γ-deletion, result from in-frame deletions of exonic sequences in exon 6 and 11, respectively, and the β-deletion variant derives from an exon 7 and 8 deletion.

Some of these hTERT inactive mutants can negatively influence telomerase activity such as the α-and β- deletion variants [301,302]. It was postulated a role of this mutants in binding most of the components needed to form the ribonucleoproteins such as hTR, resulting in a sort of competition with the wild-type forms for binding to the telomeres. Moreover the

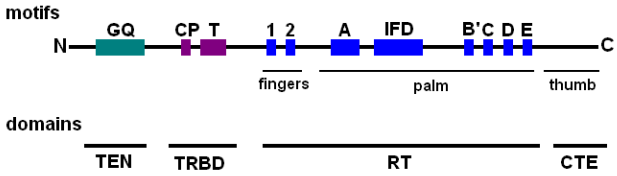
dimerization of the wild-type and mutant telomerase may create a non-functional heterodimer more susceptible to degradation [303].

Mitomo and coll. [304] demonstrated that also miRNAs can play a critical role in telomerase regulation. In particular miR-138 targets specificity the *hTERT* 3'-untranslated region consequently inducing a reduction in hTERT protein expression [304]. On the contrary the loss of miR-138 expression may partially contribute to the gain of hTERT protein expression.

Telomerase structure and domain organization

The telomerase RNA and protein subunit form the enzyme catalytic core, being sufficient to reconstitute catalytically-active telomerase *in vitro*. The telomerase RNA has diverged considerably in size and sequence during evolution, nonetheless conserving some structural elements. The amino acid sequence of telomerase catalytic subunits is more conserved among species, especially in residues involved in important functions such as catalysis, nucleotide binding, and ribo- and deoxynucleotide recognition [229].

Figure 8. Telomerase domain structure



hTERT contains four major functional domains (Fig. 8):

- N-terminal TEN domain containing moderately conservative GQ motif (hypomutable domain I) [305], the TEN domain participates in the interaction with DNA primer and influences the enzyme activity [306].
- RNA-binding domain (TRBD domain) contains the conservative motives CP, QFP, and T (hypomutable domains II, III, and IV). Motifs CP and T directly participate in RNA binding while motif QFP has a structural function.
- Reverse transcriptase domain (RT domain) containing seven conservative domains and an IFD site (Insertion in Fingers Domain) which is located between motifs A and B and it is a distinctive feature of telomerases [307].
- Lowly conserved C-terminal domain (CTE domain) that binds the RNA/DNA hybrid and catalyze the addition of DNA repeats onto the 3' end [308].

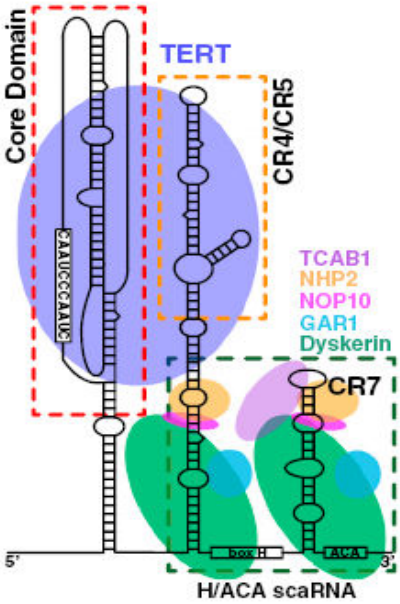
Such organization of hTERT domains results in formation of a central "hole" of sufficient width for the accommodation of a 7-8 bp long nucleic acids double-strand. In yeast telomerase was also detected an endonuclease activity however, the telomerase domain responsible for nuclease activity has not been identified.

hTR contains secondary structure elements necessary for catalytic functions, type I and II processivity, as well as elements necessary for maturation, telomerase stability, and hTR localization.

hTR contains four conserved structural domains:

- The pseudoknot, the core domain, which includes the template.
- The conserved regions 4 and 5 (CR4/CR5), which together comprise the catalytic core of the TR.
- The box H/ACA that binds the H/ACA RNP proteins (dyskerin, Gar1, Nop10, Nhp2) the CR7 [309] (Fig. 9).

Figure 9. Architecture of human telomerase RNA.



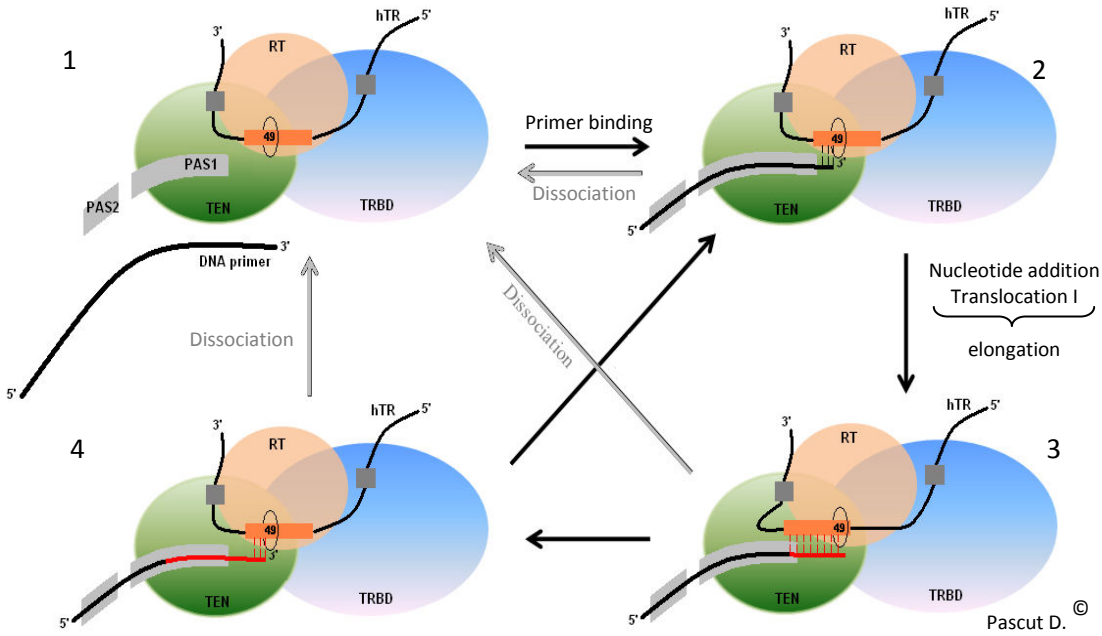
Secondary structure and known protein components of the human telomerase RNA (hTR). The hTR core and CR4/CR5 domains independently bind the hTERT (blue ellipse). The hTR scaRNA domain binds two sets of the four H/ACA RNP proteins: dyskerin (green), Gar1 (cyan), Nop10 (magenta), and Nhp2 (orange). The protein TCAB1/WDR79 (purple) binds both the dyskerin and the CAB box located at the CR7 region within the H/ACA scaRNA domain. Taken from Zhang *et al*., (2011) [309].

In mammals hTR is synthesized by RNA polymerase II, then it is capped at the 5' end, modified, and processed at the 3' end [194,216]. hTR processing and stability depends on H/ACA that associates dyskerin, hGAR1 hNHP2 and hNOP10 [187,189,190]. Although the H/ACA motif is necessary to hTR accumulation it is not sufficient, there is another motif at the distal end of the 3' H/ACA motif hairpin that is also required for RNA stability *in vivo* [310,311]. The complexes protein/hTR are generally referred as "telomerase RNA" and they accumulate ubiquitously in cells regardless of the presence of telomerase activity in cell extracts [216]. Once hTR is preassembled into stable telomerase RNP, hTERT associates by interactions with two independent regions of telomerase RNA: The template region (including nucleotides 44 ± 186) and a putative double hairpin element in the 5' stem of the H/ACA domain (a region within nucleotides 243 ± 326). The functional telomerase enzyme assembly in humans is dependent from chaperones such as, heat-shock protein-90 (HSP90) and p23 chaperones that seem to participate in the assembly, disassembly and degradation of telomerase complexes [197,312].

The catalytic cycle of telomerase

The cycle of *in vitro* telomerase reactions (Fig. 10) includes the following stages: primer binding, elongation, translocation, and dissociation. The first step of the telomerase cycle is the recognition of the template from the enzyme. However the mechanisms that underlie the recognition of a single-stranded-DNA substrate seem surprisingly variable between holoenzymes and have not yet been well characterized [313,314]. Interaction assays and high-resolution structure have evidenced the presence of a binding surface for single-stranded DNA, termed PAS1 (primer/product alignment/anchor site-1), that is partially located in the TEN domain and adjacent to the template hybrid (Fig. 10).

Figure 10. Telomerase reaction cycle.



The figure represents the telomerase reaction cycle. In orange the reverse transcriptase domain (RT), in green the TEN domain and in blue the TRBD domain. The telomerase RNA (hTR) with the template site (orange rectangle) is maintained in the correct position through interaction between the telomerase domains in the anchor sites. 1) Enzyme is not bound to primer. 2) Primer annealing in PAS1, the primer 3' end is positioned in correspondence of residue 49 of hTR (catalytic site). 3) Elongation stage. 4) Completion of a single telomeric repeat synthesis and enzyme translocation along the primer. In red the newly synthesized DNA portions. Grey arrows point to possible processes of primer dissociation during enzyme functioning.

From ciliate and vertebrate models was discovered another DNA-interaction specificity domain termed PAS2. PAS2 sites could be contiguous with or separated from PAS1 and are proposed to account for the enhanced binding affinity of longer primers with the telomerase holoenzyme [313,314]. Single-stranded DNA binds to PAS1 with the 3' end near the hTR's

template region. The hRT is maintained in the correct position by interactions with the TEN and the TRBD domains of the hTERT subunit. In particular the 3' and the 5' anchor sites of hTR are located in the TEN and TRBD domains respectively (Fig. 10). These interactions allow the positioning of the residue 49 of the hTR in the active site. The 3' end the DNA primer locates in correspondence of residue 49, once the first 6 bases are added the enzyme translocates along the template to add the others 6 base repetitions without separation from the primer.

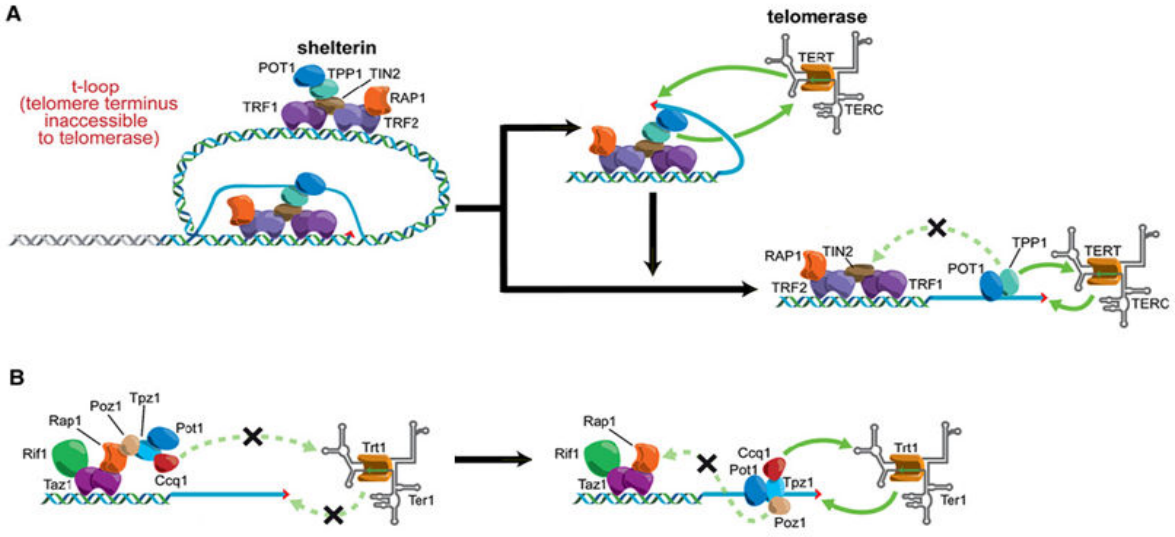
The ability for translocation is connected with enzyme processivity. Two types of telomerase processivity are distinguished [314]. Processivity I is the telomerase capability for RNA-DNA duplex translocation in the active center after each nucleotide addition at the stage of elongation. Processivity II is telomerase capability for translocation relative to the bound DNA primer after addition of one telomeric repeat, after which the primer again becomes capable of elongation. Human and protozoan telomerases *in vitro* exhibit type II processivity. They are able to add hundreds of nucleotides to telomeric substrate via multiple completions of telomeric repeats along their RNA template [315].

Telomerase recruitment to telomeres and telomerase regulation

Telomerase is regulated in cis at individual chromosome ends by the telomeric protein/DNA complex in a manner dependent on telomere repeat-array length. A dynamic interplay between telomerase-inhibiting factors bound at duplex DNA repeats and telomerase promoting ones bound at single-stranded terminal DNA overhangs appears to modulate telomerase activity.

Telomeres structure by itself act as an inhibitor of telomerase activity since the terminal t-loop structures sequester the 3' telomeres end avoiding the interaction with telomerase. The t-loop is formed when the G-reach 3'-single-stranded telomeric end penetrates the double stranded region where the displaced second strand forms an internal D-loop [157,316]. Nevertheless there may be an interval within S phase when t-loops are disassembled by the DNA replication machinery which provides the best opportunity for telomerase access to a chromosome 3' end.

Figure 11. Telomeres and telomerase recruitment



Schematic representation of the telomeric complexes responsible for telomerase regulation in mammals (A) and yeasts (B). (A) In mammalian cells, t-loops are proposed to be non-permissive for telomerase activity due to sequestration of the telomere terminus. Opening of the t-loop could be in itself sufficient to allow telomerase to act (middle). Alternatively, even in this unfolded state, the 3' end might conceivably be made unavailable as a substrate for telomerase by (for example) interaction with shelterin-bound TPP1-POT1. If so, possibly a structural transition might have to take place that would unlock the telomerase-stimulatory activity of POT1-TPP1.
(B) Similarly, in fission yeast, the Pot1/Tpz1/Ccq1 complex might be conductive to telomerase recruitment/stimulation only when in a proper configuration (i.e., when bound directly to the overhang) and/or postranslationally modified state. Poz1-mediated binding of the complex to Taz1-Rap1 is proposed not to be conductive of telomerase-promoting action. Modified from Bianchi *et al.* (2008) [317].

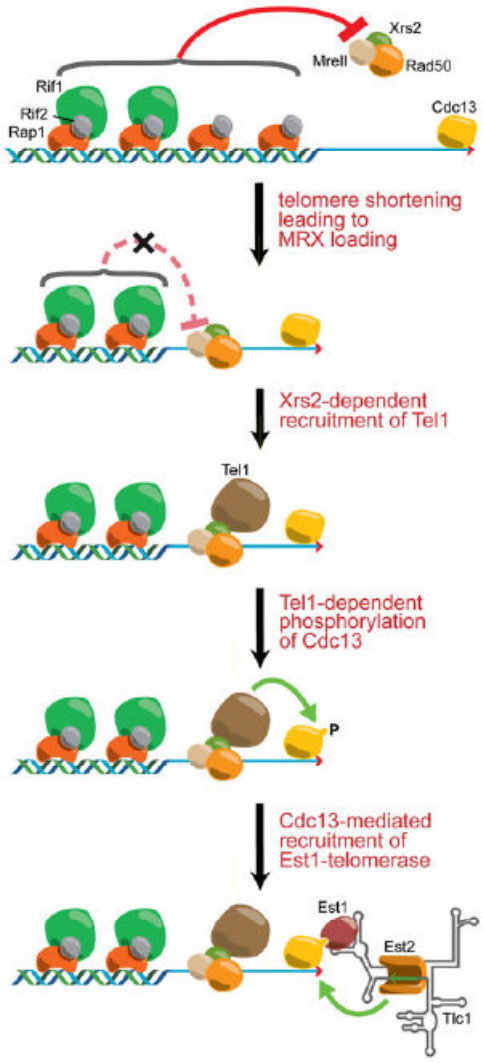
In budding yeast telomerase activation is telomere length dependent. The protein Rap1 is the main responsible of telomerase inhibitors recruitment due to its ability to bind double-stranded telomeric repeats via Taz1 (Fig. 11B). The Rap1 C-terminal domain (RCT) interacts with Rif1 and Rif2 that independently relay the inhibitory signal to telomerase [317,318]. Longer telomeres, by carrying a larger number of Rap1 binding sites, allow increased association of telomerase repressors that inhibit the MRX (Mre1/Rad50/ Xrs2) complex binding to telomeres and as a consequence telomerase is largely inhibited at these ends.

The telomere shortening reduce the binding sites for telomerase inhibitory complexes (Fig. 12) allowing, during S phase, the association of the MRX (Mre1/Rad50/ Xrs2) to telomeres leading to Tel1 kinase recruitment through an interaction with the C-terminus of Xrs2 and subsequent phosphorylation of Cdc13 on serine residues. The protein Cdc13, a single-stranded DNA-binding protein associates with TG-rich telomeric repeats, and its expression peaks in late S phase concomitant with the appearance of long overhangs [319] (Fig. 12). Recently, Cdc13 has indeed been shown to be phosphorylated *in vitro* by the Tel1 and Mec1 checkpoint kinases (orthologs of mammalian ATM and ATR, respectively) on several serine residues, two of which are required for telomere maintenance [320]. Indeed cells lacking both

kinases undergo telomere shortening and senescence [321]. Phosphorylated Cdc13 interacts with Est1 [322] which itself associates with the telomerase RNA (Tlc1) and then Est2 (the catalytic subunit of yeast telomerase) (Fig. 12).

In mammals the DNA binding function is supplied by two orthologs of Taz1, TRF1 and TRF2, that bind as homodimers to double-stranded telomeric repeats (Fig. 11A). TRF1 and TRF2 interact with each other via TIN2 protein that recruits TPP1 and its partner POT1; TRF2 also binds RAP1. This overall structure on mammalian telomeres forms a six-protein-complex TRF1/TRF2/RAP1/TIN2/TPP1/POT1) named shelterin complex [156] (Fig. 11A). Shelterin has an inhibitory effect on telomere lengthening, which appears to be exerted largely by structural changes at telomeres that may alter telomerase accessibility. Indeed TRF2 promotes a t-loop formation *in vitro* [323]. Moreover the POT1-TPP1 complex binds telomeres terminal overhangs with higher affinity and can also bind the shelterin complex via TIN2 interaction. The simultaneous interaction with overhangs and TIN2 could sequester the telomere terminus in a conformation non-accessible for telomerase and this interaction could be removed following secondary modification (for example, phosphorylation) [324-326] (Fig. 11A). The inhibitory effect of the shelterin complex increases with the number of complexes bound to the telomere arrays which clearly depends on telomere length [325,327]. Several studies, primarily using RNA-interference and dominant-negative alleles, have revealed the importance of every single component of the shelterin as negative regulator of telomerase including TRF1 and TRF2 [327], RAP1 [328-330], TIN2 [331,332], and POT1/TPP1 [325,332-335]. The switch from an inhibitory state to a telomere elongation promoting one depends on the balance between repressing and promoting factors. When telomeres reach the critical length some not well defined signaling pathways leads to telomerase activation. By analogy with the yeast

Figure 12. Telomeres shortening and telomerase activation in yeasts.



Taken from Bianchi *et al.* (2008) [317].

systems, human RAP1 interacts, probably directly, with the MRN complex (MRE11/RAD50/NBS1) and Ku86 [330] which recruitment to telomeres leads to ATM-dependent phosphorylation of TRF1 and to its dissociation from telomeres [336]. In an *in vivo* repressing state TRF2 binds to and inhibits ATM (ataxia telangiectasia mutated), Tel1 homologue [337], the effect of TRF2 on ATM depends on the number of TRF2 bound at the telomeres. Telomeres shortening and TRF2 removal might activate [337] the ATM-dependent phosphorylation of TRF1 that dissociate from telomeres and further exacerbate the loss of TRF molecules from telomeres, with consequent amplification of the signal for the activation of telomerase.

In human breast cancer telomerase was shown to be regulated by phosphorylation in both TERT and TEP1 subunits by Protein phosphatase 2A and protein kinase Ca [196,338]. Phosphorylation is associated with high telomerase activity, and dephosphorylation with low.

Telomerase: the extratelomeric effects

One of the most interesting findings in recent years is the discovery that telomerase functions are not limited to telomeres maintenance in cancer cells. A wide variety of non-canonical effects of telomerase that are independent of telomere lengthening have been discovered. In particular, inhibition of apoptosis seems to be a general function of this enzyme [339], telomerase expression directly inhibits apoptosis by blocking both the mitochondrial [340] and the death receptor pathway [341] through unknown mechanisms. The anti-apoptotic effect can also be conferred by catalytically inactive forms of hTERT and is therefore activity-independent [342-344]. Telomerase seems to actively promote cell growth; indeed it induces growth-related proteins such as epidermal growth factor receptor (EGFR) in mammary epithelial cells [345] and interferes with the TGF-beta network of growth factors in primary murine cell lines [346].

Telomerase has a clear effect on the DNA repair machinery; cells in which telomerase activity was suppressed had a significantly impaired DNA damage repair [347]. The effect on DNA repair is dependent on catalytic activity, although the precise mechanism is not known [347].

Telomerase has also a mitochondrial targeting sequence [348] but its role in the mitochondria is unclear at present. Notably, Ahmed and coll. [349] found that oxidative stress induces 80-90% of all telomerase molecules to enter the mitochondria where it has been suggested to directly bind the mtDNA [349].

The telomerase that remains in the nucleus is insufficient to maintain telomere length and only a re-introduction of this enzyme into the nucleus can rescue telomeres lengthening [349]. Also single telomerase components have been proven to exert some specific functions; hTR, for example, was shown to modulate the DNA damage responses and increases cell viability after UV irradiation by impairing damage checkpoint activation [350]. This may explain why hTR is widely expressed in somatic cells and why it is upregulated in cancers and promotes growth even before hTERT is activated [351].

Dyskeratosis congenita

Telomerase deficiency in humans was first described in the disease dyskeratosis congenita (DC). Patients with DC share signs of insufficient cellular renewal in the skin with an excess in skin pigmentation due to a decrease in keratinocyte turnover, an increase in melanin synthesis associated with melanocyte senescence [352] or aberrant melanin uptake, an exhaustion of epithelial stem cells that cause nail dystrophy. Moreover DC is characterized by bone-marrow failure with an insufficient renewal in blood cell counts that cause the premature dead of affected patients [353,354]. Interestingly long surviving DC patients show an increased risk of cancer [355], indeed prematurely short epithelial cell telomeres would enhance genomic instability [356].

The predominant X-linked inheritance of DC arises from substitutions in the RNA-binding protein dyskerin. The inability of mutated DKC1 to bind hTR determines the accumulation of the telomerase RNA subunit which is not assembled with hTERT to form active enzyme. Telomerase deficiency leads to premature telomere shortening, which in turn limits the renewal capacity of highly proliferative cell types in skin and blood. The less common, and generally less severe, autosomal dominant (AD) inheritance of DC depends on mutations in the H/ACA region of human telomerase RNA [357]. Other autosomal diseases, detected in patients with blood diseases, are associated with mutations in hTERT [358].

The phenotypic and molecular differences between X-linked and AD disease suggest that the greater the telomerase deficiency, the greater the disease severity, indeed the twofold reduction in hTR predicted in AD DKC would limit maximal catalytic activation to 50% of actively growing cells that usually employ more than 50% of the holoenzyme maximum in the effort of telomere maintenance.

AIMS OF THE STUDY

HCC is currently one of the most common worldwide causes of cancer death counting 560,000 new cases per year. OLT or liver resection represent the best treatments for HCC. However, most patients cannot be subjected OLT or resection, and a good alternative is represented by chemotherapy or chemoembolization. Unfortunately the development of the MDR phenotype could lead to unsuccessful drug therapy.

To understand and define ways to overcame this problem we developed a multitasking study in which the main objectives are:

- Analyse the drug-influenced expression profile of the main ABCs involved in MDR in HCC derived cell lines, in order to clarify the role of these transporters in liver malignancies.
- Develop a new therapeutic approach that clear the hurdle of MDR. In particular targeting an essential and specific cancer related gene, such as telomerase could represent the new challenge in anticancer molecular techniques flanking conventional treatments.

MATERIALS & METHODS

Chemicals and Reagents

2X iQ ™ SYBR Green Supermix - 170-8885 - Bio-Rad Laboratories - Hercules, CA, USA
Anti-ABCB1 antibody - C219- Abcam plc, Cmmbridge, UK
Anti-ABCC1 antibody - A23 - Alexis Biochemicals, San Diego, CA
Anti-ABCG2 antibody – BXP-53 - Abcam plc, Cmmbridge, UK
Anti-Actin antibody - A2066 - Sigma-Aldrich, St.Louis, MO
Anti-mouse secondary antibody - P0260 - Dako, Glostrup, Denmark
Anti-rabbit secondary antibody - P0448- Dako, Glostrup, Denmark
Anti-rat secondary antibody – P0450 - Dako, Glostrup, Denmark
Bicinchoninic Acid Solution-KIT - B-9643 and 209198 - Sigma-Aldrich, St.Louis, MO
Bovine pancreas insulin - I1882 - Sigma-Aldrich, St.Louis, MO
bovine serum albumin - A7906 - Sigma-Aldrich, St.Louis, MO
Cell Lysis Buffer (10X) – 9803 - Cell Signaling technology, Boston, MA
Dexamethasone - D4902 - Sigma-Aldrich, St.Louis, MO
DMEM high glucose - EC B7501L - EuroClone Milano Italy
DMEM/F-12 (1:1)(Ham) 1X - 11039-021 – Invitrogen, Life Technologies Corporation, Grand Island, NY
DMSO (dimethyl sulfoxide) - D5879-L - Sigma-Aldrich, St.Louis, MO
Doxorubicin - D9891 - Sigma-Aldrich, St.Louis, MO
EDTA (Ethylenediaminetetraacetic Acid) - *405497* - Carlo Erba Reagents, Milan, Italy
Ethanol - 34852 - Sigma-Aldrich, St.Louis, MO
FBS (Foetal bovine serum) - F7524 - Sigma-Aldrich, St.Louis, MO
FITC (Fluorescein 5(6)-isothiocyanate) - F3651 - Sigma-Aldrich, St.Louis, MO
FITC conj. anti-rabbit secondary antibody (Alexa Fluor®) - A11008 - Invitrogen, Life Technologies Corporation, Grand Island, NY
Glycine - G4392 - Sigma-Aldrich, St.Louis, MO
Hepes - H3375 - Sigma-Aldrich, St.Louis, MO
Hoechst H33258 - H6024 - Sigma-Aldrich, St.Louis, MO
Laemmli Buffer 5X - 161-0737 - Bio-Rad Laboratories - Hercules, CA, USA
L-glutamine 100X - EC B3000D - EuroClone, Milano Italy
Luminata™ Western HRP substrate - WBLUC 05500 – Millipore, Billerica, MA
methanol - 34860 - Sigma-Aldrich, St.Louis, MO
MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide) - M2128 - Sigma-Aldrich, St.Louis, MO
Nitrocellulose membranes - 162-0177 - Bio-Rad Laboratories - Hercules, CA, USA
Normal Goat Serum - G9023 - Sigma-Aldrich, St.Louis, MO
Paraformaldehyde - P6148 - Sigma-Aldrich, St.Louis, MO
PBS (Dulbecco's Phosphate Buffered Saline) - D5652-50L - Sigma-Aldrich, St.Louis, MO
Penicillin/streptomycin 100X - EC B3001D - EuroClone Milano Italy
Phenylmethylsulfonyl Fluoride - P-7626 - Sigma-Aldrich, St.Louis, MO
PI (Propidium Iodide) - P4170 - Sigma-Aldrich, St.Louis, MO
RNAse A – R6513 - Sigma-Aldrich, St.Louis, MO
RPMI 1680 - EC B9006L - EuroClone, Milano Italy
Silencer® siRNA Construction Kit AM1620 - Ambion, Invitrogen, Life Technologies Corporation, Grand Island, NY
siLentFect Lipid Reagent for RNAi - 170-3360 - Bio-Rad Laboratories - Hercules, CA, USA
Sodium acetate - S2889 - Sigma-Aldrich, St.Louis, MO
Sodium bicarbonate - S5761 - Sigma-Aldrich, St.Louis, MO
Sodium bicarbonate - S5761 - Sigma-Aldrich, St.Louis, MO
Sucrose - S1888 - Sigma-Aldrich, St.Louis, MO
the iScript ™ cDNA Synthesis Kit - 170-8891- Bio-Rad Laboratories - Hercules, CA, USA
TRAPeze® Telomerase Detection Kit – S7700 - Millipore Corporation, Billerica, MA
Tri-Reagent Kit - T9424 - Sigma-Aldrich, St.Louis, MO
Tris Base - T6066 - Sigma-Aldrich, St.Louis, MO
TritonX100 – T8787 - Sigma-Aldrich, St.Louis, MO
TWEEN 20 - P7949 - Sigma-Aldrich, St.Louis, MO
Williams E medium - W4128 - Sigma-Aldrich, St.Louis, MO
β-mercaptoethanol – M3148 - Sigma-Aldrich, St.Louis, MO

Table 1. List of the oligonucleotides used for Real Time PCR.

Gene	Accession number	Forward Primer	Reverse Primer
18S	NR_003286.2	5'-TAACCCGTTGAACCCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'
β-actin	NM_001101.3	5'-CGCCGCCAGCTCACCATG-3'	5'-CACGATGGAGGGGAAGACGG-3'
ABCB1	NM_000927	5'-TGCTCAGACAGGATGTGAGTTG-3'	5'-AATTACAGCAAGCCTGGAACC-3'
ABCC1	NM_004996	5'-GCCAAGAAGGAGGAGACC-3'	5'-AGGAAGATGCTGAGGAAGG-3'
ABCG2	NM_004827	5'-TATAGCTCAGATCATTGTCACAGTC-3'	5'-GTTGGTCGTCAGGAAGAAGAG-3'
Albumin	NM_000477	5'-GTGGAAGAGCCTCAGAAT-3'	5'-TTGGTGTAACGAACTAATAGC-3'
Aurora Kinase A	NM_198433	5'-GAGAATTGTGCTACTTATACTG-3'	5'-GGTACTAGGAAGGTTATTGC-3'
IL-6	NM_000600	5'-ACAGATTTGAGAGTAGTGAGGAAC-3'	5'-GGCTGGCATTTGTGGTTGG-3'
IL-8	NM_000584	5'-GACATACTCCAAACCTTTCCAC-3'	5'-CTTCTCCACAACCCTCTGC-3'
Midkine	NM_001012334	5'-AAAGCCAAGAAAGGGAAG-3'	5'-CTAACGAGCAGACAGAAG-3'
Survivin	NM_001168	5'-CTAAGTTGGAGTGGAGTCTG-3'	5'-GCTTGCTGGTCTCTTCTG-3'
Telomerase hTERT subunit	NM_198253	5'-CGTCTGCGTGAGGAGATC-3'	5'-AAGTGCTGTCTGATTCCAATG-3'
TNF-α	NM_000594	5'-GTGAGGAGGACGAACATC-3'	5'-GAGCCAGAAGAGGTTGAG-3'

Table 2. Real Time PCR amplification protocol.

Step	Cycle repeats	Temperature	Time
PCR Reaction	Cycle 1: (1X)	95°C	03:00
	Cycle 2: (40X)	95°C	00:20
		60 °C	00:30
	Cycle 3: (1X)	95 °C	01:00
Melting Curve	Cycle 4: (1X)	55 °C	01:00
	Cycle 5: (80X)	55 °C-94.5 °C	00:10
	Cycle 6: (1X)	4°C	for Hold

General procedures

Cell lines and culture conditions

SV-40 Immortalized Human Hepathocytes (IHH) were kindly provided by Dr. T.H. Nguyen [359] and were cultured in DMEM/F-12 (1:1) DMEM/F-12/(Ham) 1X with 10% (v/v) FBS, 1% L-glutamine 100X, 1% penicillin/streptomycin 100X, 5µg/mL bovine pancreas insulin, 1µM dexamethasone.

HuH-7 cells (differentiated human hepatoma) were obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB0403) and were cultured in DMEM high glucose with 10% (v/v) FBS, 1% L-glutamine 100X, 1% penicillin/streptomycin 100X.

JHH6 (undifferentiated human hepatocellular carcinoma) were obtained from Japan Health Science Research Resources Bank (HSRRB, JCRB1030) and were cultured in Williams E medium with 10% (v/v) FBS, 1% L-glutamine 100X, 1% penicillin/streptomycin 100X.

The three cell lines were grown as monolayer culture at 37°C in 5% CO_2 and 95% humidity.

RNA extraction and reverse transcription-qPCR

Total RNA was extracted by using Tri-Reagent Kit according to manufacturer's instructions. Briefly, cells were lysed with the reagent, chloroform was added and cellular RNA was precipitated by isopropyl alcohol. After washing with 75% ethanol, the RNA pellet was dissolved in nuclease-free water and stored at -80°C until further analysis. The total RNA concentration and the purity were assessed by spectrophotometric analysis in a Beckman DU730 spectrophotometer.

The integrity of RNA was assessed on standard 1% agarose/formaldehyde gel. Total RNA (1µg) was reverse-transcribed using the iScript™ cDNA Synthesis Kit according to manufacturer's instructions and retrotranscription was performed using the iQ5 ™ Multicolor Real-Time Detection system (Bio-Rad Laboratories, Hercules, CA, USA) in agreement with the reaction protocol proposed by the manufacturer's: 5 min at 25˚C (annealing), 45 min at 42˚C (cDNA synthesis), and 5 min at 85˚C (enzyme denaturation).

Real Time quantitative PCR was performed using the iQ5 ™ Multicolor Real-Time Detection system (Bio-Rad Laboratories, Hercules, CA, USA). All primers pairs were synthesized by Sigma-Aldrich (Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103) and were designed

using the software Beacon Designer 7.91 (PREMIER Biosoft International, Palo Alto, CA USA), β-actin and 18S were used as reference genes [360] (Table 1).

PCR amplification was carried out in 25μL reaction volume containing 25ng of cDNA, 1x iQ SYBR Green Supermix [100 mM KCl; 40mM Tris–HCl, pH 8.4; 0.4mM each dNTP; 50U/mL iTaq DNA polymerase; 6mM MgCl2; SYBR Green I; 20nM fluorescein; and stabilizers] and 250nM gene specific sense and anti-sense primers and 100nM primers for 18S. All real-time PCR reactions were performed in triplicate using the three-step PCR program shown in Table 2.

Standard curves using a "calibrator" cDNA (chosen among the cDNA samples) were prepared for each target and reference gene. In order to verify the specificity of the amplification, a melt-curve analysis was performed, immediately after the amplification protocol. Non-specific products of PCR were not found in any case. The relative quantification was made using the Pfaffl modification of the ΔΔCt equation, taking into account the efficiencies of individual genes. The results were normalized to 18S and beta-actin, the initial amount of the template of each sample was determined as relative expression versus one of the samples chosen as reference (in this case the control sample) which is considered the 1x sample.

The data were analyzed using iQ5^{TM} optical system software version 2.0 (Bio-Rad Laboratories, Hercules, CA, USA).

Total protein extraction

After the Doxorubicin (Dox) treatment cells were washed twice with cold PBS and than lysed with 400μL of Cell Lysis Buffer (10X) and maintained on ice for 5 min. The cells were then scraped and sonicated briefly (3 pulses of 5s at 10W) using a sonicator UW3100 (Bandelin electronics, Berlin). The extracts were centrifuged at 14000 x g for 10 min at 4°C. The supernatant was collected for protein quantification by reaction with Bicinchoninic Acid Solution-KIT [361] according to manufacturer's instructions.

Preparation of crude membranes

After the incubation cells were washed twice with cold PBS, treated with 5mL of a PBS solution 2mM EDTA, 200μM Phenylmethylsulfonyl Fluoride and scraped.

The cells were collected and centrifuged in 4°C at 1000 g for 5 min. The pellets were lysed by agitation for 40 min on ice with an hypotonic solution 1mM Sodium bicarbonate pH 7.4, sonicated with 10 pulses of 5 sec at 10W (UW3100 sonicator, Bandelin electronics, Berlin) and centrifuged for 1h at 33000 rpm at 4°C.
The resulting membrane pellets were resuspended with 300-400µL of 250mM sucrose, 10mM Hepes pH 7.4 and homogenized. The supernatant was collected for protein quantification by reaction with Bicinchoninic Acid Solution-KIT [361] according to manufacturer's instructions.

MTT assay

The cell viability in terms of mitochondrial activity was determined by the MTT assay [362,363]. Typically the cells were cultured in a 6 multiwell plate. After the treatment the cell culture medium was removed the wells were washed with a 9.6g/L solution of PBS pH 7.2-7.6. 50µL of a MTT stock dye solution (5mg/ml in PBS) was added to each well containing 450µL of fresh medium. The plates were incubated at 37 °C under 5% CO2 for 1h. The medium from each well was removed and 500µL of DMSO (Sigma-Aldrich D5879-L) were added to dissolve the purple formazan crystals. The plates were shaken for 10min and the absorbance for each well was read on microplate reader (Beckman Coulter LD 400C Luminescence detector) at 570nm. The fractional absorbance was calculated by the following formula: % Cell survival = (mean absorbance in test well)/(mean absorbance in control wells) * 100 as described by Chearwae and coll. [364,365].

TASK 1. MULTIDRUG RESISTANCE IN HEPATOCELLULAR CARCINOMA

LC_{50} determination and MTT assay

A 24 multiwell plate for each cell line was prepared seeding 40000 cells/cm^2, cells were incubated at 37°C under 5% CO2 for 24h and then treated for 24 and 48h with different concentrations of Dox 0.5-1-5-15-30-60-120μM.
The cell viability in terms of mitochondrial activity in each well was determined by the MTT assay [362,363] as previously described. Dox dose-response curve was plotted with SigmaPlot version 11 [366] the LC_{50} (the drug concentration where 50% of cells die) was calculated for each experiment based on the best curve fit. From each curve equation the value of the LC50 was extrapolated. With the same equation the experimental drug concentrations were selected in order to kill approximately 30% of the cells (LC_{30}).

Drug treatments

The cells were seeded at the proper density (Table 3) and incubated at 37°C in a 5% CO2 incubator overnight. The day after the three cell lines were treated with their respective LC_{30} (2μM for IHH, 1μM for HuH7, 3μM for JHH6). In control samples Dox was substituted with the same amount of physiological solution. After 24h and 48h the total RNA, total proteins and crude membrane proteins were extracted as previously described.

Table 3. Seeding density for each cell line.

Cell line	Seeding density
IHH	40000 cell/cm^2
HuH7	40000 cell/cm^2
JHH6	30000 cell/cm^2

SDS-page Western Blot analysis

Total proteins (10μg for IHH and JHH6; 50μg for Huh7) and crude membrane proteins (5μg for IHH and JHH6; 15μg for Huh7) were solubilised in Laemmli Buffer 5X and 10% β-mercaptoethanol, separated with 10% SDS/PAGE gel and transferred to nitrocellulose

membranes by electroblotting, using 25mM Tris Base, 192mM glycine, 20% methanol as transfer solution.

After the transfer the membranes were blocked for 1h in 4% bovine milk/bovine serum albumin in TTBS (100mMTris Base, 2.5M NaCl, TWEEN 20 1% pH 7.5). Subsequently the membranes were incubated overnight at 4°C with the respective primary antibodies in blocking solution at the dilution reported in Table 4. After washing 3 times for 10 min in blocking solution, immune-complexes were detected with the respective secondary antibodies after 60 min incubation (Table 4).

Latter, membranes were washed (3 x 5 min Blocking solution, 1 x 5 min T-TBS 1 x 5 min TBS) and the bands were visualizated using Luminata^{TM} Western HRP substrate by following the manufacturer's protocol.

Table 4. Specific antibodies used for the SDS-page Western Blot analysis.

Protein	Blocking Solution	Primary Antibody	Dilution	Secondary Antibody	Dilution
ABCB1	Milk in T-TBS	C219	1:100	anti-mouse	1:4000
ABCC1	Milk in T-TBS	A23	1:600	anti-rabbit	1:2000
ABCG2	BSA in T-TBS	BXP-53	1:100	anti-rat	1:6000
ACTIN	Milk in T-TBS	A2066	1:4000	anti-rabbit	1:4000

Fluorescence microscopy

The cells were allowed to grow on glass coverslips, after the drug treatment cells were fixed with paraformaldehyde 3% in PBS for 20 min at room temperature(RT), they were washed twice with PBS, blocked for 2h at RTwith blocking solution (PBS, 5% v/v Normal Goat Serum, 1% w/v Bovine Serum Albumin, 0,3% v/v TritonX100) and incubated for 10 min at RT with Hoechst H33258 stain solution, washed twice with PBS and once with water, mounted and analysed under a fluorescence microscope (Leica DM2000, Wetzlar Germany).

Confocal analysis

JHH6 cells were treated with 3μM of Dox. Confocal images were acquired with Nikon C1 laser scanning confocal microscope (Nikon D-eclipse C1Si, Japan) with 100_/1.49 oil Apo TIRF objective (Nikon, Japan) at different time points (10min and 1h). The fluorophore excitation was performed with an air-cooled argon laser at 488 nm and appropriate filter sets were used to collect the fluorescence emission. Images were acquired and analyzed using the Nikon provided operation EZ-C1 software.

TASK 2. TELOMERASE SILENCING EFFECTS IN HCC

Tissue samples screening

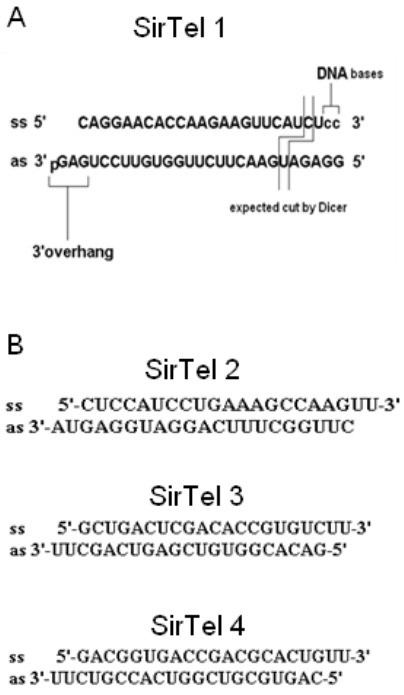
From the same diseased liver three samples were collected: tumoral (neoplastic lesion), pheripheral (lesion surrounding tissue) and distal. A total of 57 HCC samples (21 tumoral, 18 peripheral, 18 distal) and 11 non tumoral liver samples were collected from Cattinara Hospital in Trieste. Tissues were homogenized, total RNA was extracted and reverse transcription-qPCR was performed as previously described.

siRNA design

Homo sapiens telomerase reverse transcriptase (hTERT) transcript variant 1 (NM_198253.2) and 2 (NM_001193376.1) mRNA sequence were obtained from Nucleotide database (www.ncbi.nlm.nih.gov). Predicted mRNA secondary structure were obtained by using mfold Web server version 2.3 [367] and UNAfold Software [368].

siRNA against Telomerase were designed using SiDE [369], siRNA Target Finder [370], BLOCK-iT^{TM} RNAi Designer (Invitrogen, Life Technologies Corporation, Grand Island, NY), DEQOR [371]. The best output sequences were manually mapped in the predicted mRNA secondary structure. Only the antisense sequences targeting 3'- and 5'-loop and loop structures where proceeded for further analysis.

Figure 1. siRNA designed.



There were selected four siRNA sequences, one of them (SirTel 1) was manually modified to create the final siRNA sequence reported in Fig. 1A. The siRNA was synthesized by Sigma-Aldrich (Sigma-Aldrich 3050 Spruce St. St. Louis, MO 63103). The remaining siRNAs (SirTel 2, SirTel 3 and SirTel 4) (Fig. 1B) were synthesized with *Silencer*® siRNA Construction Kit according to manufacturer's instructions.

Silencing experiments

siLentFect toxicity

In order to evaluate siLentFect Lipid Reagent for RNAi toxicity the day before transfection, JHH6 cell were seeded ($9x10^3$ cells/cm^2) in a 24-well plates in serum-containing William's E medium. The cells were incubated at 37°C in a 5% CO_2 incubator overnight. Sixty minutes prior to transfection, medium from each well was carefully removed and replaced with 250µL of fresh growth medium. Different concentrations of siLentFect Lipid Reagent for RNAi (0- 0.25-0.5- 1- 1.25-1.5-1.75µL) were added to 250µL of serum-free William's E medium and then dispensed into the wells (total volume 0.5mL). After 72h cell viability was determined by the MTT assay [362,363] as described previously. All experiments were performed in triplicate.

Silencing

The day before transfection, JHH6 cell were seeded ($9x10^3$ cells/cm^2) in a 6-well plate in serum-containing William's E medium. Sixty minutes prior to transfection, medium from the wells was carefully aspirated and replaced with 1mL of fresh growth medium to each well. For each well to be transfected, 500µL of serum-free medium containing 1.2µL of siLentFect Lipid Reagent for RNAi and 500µL of serum-free medium containing siRNA (final concentration 100, 200, 400nM) were prepared. The diluted siRNA and the diluted siLentFect were mixed and incubated at RT for 20 min (final volume 1mL). The complexes were directly added to cells in serum-containing medium (final volume 2mL; final siRNA concentration 25, 50, 100nM). In each plate three controls were included: cells -siRNA/- siLentFect; cells -siRNA/+siLentFect; cells +siRNA/-siLentFect. The plate was incubated at 37°C in a 5% CO2 incubator for 72h. All experiments were performed in triplicate for all the four siRNAs designed. After 72h silenced cells were harvested, the total RNA was extracted using Tri-Reagent Kit according to manufacturer's instructions (see general procedures section) and reverse transcription-qPCR was performed as previously described.

Fitch conjugation transfection efficiency

To 5µg of siRNA were added 1.5 volumes of nuclease-free water, 1 volume of sodium bicarbonate 1.2M, 2 volume of FITC solution 40mM. The mixture was incubated at RT for 24h in the dark with occasional vortexing. The labelled siRNA was precipitated overnight at -20°C by adding 1/10volume of sodium acetate 3M pH 5.2 and 2 volumes of absolute ethanol. The mixture was centrifuged at 8000g for 15 min at 4°C and washed with 70% ethanol. The pellet was dried at RT and then resuspended in nuclease-free water.

The base/FITC ratio was calculated by spectrophotometric analysis in a Beckman DU730 spectrophotometer.

JHH6 cells were silenced with 25nM and 100nM of FITC labelled siRNA as mentioned before. Cells were analysed 24h, 48h and 72h post-transfection with Nikon C1 laser scanning confocal microscope (Nikon D-eclipse C1Si, Japan) with 100/1.49 oil Apo TIRF objective (Nikon, Japan). The fluorophore excitation was performed with an air-cooled argon laser at 488 nm and appropriate filter sets were used to collect the fluorescence emission. Images were acquired and analyzed using the Nikon provided operation EZ-C1 software.

FITC intracellular fluorescence was also determined by flow cytofluorimetry using a Becton Dickinson FACSCalibur System, following excitation with an argon ion laser source at 488 nm and appropriate filter sets. Data were collected in 10,000 cells and analyzed using Cellquest software from BD Biosciences (San Jose, CA, USA).

Silenced fibroblast viability

Fibroblast primary cell culture was used as a telomerase negative control for the silencing experiments. Primary fibroblasts cultures were obtained from human healthy donors. Cells were cultured in RPMI 1680 medium with 10% (v/v) FBS, 1% L-glutamine 100X, 1% penicillin/streptomycin 100X. The day before transfection, fibroblasts were seeded ($3x10^4$ cells/cm^2) in a 6-well plate in serum-containing RPMI 1680 medium.

The cells were incubated overnight. Cells were treated with 25-50-100nM of SirTel 1 following the same procedure used for JHH6 cells. Control cells without treatment were included into analysis. After 72h of incubation cell viability was determined by the MTT assay [362,363] as previously described.

TRAP

The day before transfection, JHH6 cell and human primary fibroblasts were seeded ($9x10^3$ cells/cm^2) in a 6-well plate. The cells were transfected with 25nM of SirTel 1 as mentioned before. After 72h telomerase activity was assessed using TRAPeze® kit (Millipore Corporation, Billerica, MA) according to manufacturer's instructions.

Time course experiments

The day before transfection, JHH6 cell were seeded ($9x10^3$ cells/cm^2) in a 6-well plate in serum-containing William's E medium. The cells were transfected with 25nM of SirTel 1 as previously described. The hTERT and albumin mRNA expression was followed in time by quantitative Real time PCR at 24-48-72-96-120-168-216-264-312h post-treatment.
In re-exposure experiments silenced cells were re-exposed at 72h with 25nM of SirTel 1, hTERT expression was followed in time by quantitative Real time PCR at 120-168-216-264h post-treatment.

SirTel-1 *vs.* Dox cell viability

The day before transfection, JHH6 were seeded ($9x10^3$ cells/cm^2) in a 6-well plate in serum-containing William's E medium.
The cells were incubated at 37°C in a 5% CO_2 incubator overnight. Cells were silenced either with 25-50-100nM of SirTel 1 either with 25-50-100nM Dox as previously described. Control cells without treatment were included into analysis. After 72h of incubation cell viability was determined by the MTT assay [362,363] as previously described.

Cell cycle FACS analysis

After 72h, silenced cells were detached, pelleted, washed twice with PBS and then resuspended in 500µL PBS. With a glass Pasteur pipet the cells were homogenized in order to disaggregate the eventual groups of cells for obtaining a single cell suspension, subsequently

they were transferred to a fresh tube containing 4.5mL of ethanol 70%. Fixed cells were pelleted, to remove the ethanol, and resuspended in 1mL of staining solution (0.1% v/v TritonX-100 in PBS; 0.02 mg/mL PI; 0.2 mg/mL RNAse A). After 30 min of RT incubation, cellular DNA content was measured by flow cytofluorimetry using a Becton Dickinson FACSCalibur System, following excitation with an argon ion laser source at 488nm and appropriate filter sets. Data were collected in 10,000 cells and analyzed using Cellquest software from BD Biosciences (San Jose, CA, USA). The percentage of cells in G0/G1, S and G2/M was determined from DNA content histograms.

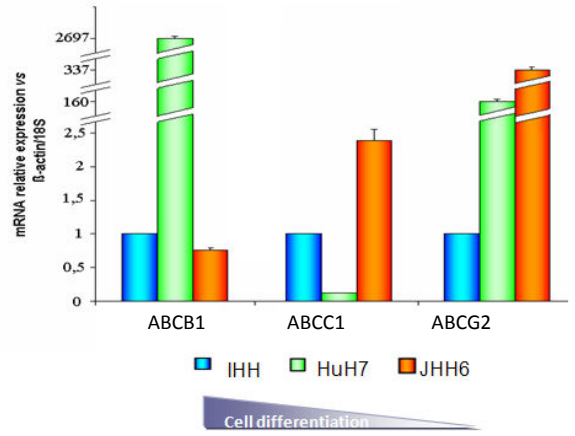
RESULTS

TASK 1. MULTIDRUG RESISTANCE IN HEPATOCELLULAR CARCINOMA

ABCs basal mRNA expression levels

Our *in vitro* model for studying the role of ABC transporters in MDR in HCC comprehends three hepatic cell lines: IHH (immortalized hepatocytes, non tumoral control), HuH7 (well differentiated hepatocyte derived cellular carcinoma *cell line*), in spite of the tumoral origin of this cell line, some of the main hepatocyte's characteristics are conserved (albumin production), JHH6 (poorly differentiated hepatocytes derived cellular carcinoma *cell line*). The basal mRNA expression levels of ABCB1, ABCC1 and ABCG2 were assessed in all the three cell lines and are reported in Figure 1. ABCB1 is mainly expressed in Huh7 cells which have also the lowest ABCC1 expression in comparison with IHH and JHH6. ABCG2 mRNA expression is inversely correlated with the differentiation grade of the cells.

Figure 1. ABCs basal mRNA expression levels.



Drug treatments and LC_{50} determination

In order to establish the cellular sensitivity to Dox viability/dose (0.5-1-5-15-30-60-120μM) curves were plotted for each cell line under study and the lethal dose 50 (LC_{50}) was calculated for each experiment based on the best curve fit. From each curve equation the value of the LC_{50} was extrapolated (Table 1). With the same equation the experimental drug concentrations were selected in order to kill approximately 30% of the cells (LC_{30}) (Table 1).

Table 1. LC_{50} and Dox experimental concentrations.

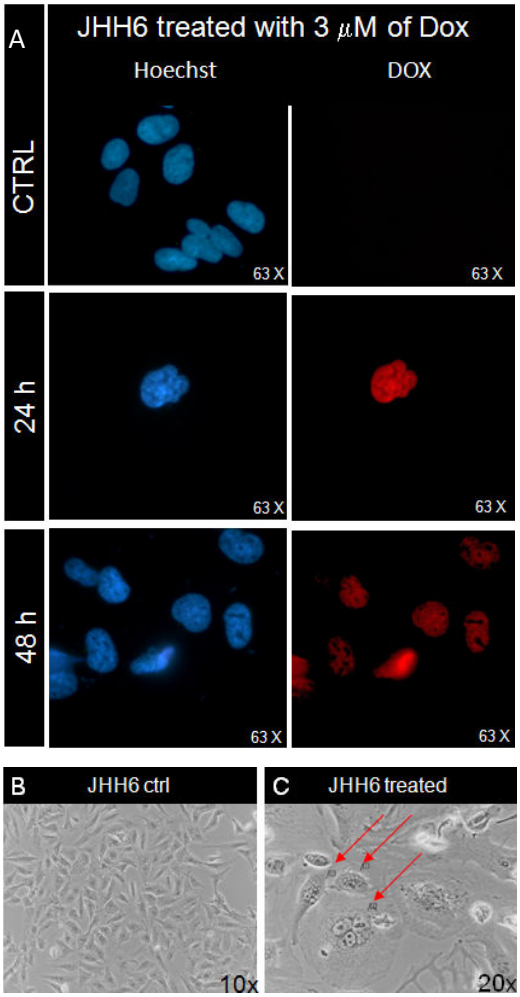
Cells	LC_{50} μM S.D. (24h)	Exp. concentrations (LC_{30} μM)
IHH	4.5±0.3	2.0
Huh7	4.3±0.3	1.0
JHH6	4.5±0.8	3.0

Dox cellular uptake and induced damages

Dox fluorescent properties ($l_{ex.}$470nm $l_{em.}$ 585nm) allow to detect its presence within the cell. Confocal analysis revealed that JHH6 cells treated with 3µM of Dox showed a nuclear Dox staining evident after 10 min (data not shown).

For the fluorescence microscopical analysis cells were exposed at their respective experimental Dox concentration. The cell nuclei were stained with Hoechst. After 24h was evident a co-localization of Dox and Hoechst denoting the nuclear drug accumulation. After 48h morphological alterations were evident denoting a marked cell damage (Fig. 2). After Dox exposure bright field microscopy analysis leaded to the identification of altered cellular shapes with unequal cell size (35-345µm) and unclear cellular profile (Fig. 2C). Moreover multinuclear cells were also observed. These features contrast with the normal cell morphology with fusiform shapes, homogeneous and well-distributed sizes (35-55µm) and distinct boundaries (Fig. 2B).

Figure 2. nuclear Dox accumulation and morphological changes.



(A) Flourescence microscopy image of JHH6 cells treated with 3µM of Dox. (left) Cell nuclei are stained with hoescht (blue), (right) nuclei containing Dox. (B) Bright field microscopy of control JHH6 cells and treated cells (C), red arrows indicate multinucleated cells or apoptotic cells.

ABC mRNA and protein expression in IHH cells

IHH cells were exposed to 2µM of Dox for 24h and 48h. ABCC1 mRNA expression significantly decreases both at 24h ($p<0.05$) and 48h ($p<0.001$) (Fig. 3B).

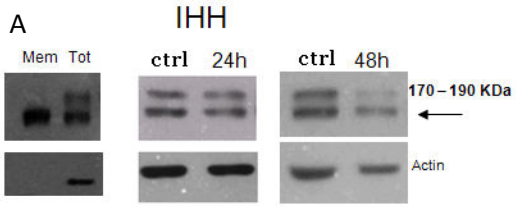
ABCC1 protein expression significantly decreases only after 48h Dox exposure ($p<0.05$) (Fig. 3B).

Table 2. ABCB1 and ABCG2 mRNA expression.

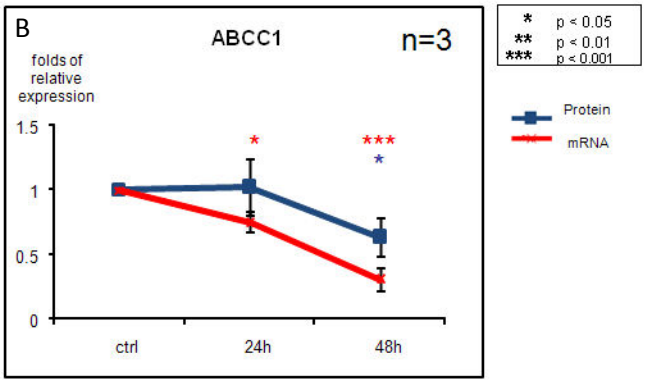
ABC protein	Time	Folds of relative expression *vs.* ctrl
ABCB1	24h	**15.01**±1.69 ($p<0.001$)
	48h	**13.28**±2.18 ($p<0.001$)
ABCG2	24h	**4.72**±1.08 ($p<0.01$)
	48h	**3.51**±1.53 ($p<0.05$)

ABCB1 and ABCG2 proteins were undetectable by SDS-page western blot due to their very low expression levels, consequently the analysis was performed only at mRNA level (Table 2). Both ABCB1 and ABCG2 mRNA expression significantly increases after 24h ($p<0.001$ and $p<0.05$ respectively). After 48h the mRNA expression remains significantly higher when compared with controls for both genes ($p<0.001$ and $p<0.01$ respectively), although there is a trend of decrease both cases. Results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Figure 3. ABCC1 mRNA and protein expression in IHH cells.



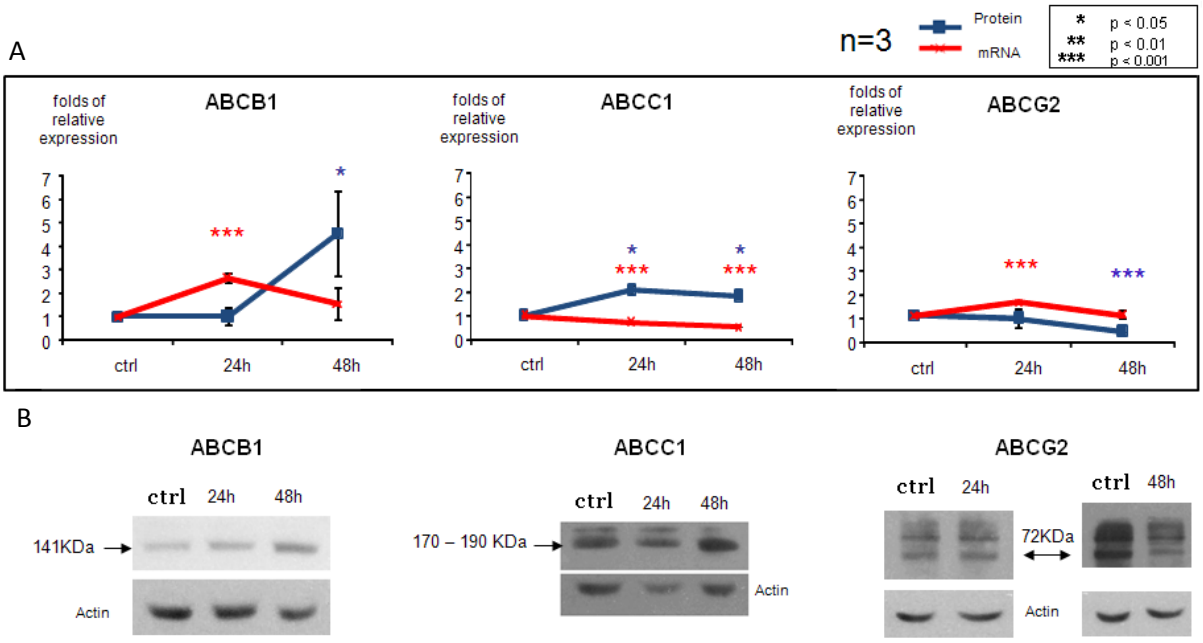
(A) Representative western blot of ABCC1 at 24h and 48h *vs.* controls (ctrl). 10µg of total protein and 5µg of membrane protein extracts were loaded. The row indicates the ABCC1 corresponding band at the predicted molecular weight. (B) ABCC1 mRNA and protein relative expression. mRNA levels (red line) are reported as folds of expression *vs.*β-actin/18S. Protein expression (blue line) was normalized to actin.



ABC mRNA and protein expression in Huh7 cells

HuH7 cells were treated with 1µM of Dox for 24h and 48h. ABCB1 mRNA expression significantly increases at 24h ($p<0.001$) before restoring the basal levels after 48h. Protein expression significantly increases only after 48h ($p<0.05$) (Fig. 4A). Regarding ABCC1 mRNA expression significantly decreases both at 24h ($p<0.001$) and 48h ($p<0.001$), on the contrary protein levels increases after 24h ($p<0.05$) and remain higher also at 48h ($p<0.05$) (Fig. 4A). For ABCG2 mRNA expression significantly increases at 24h ($p<0.001$) before returning to basal levels after 48h. There is a ABCG2 protein downregulation which reaches the maximum at 48h ($p<0.001$) (Fig. 4A). Results are expressed as mean ± S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Figure 4. ABCs mRNA and protein expression in HuH7 cells.



(A) ABCB1, ABCC1 and ABCG2 mRNA and protein relative expression. mRNA levels (red lines) are reported as folds of expression *vs.*β-actin/18S. Protein expression (blue lines) was normalized to actin. (B) representative western blot 24h and 48h *vs.* controls (ctrl). 30µg, 50µg and 60µg of total protein extracts were loaded for ABCB1, ABCC1 and ABCG2 detection respectively. The black rows indicates corresponding band at the predicted molecular weight for each ABC protein.

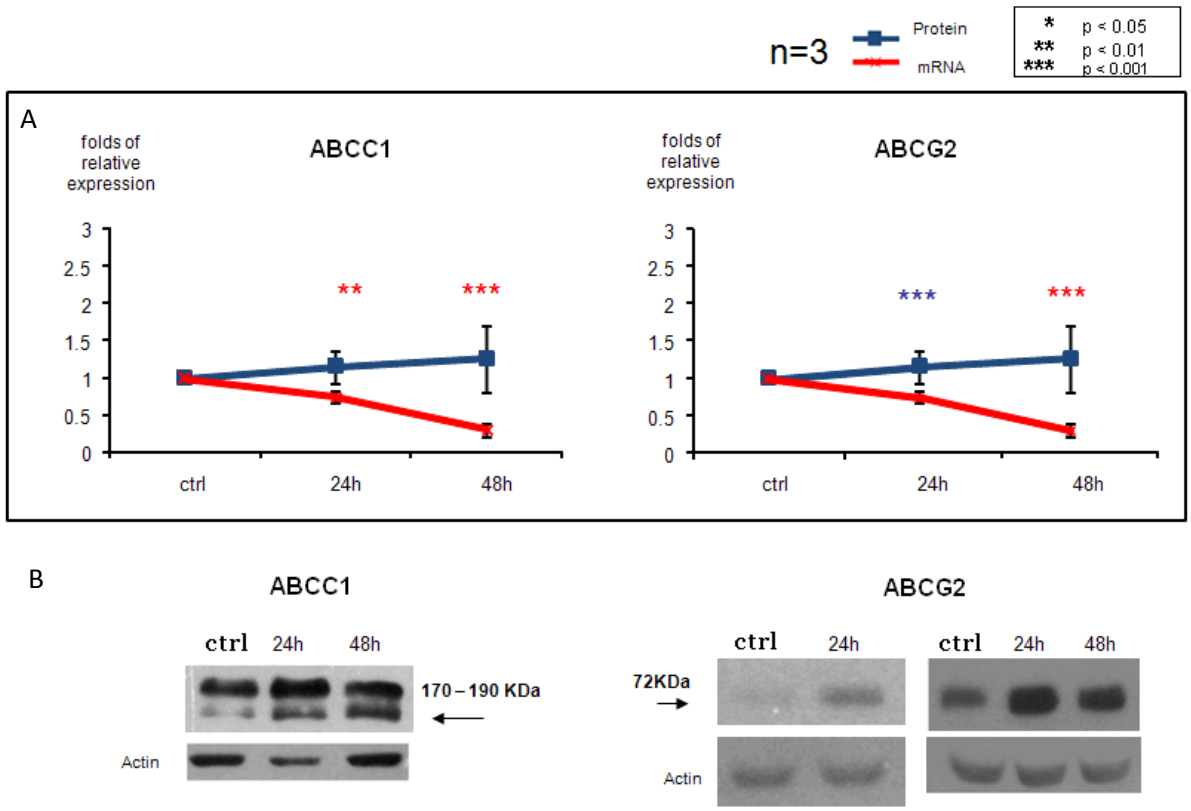
ABC mRNA and protein expression in JHH6 cells

JHH6 cells were treated with 3µM of Dox for 24h and 48h. ABCB1 protein expression was undetectable by SDS-page western blot due to the very low expression level consequently only mRNA expression data are available (Table 3). ABCB1 mRNA levels significantly increase after 24h ($p<0.001$) as well as after 48h treatment ($p<0.001$). ABCC1 mRNA expression in JHH6 cells significantly decreases both at 24h ($p<0.01$) and 48h ($p<0.001$). On the contrary ABCC1 protein expression increases at both times, although its values does not reach significant (Fig. 5). ABCG2 mRNA expression decreases at both experimental times being significant only at 48h ($p<0.001$). On the contrary the protein expression increases both at 24h and 48h being significant only at 24h ($p<0.001$) (Fig. 5). Results are expressed as mean ±S.D. from 3 independent experiments and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Table 3. ABCB1 mRNA expression.

ABC protein	Time	Folds of relative expression *vs.* ctrl
ABCB1	24h	**8.71**±1.28 ($p<0.001$)
	48h	**30.28**±0.04 ($p<0.001$)

Figure 5. ABCs mRNA and protein expression in JHH6 cells.



(A) ABCC1 and ABCG2 mRNA and protein relative expression. mRNA levels (red lines) are reported as folds of expression *vs.*β-actin/18S. Protein expression (blue lines) was normalized to actin. (B) Representative western Blot 24h and 48h *vs.* controls (ctrl). 10µg and 50µg of total protein extracts were loaded for ABCB1 and ABCG2 detection respectively. The black rows indicates corresponding band at the predicted molecular weight for each ABC protein.

TASK 2. TELOMERASE SILENCING EFFECTS IN HCC

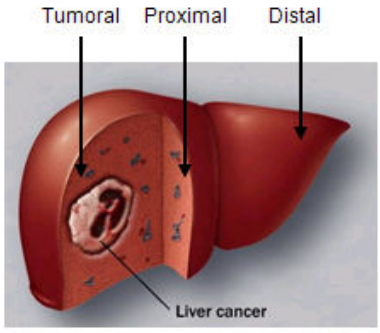
This study is part of a macro-project whose main objective is to use a liver specific delivery system to target tumoral genes. In particular the aim of this section is to selectively silence a cancer related and specific gene in HCC. Gene silencing could represent a future alternative for cancer treatments as an alternative of chemotherapy.

Target selection

Based on literature, a set of 4 genes (aurora kinase A, midkine, survivin and telomerase) was selected as possible candidates for gene silencing. The selection criteria taken into consideration were that the target gene should:

- ✓ Be essential for cancer cell survival, growing and maintenance.
- ✓ Be over-expressed or exclusively expressed in tumoral cells.
- ✓ Be involved in as less pathways as possible.

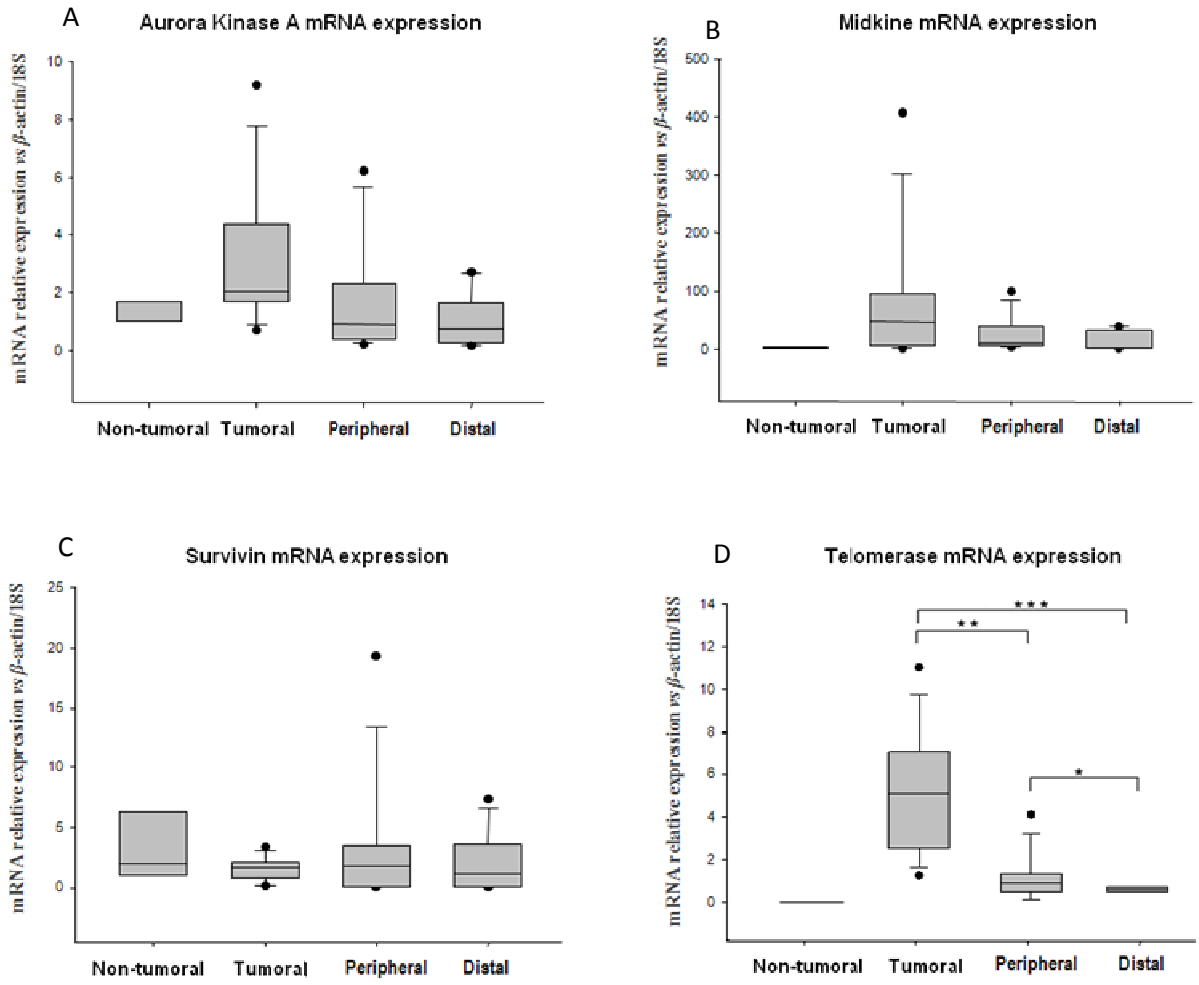
Figure 6. Liver cancer sampling.



For each gene the mRNA expression was assessed in human HCC derived samples (n=22 tumoral, n=18 peripheral and n=18 distal; see Figure 6 for details) as well as in normal liver (n=11).

Aurora kinase A resulted upregulated in tumoral samples although its levels did not reach the significance (Fig. 7A). A very high midkine mRNA expression was observed in tumoral samples in comparison with controls (Fig. 7B). However there were no statistically significant differences among the considered groups due to the high standard deviations. Survivin was not differently expressed among controls, tumoral, peripheral and distal samples (Fig. 7C). In contrast with all the potential target genes under study, telomerase was not expressed in the eleven non-tumoral samples, moreover a statistically significant increased mRNA expression in the tumoral portion of the diseased liver compared with the peripheral ($p<0.01$) and the distal ($p<0.001$) ones was observed (Fig. 7D). This results pointed to the conclusion that telomerase would represent the appropriate gene since is exclusively expressed in the tumoral portions, with no detectable levels in healthy tissue. For this reason, the following experiments include this gene.

Figure 7. mRNA relative expression in HCC human samples.



mRNA levels are reported as folds of expression *vs.*β-actin/18S. For (A) aurora kinase A, (B) midkine and (C) surviving the expression in tumoral, peripheral and distal samples is reported in relation to the non-tumoral samples. (D) Telomerase tumoral and peripheral mRNA expression is reported as relative to the distal samples.

siRNA design

The siRNAs against telomerase were disegned on the catalytic subunit of the enzyme (hTERT) which is the activity rate limiting subunit. The siRNAs were designed in the homology region of the two known hTERT mRNA sequences (NM_001193376.1; NM_198253.2).

For the design the siRNAs were taken into consideration both sequence-based limitations (siRNA and mRNA) [369,372-375] and structure-based limitations (target mRNA) [376,377]. siRNA sequence-based limitations are:

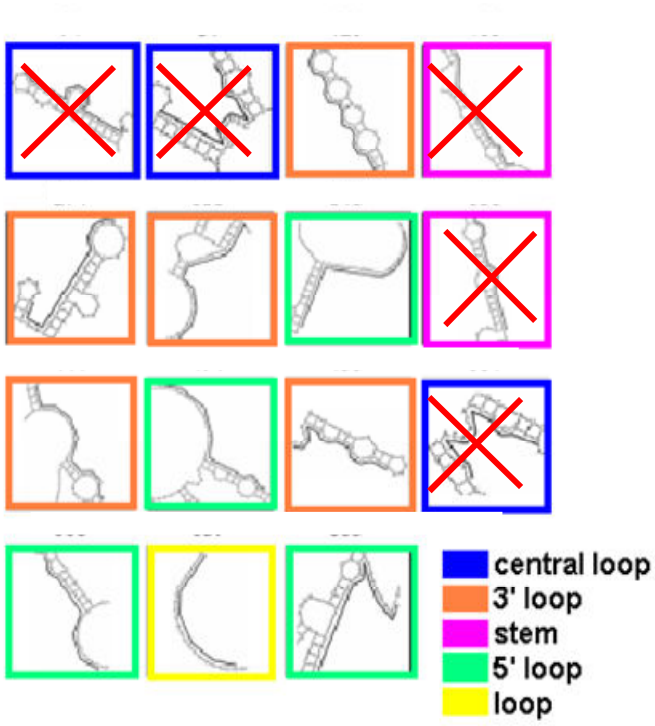
- G/C 36-53%
- base at 5' AS(antisense strand) should be A/U
- base 13-19 AS A/U at least 3/4
- base 16 AS A/U
- base 1 at 5' SS G/C
- avoid AAAA TTTT GGG/CCC motifs and GCs stretches
- G/C region 13-19 ≤19% 2-12 ~ 52%
- ΔG Kcal/mol at 5'-AS terminal, region 9-14 less than -8,5
- no ΔG < 13 Kcal/mol in region 7-12

mRNA sequence-based limitations are:

- avoid 3' UTR or 5' UTR
- avoid regions closer to STOP codons, and splicing sites
- keep 75nt distance from the start site
- avoid AAAA TTTT GGG/CCC motifs and GCs stretches

Based on these consideration of the sequence-based rules, siRNAs were designed using free web tools such as RNAi Target Sequence Selector, BLOCK-iT™ RNAi designer, SIDE, Deqor, siRNA Target Finder. There is evidence that target's secondary structures can influence silencing efficiency [376,377]. For instance, mRNA secondary structures such as stem and loop derived structures can influence accessibility of the siRNA to the target, siRNA/mRNA annealing and duplex stability (Fig. 8). In particular

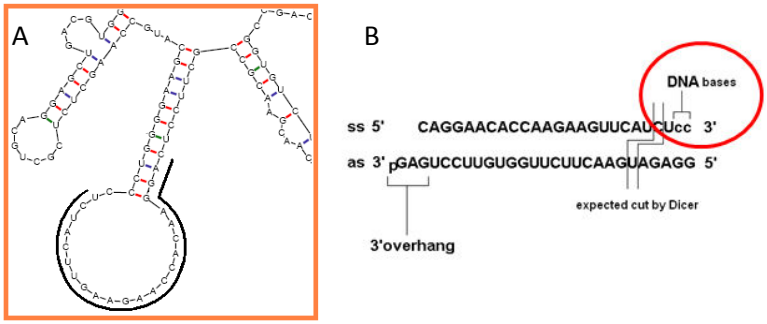
Figure 8. mRNA secondary structures.



central loops and stem structures seem to inhibit the silencing efficacy [376,377]. For these reasons the target mRNA sequence was folded using mFold, a web server tool for nucleic acid folding (http://mfold.rna.albany.edu/?q=mfold/ RNA-Folding-Form) and UNA-fold, a software for nucleic acid folding and hybridization. The target sequence was fractioned in 800 bases strings with each one with 200 bases of overlap. The strings were folded as well as the whole sequence. Only the secondary structures that were conserved in both string and whole sequence were considered suitable siRNA target regions.

All the siRNAs selected using the web tools mentioned above were manually alligned to the secondary target structure. Only one sequence, targeting a loop structure (Fig. 9A), was selected for further modifications such as the addition of two DNA bases at 3'end of the sense strand (ss) and a 3' overhang of the antisense strand (as) to became a direct Dicer substrate (Fig. 9B). Dicer is a RNase III class endonuclease which *in vivo* cuts long dsRNA involved in the RNAi pathways [378]. The siRNA designed was called SirTel 1 and synthetized by Sigma-Aldrich, St.Louis, MO.

Figure 9. SirTel 1 features.



(A) ss targeting a loop structure in the hTERT mRNA (NM_198253.2). (B) SirTel 1 double strand siRNA with two DNA base at the 3' end of the ss (red circle). The expected Dicer cutting is also shown.

Other three siRNAs were designed not following the rules mentioned above due to the restrictions imposed by the kit used for the synthesis (*Silencer*® siRNA Construction Kit; Ambion, Invitrogen, Life Technologies Corporation, Grand Island, NY). The three siRNAs are reported in Table 4.

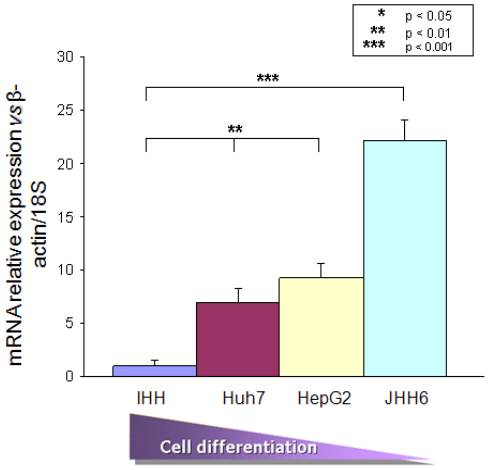
Table 4. siRNA features.

siRNA Name	siRNA sequence	Programs used for siRNA identification	Targeting structure	Position in the seq. NM_198255.2
SirTel 2	ss 5'-CUCCAUCCUGAAAGCCAAGUU-3' as 3'-AUGAGGUAGGACUUUCGGUUC	RNAi Target Sequence Selector	3' loop	3154-72
SirTel 3	ss 5'-GCUGACUCGACACCGUGUCUU-3' as 3'-UUCGACUGAGCUGUGGCACAG-5'	siRNA Target Finder; BLOCK-iT™ RNAi designer	stem	3265-83
SirTel 4	ss 5'-GACGGUGACCGACGCACUGUU-3' as 3'-UUCUGCCACUGGCUGCGUGAC-5'	Deqor	stem	434-51

Choice of *in vitro* cell model

The first approach for selecting a suitable cell model was to assess hTERT expression in several hepatic cell lines like human immortalized hepatocytes (IHH) and in three different HCC derived cell lines with various stages of differentiation Huh7, HepG2 (moderately differentiated HCC derived cells) and JHH6 (poorly differentiated HCC derived cell line).

Figure 10. hTERT mRNA relative expression in different cell lines.



mRNA levels are reported as folds of expression *vs.* β-actin/18S. hTERT mRNA expression is reported as relative to IHH cells.

From the data reported in Figure 10, is possible to conclude that the hTERT mRNA expression is higher in the most undifferentiated cell line, whereas in IHH its expression is minimal.

It worth to be noticed that the IHH cell line constitute a non tumoral cell line, however they express telomerase since they were immortalized with a SV40 viral derived construct containing telomerase gene. All together these results point to the conclusion that JHH6 constitute a valid model for the hTERT silencing, and for this reason this cell line will be used for the future experiments. Results are expressed as mean ± S.D. from at least 3 independent samples and statistical analysis was performed using the Tukey-Kramer Multiple Comparisons Test.

Setting-up the working conditions

siLenFect toxicity, siRNA working concentrations and silencing timing were evaluated in preliminary setting-up experiments (data not shown).

Table 5. Sample organization and controls included in the experimental set-up.

	Cells 9000 cells/cm^2	siLentFect 0.8µL/mL	siRNA 25nM, 50nM, 100nM
Ctrl	√		
Ctrl vehicle	√	√	
Ctrl siRNA	√		√
Treated cells	√	√	√

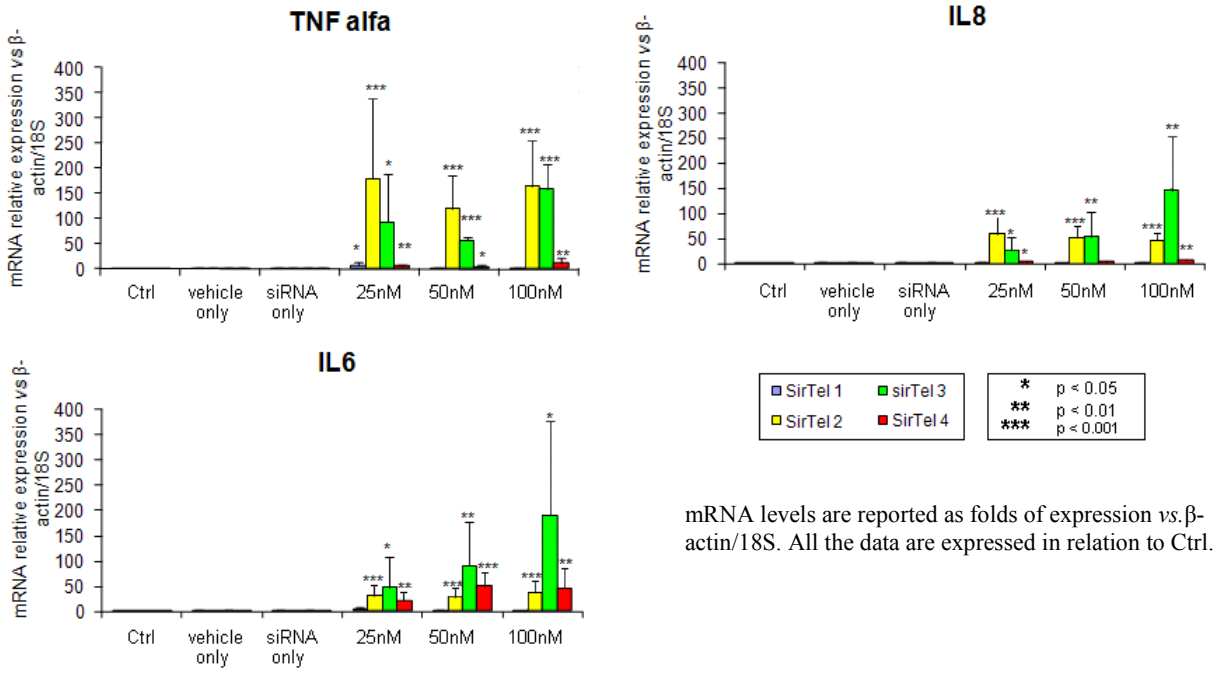
The following working conditions were chosen: 0.8µL/mL of siLenFect and 25, 50, 100nM of siRNA for 72h silencing. For each experiment three controls were included

(Table 5). There was no statistically significant difference in cell viability among the control and cells treated with 0.8µL/mL of siLenFect (data not shown). Transfection efficiency was calculated by Flow cytometry using FITCH conjugated siRNA and it was higher than 75%.

Cytokines mRNA expression

JHH6 were transfected with 25, 50 and 100nM of SirTel 1, SirTel2, SirTel 3 and SirTel 4 and after 72 hours proinflammatory cytokines mRNA expression was assessed (Fig. 11).

Figure 11. Proinflammatory cytokines mRNA relative expression.



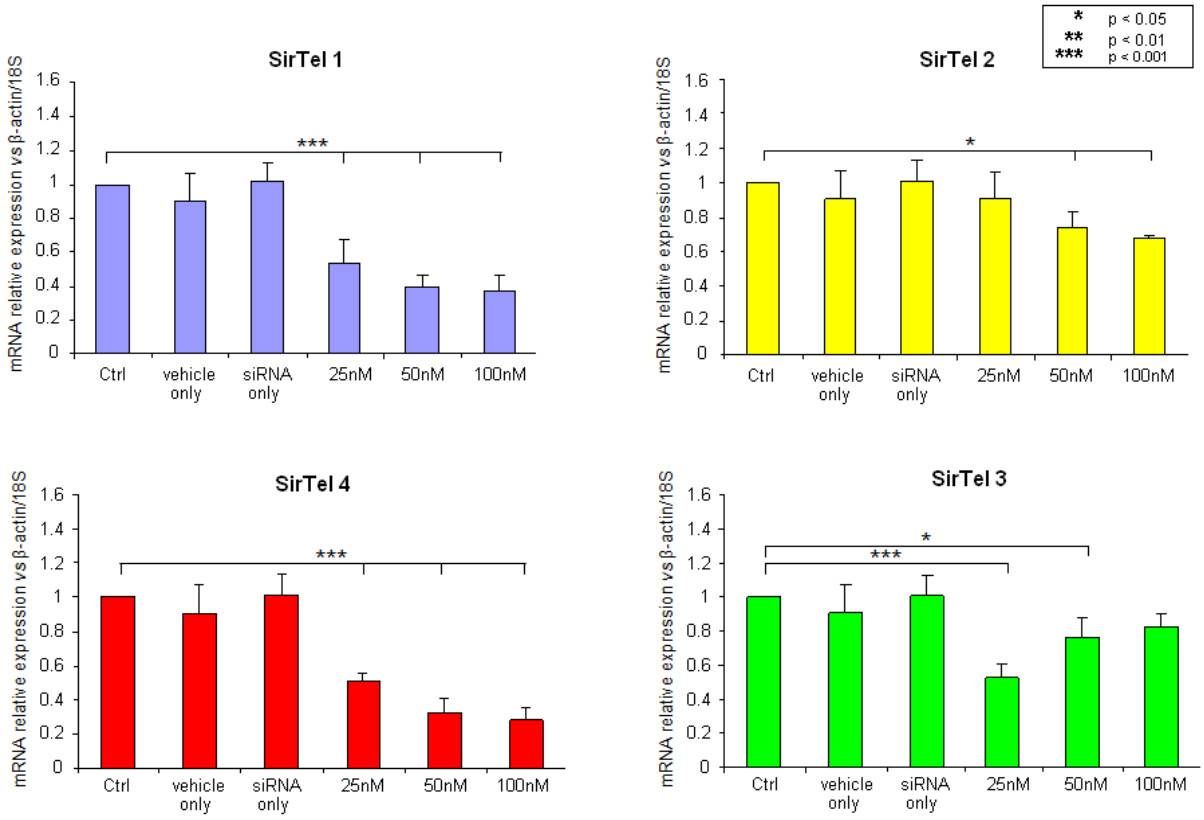
mRNA levels are reported as folds of expression *vs.*β-actin/18S. All the data are expressed in relation to Ctrl.

It was observed an overall significant increased mRNA expression after SirTel 2, SirTel 3 and SirTel 4 transfection at every considered concentration. SirTel 1 only induced a significant increased TNFα mRNA expression at 25nM concentration ($p<0.05$) (Fig. 11). As expected the vehicle and the siRNAs alone have no effect in modulating TNFα, IL6 and IL8 mRNA expression. Results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Telomerase silencing

Telomerase mRNA expression was assessed in JHH6 cells after 72h of SirTel 1, SirTel2, SirTel 3 and SirTel 4 transfection (Fig. 12).

Figure 12. hTERT mRNA relative expression in JHH6 cells



mRNA levels are reported as folds of expression *vs.* β-actin/18S. All the data are expressed in relation to Ctrls.

SirTel 1 and Sirtel 4 significantly reduce the hTERT mRNA expression after 72h silencing at all the considered concentrations ($p<0.001$). SirTel 2 had a silencing effect limited to higher doses, 50nM and 100nm ($p<0.05$). The silencing effect of SirTel 3 is inversely correlated with its concentrations, indeed the higher silencing effect was assessed at 25nM ($p<0.001$). At 50nM the decrease in the mRNA expression is still significant ($p<0.01$) whereas at 100nM there was no more statistically significant difference among the control and the treated sample.

All together these data show that there is non statistically significant difference in hTERT mRNA expression among the three controls considered. Moreover SirTel 1, SirTel 2 and

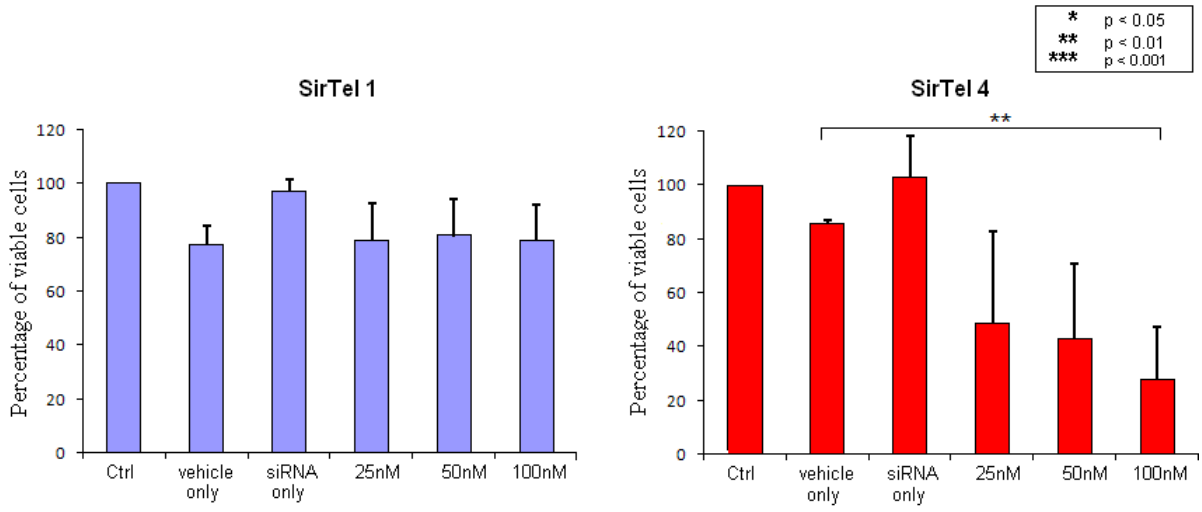
SirTel 4 have a dose dependent silencing effect. However due to the low silencing effect, SirTel 2 and SirTel 3 were excluded for the future determinations.

All the results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Off-target effects assessment

After few years from the siRNA discovery, it became evident that sometimes siRNAs can cross-react with other unspecific mRNA different from their target (off-target) causing, in this way, undesired effects [379]. To evaluate the off-target effect of SirTel 1 and SirTel 4, fibroblast primary cultures were silenced since fibroblasts do not express telomerase. The presence of off-target effect was evaluated in terms of cell viability (Fig. 13).

Figure 13. Cell viability in siRNA treated primary fibroblasts.



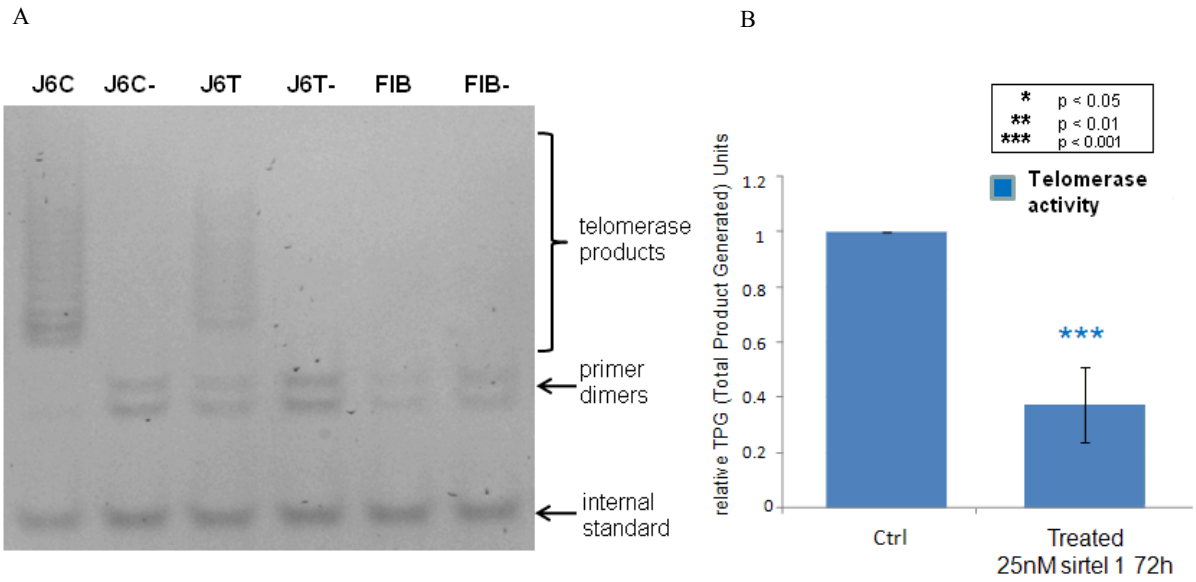
In cells treated with SirTel 1 the variation in cell viability was due to the vehicle by itself, as a matter of fact there was no significant difference between the vehicle control and the treated samples. On the contrary SirTel 4 caused a dose dependent reduction in cell viability which resulted significantly considerable in respect of the vehicle control ($p<0.01$ for 100nM of siRNA). Results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Due to the intrinsic cell toxicity SirTel 4 was discarded. Only SirTel 1 was used for further experiments.

Telomerase enzymatic activity

JHH6 and human derived fibroblasts primary cultures were treated with 25 nM of SirTel 1 for 72h. Protein lysates were collected and Telomeric Repeat Amplification Protocol (TRAP) was performed. Briefly, telomerase present in the lysates elongates oligonucleotides that mimic the telomeres ends. The products generated are then amplified by PCR. In the experiment telomerase heat inactivated samples were included. After hTERT silencing there was a statistically significant decrease in telomerase activity ($p<0.001$) (Fig. 14B). There was no telomerase activity in heat treated cells and in human primary fibroblasts (Fig. 14A). Results are expressed as mean ± S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Figure 14. Telomerase enzymatic activity.

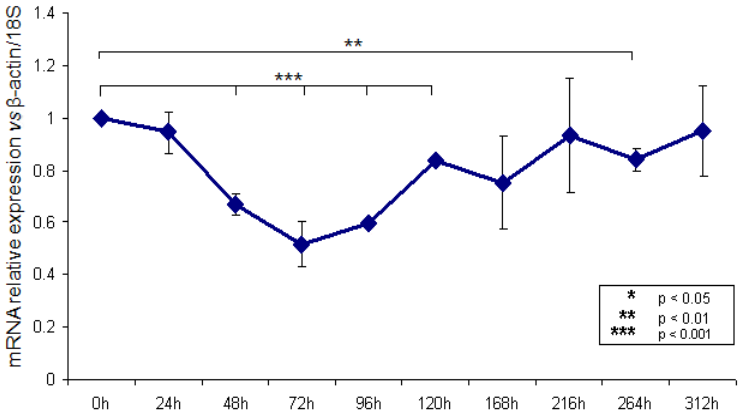


(A) PCR products of telomerase activity were loaded into a polyacrylamide gel (10%). J6C: control JHH6 cells; J6C-: heat treated control JHH6 cells; J6T: silenced JHH6 cells; J6T-: heat treated silenced JHH6 cells; FIB: human primary fibroblast; FIB-: heat treated primary fibroblasts. (B) Telomerase activity was expressed as relative total product generated (TPG) units. Data were normalized using an internal standard control.

Silencing time course

JHH6 cells were treated with 25nM on SirTel 1 and the hTERT mRNA expression was followed along time. hTERT mRNA downregulation reached its maximum after 72h treatment (Fig. 15). After 96h hTERT mRNA expression start to increase to restore the initial levels at 312h after the treatment. Results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the unpaired t-test.

Figure 15. mRNA hTERT relative expression: time course.

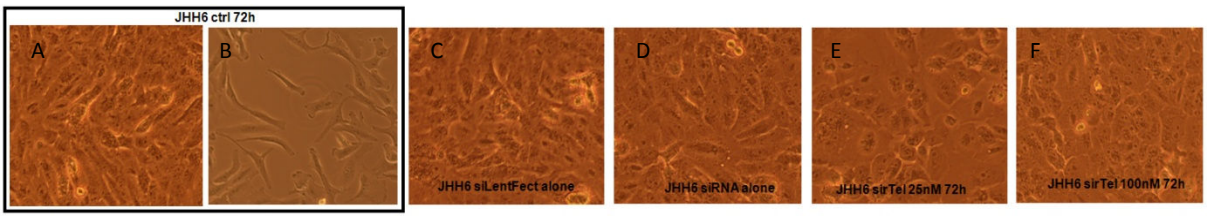


mRNA levels are reported as folds of expression *vs.*β-actin/18S. All data are expressed in relation to the control (untreated cells).

Investigating the hTERT silencing effects

Cell morphology. The first evident effect after 72h of SirTel 1 transfection was a change in cell morphology. By observations with bright filed microscope was appreciated a change in JHH6 cell morphology from a fibroblastic-like shape to an hepatocyte-like shape (Fig. 16).

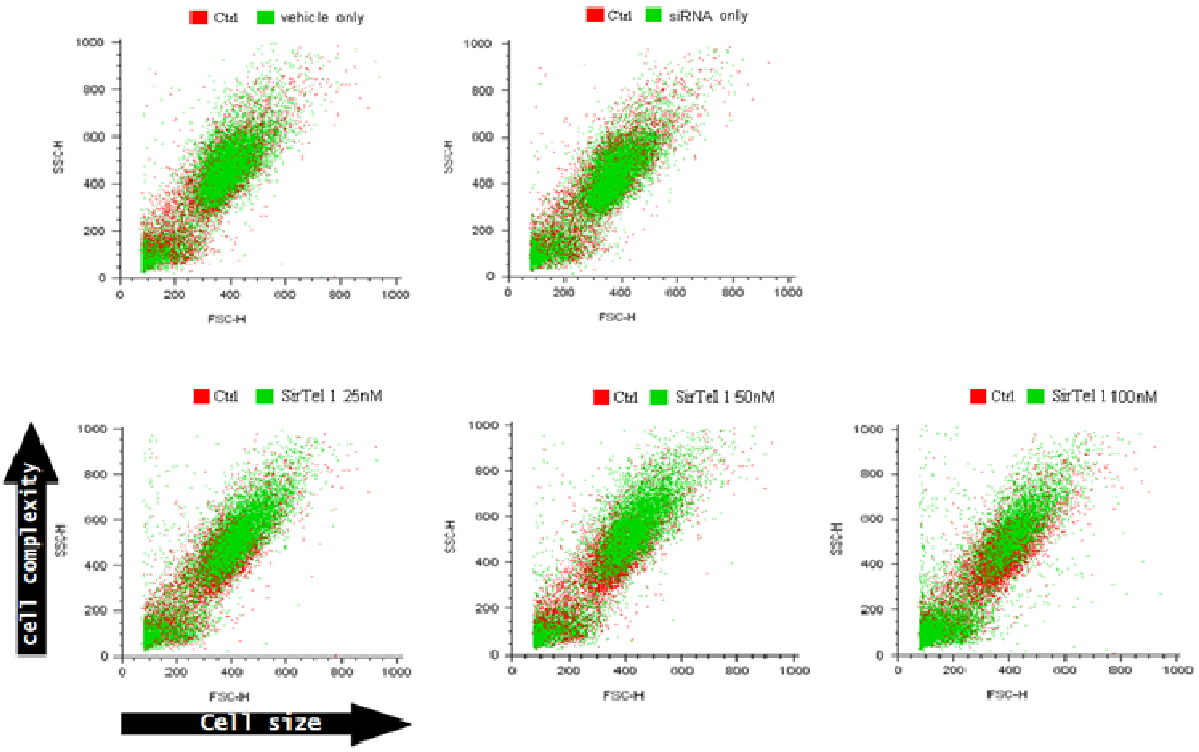
Figure 16. Optical images of JHH6 cells after 72h treatment



Cells were observed under optical microscope (10X). (A)(B) Untreated cells at different confluences. (C)(D) Vehicle and siRNA controls respectively. (E)(F) Treated cells.

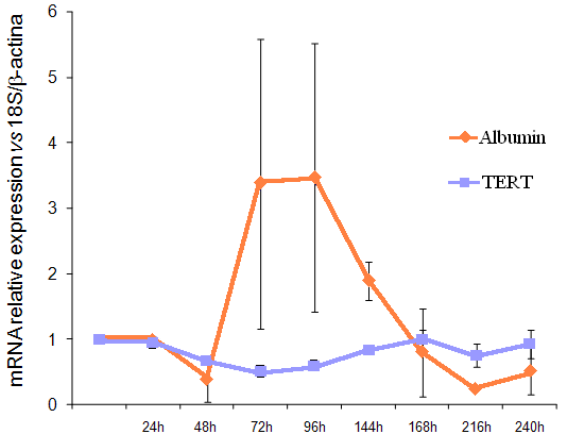
The alteration in cellular morphology was also observed by a Flow Cytometry analysis comparing the forward-side scatter plot of silenced cells *vs.* controls (Fig. 17). The forward-side scatter plot gives information about the size and the internal complexity of the cells. After the hTERT silencing there was a change both in size and complexity of the cells. This is evident by the shift in the green cloud along the x and y axes in the plots reported in Figure 17 compared to the control (red cloud).

Figure 17. Forward-side scatter Plot of JHH6 cells after 72h silencing.



Albumin expression. The morphological chances of JHH6 were associated with an increase in the albumin mRNA expression. The albumin constitute an hepatic hallmark which is normally not express or express at very low levels in poorly differentiated JHH6 cells. After hTERT silencing there was an increased albumin expression, although not significant, that corresponded to the minimum in the hTERT mRNA expression (Fig. 18). Results are expressed as mean ± S.D. from 3 independent samples.

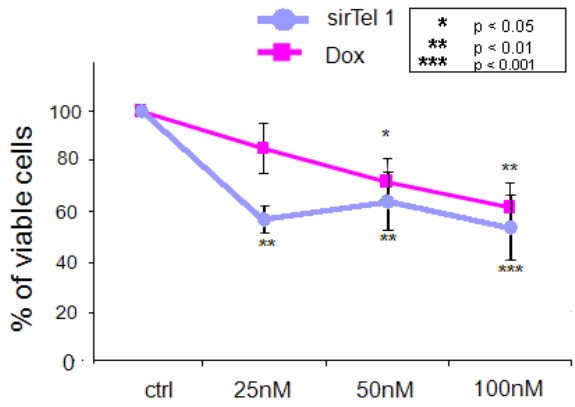
Figure 18. Time course of albumin *vs.* hTERT mRNA expression.



mRNA levels are reported as folds of expression *vs.*β-actin/18S. All the data are expressed in relation to the control (untreated cells).

Cell viability. The treatment for 72h with 25-50-100nM of SirTel 1 induced a significant reduction in JHH6 cells viability (Fig. 19). Furthermore when compared with the toxic effect of the same amount of Dox, SirTel 1 resulted more effective in reducing cell viability especially at lower concentrations with 63% of viable cells after 72h of 25nM siRNA *vs.* 85% of viable cells after 72h of 25nM Dox exposure. Results are expressed as mean ±S.D. from 3 independent samples and statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

Figure 19. JHH6 cells viability after 72h treatment.

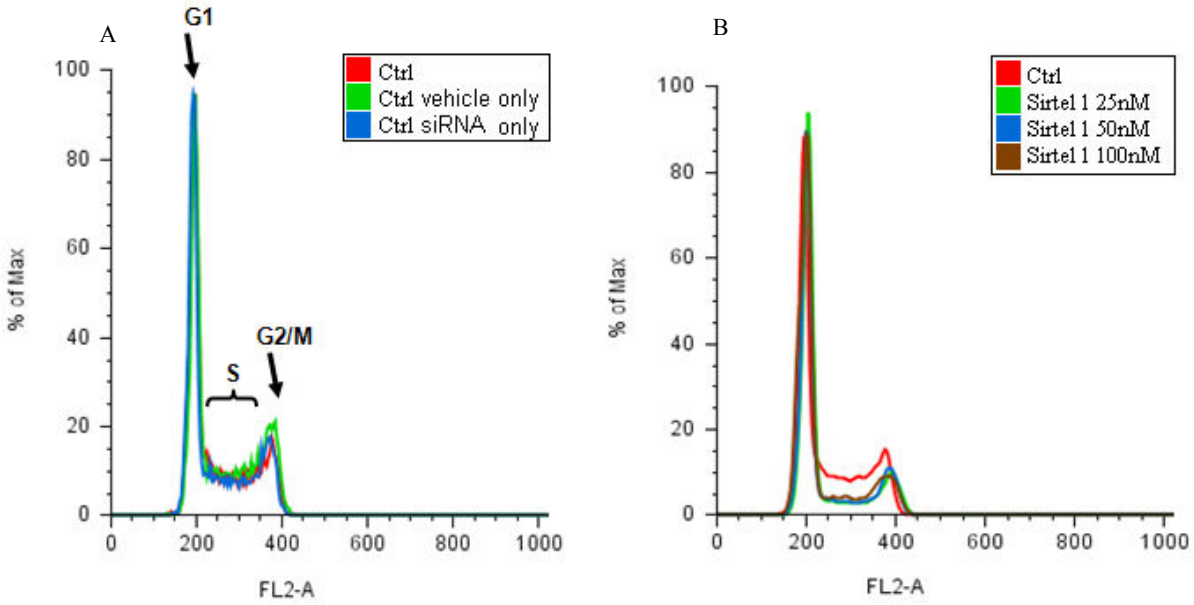


The data are expressed in percentage of viable cells in relation to controls. For Dox treated cells the control was cells treated with vehicle and for silenced cells the control was cells treated with the siLentFect (vehicle) only.

Cell cycle analysis. In order to assess the effect of SirTel 1 on the cell cycle, JHH6 were transfected with 25, 50 and 100nM of SirTel 1 for 72h and the DNA content of both treated cells and controls was analyzed by Flow Cytometry by propidium idodide DNA staining. During the analysis 10000 cells were counted and categorized as G_1-, S- or G_2/M-phase cells, based on DNA content. For the final analysis were considered only the cells that have the 100% probability to be in each cell cycle phase.

The plot in Figure 20 shows the percentage of JHH6 cell at every cell cycle phase. There is no difference in cell distribution among the controls (Fig. 20A) while there is a shift in the profile of treated cells vs. controls (red line Fig. 20B).

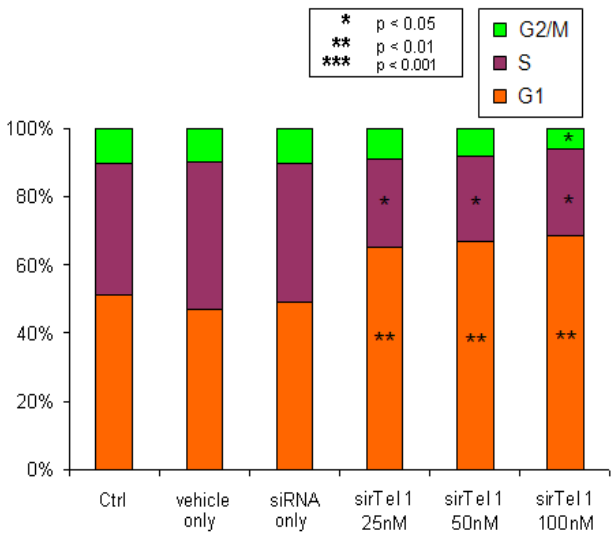
Figure 20. Cell cycle phases JHH6 distribution.



Cell cycle representative Plot. The cells were harvested and analyzed with propidium iodide staining to assess cell cycle distribution by flow cytometry analysis and the results were expressed as mean ±S.D. of three independent experiments. (A) Overlay of cell cycle distributions of controls . (B) Overlay of cell cycle distributions of JHH6 cells exposed to different concentrations of SirTel 1. On the y-axes is reported the percentage of cells in each phase of the cell cycle. On the x-axes is reported the DNA content.

In silenced samples there was a statistically significant increase of G1 arrested cells ($p<0.01$), while there was a statistically significant decrease in S phase cells ($p<0.05$) for all the considered siRNA concentrations (Fig. 21). A decrease in G2 phase cells resulted significant ($p<0.05$) only in cells treated with 100nM of siRNA. No statistically significant differences were observed among controls. Statistical analysis was performed using the Wilcoxon-Mann-Whitney test.

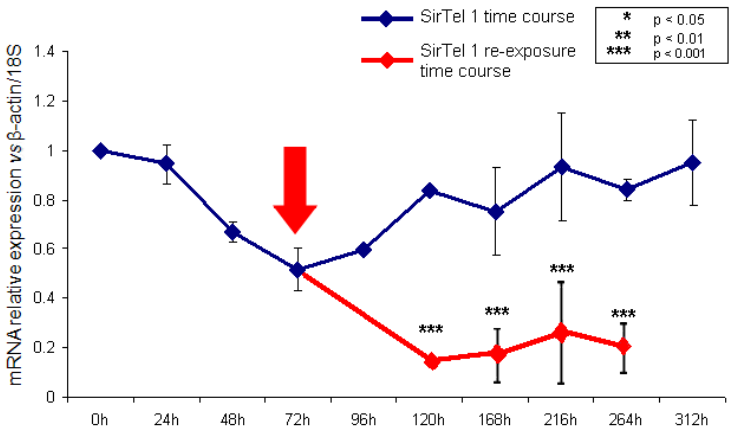
Figure 21. JHH6 cells cycle analysis after hTERT silencing.



JHH6 re-exposure to SirTel 1

After 72h silencing with 25nM of SirTel 1 JHH6 cells were re-exposed to 25nM of the same siRNA and silencing was followed during the next 168 hours. Re-exposing the cells to additional 25nM of SirTel 1 caused a reduction of mRNA levels by 76% compared to the amount already present after the first treatment (Fig. 22). The statistically significant decrease ($p<0.001$) in the hTERT mRNA was maintained at least until 168h after the re-exposure.
Results are expressed as mean ±S.D. from at least 3 independent samples and statistical analysis was performed using the unpaired t-test.

Figure 22. Re-exposure to the siRNA.



Blu line: hTERT mRNA expression time course after silencing. Red line: hTERT mRNA expression time course after re-exposure to 25nM of SirTel 1. The red arrow indicates the time point of re-exposure. mRNA levels are reported as folds of expression *vs.*β-actin/18S. All the data are expressed in relation to the control.

DISCUSSION

Hepatocellular Carcinoma (HCC) ranks fifth in frequency of cancers in the world and every year more than 700.000 people die of this disease.

HCC usually arises in the setting of HCV and HBV infections, non-alcoholic steatohepatitis and cirrhosis which cause hepatic dysfunction that limits treatment options.

Currently therapeutic guidelines are provided by the Barcelona Clinic Liver Cancer (BCLC) staging system. Orthotopic Liver Transplantation (OLT) or liver resection represent the best treatments for HCC. However, most patients cannot be subjected to potential curative OLT or resection because of extensive tumor involvement of the liver, metastasis, invasion of the portal vein or advanced underlying hepatocellular disease. Systemic chemotherapy or chemoembolization represent a palliative or down-staging alternative, but drug therapy of cancer is hampered by multidrug resistance (MDR). The establishment of the MDR leads to new therapeutically approaches such as gene therapy.

To overcome the MDR phenomena we developed two parallel studies:

- **Multidrug resistance in HCC (TASK 1),** in which we plan to clarify the role of ABC transporters in the development of MDR.
- **Gene therapy applied to HCC** in which we plan to use a liver specific lipoprotein-based structures as delivery system to target hepatic cancer cells with siRNAs. Chemotherapy is considered a double edged sword, due to the numerous side effects of drug usage, and the use of siRNAs instead of drugs could represent a good down-staging therapy alternative, avoiding MDR establishment. In this thesis we focused the attention on the **telomerase silencing effects in HCC (TASK 2)**, the developing of the delivery particle will be performed in the near future.

Task 1: Multidrug resistance in HCC

Since many years it has become evident the role of the ABC transporters in the MDR establishment [16,19,20]. Although ABCs are widely studied in many cancer types, there are contrasting and incomplete data regarding ABC expression and behavior in HCC. This is true especially for ABCG2 (also known as BCRP, breast cancer resistance protein) which is poorly studied in this kind of tumour.

The up-regulation of ABCs can compromise chemotherapy and influence therapy choices as a consequence studying the modulation of these transporters in response to Dox (the widely used antineoplastic drug) therapy could represent an important aid for conventional medicine.
For our studies we considered three cellular models with distinct grade of differentiation: IHH cells (differentiated immortalized hepatocytes [380], HuH7 cells (moderately differentiated HCC derived cell line), JHH6 cells (poorly differentiated HCC derived cell line). Each cell line has a basal distinctive ABC expression pattern with the highest levels of ABCC1 and ABCG2 expression observed in the less differentiated cells (JHH6). Furthermore ABCB1 was exclusively expressed in HuH7 cells (Fig. 1 results section).
When treating the cells with Dox doses lower than the LC_{50} (Table 1 results section) we noticed that the drug is able to enter into the cell nucleus within ten minutes after administration. After 24h, Dox is completely within the nucleus evidencing the inability of the cells to counteract its entrance and accumulation, at least at the concentrations used. The mechanism of Dox cytotoxicity is not completely understood although it appears to act principally through topoisomerase II inhibition [381]. Dox seems to stabilize catalytic intermediates of the enzyme onto target DNA thus it co-localise with chromatin [382]. After a 48h treatment Dox is not uniformly distributed into the nuclei (as observed after 24h-exposure) probably because the chromatin is starting to condensate and the apoptotic pathway has been already triggered as suggested previously [383]. However, form our results it seems that in the time-lapse between Dox entrance and apoptosis the cell is still able to modulate the mRNA and protein expression. We speculate that, in these way, the cell would be trying to limit Dox accumulation by increasing the ABC expression. Interestingly, even if the ABCC1 mRNA expression decreases in each cell line both at 24h and 48h, there is an increase in the protein expression in the tumoral cell lines compared to the immortalized hepatocytes (IHH). The differential behaviour between mRNA and protein expression suggest that the possible mechanism that determines the ABCC1 up-regulation is not the *de-novo* transcription but most probably something related to the protein turnover.
ABCG2 seems to play a role only in less differentiated cells (JHH6) where, in spite of the decrease in the mRNA expression after 48h, the protein expression increases at 24h and remains higher than controls till 48h after (Results Fig. 5). ABCG2 seems not to be involved in cellular protection in HuH7 since there is a progressive protein down-regulation at 24h and 48h (Results Fig. 4). Probably in these cells ABCG2 is not involved in anthracyclines detoxification, indeed untreated HuH7 cells express already high levels of ABCB1 and after Dox exposure the ABCB1 mRNA levels shows an subsequent additional increase after 24h,

and the protein after 48h suggesting a key role of this transporter in cellular protection. Another possible explanation about ABCG2 behaviour in these cells comes from Calcagno's and coll. studies [384] where ABCG2 expression increased after long term Dox exposure of breast, ovarian and colon cancer cells (MCF-7, IGROV-1 and S-1), suggesting that longer exposure time is needed for observing variations in the expression of this transporter. Thus it might be possible that ABCG2 is involved in the drug long term response. However in our preliminary studies (data not shown) by exposing the IHH and JHH6 cells to low Dox doses for long time did not induce the ABCG2 up-regulation. Probably in hepatic derived cell lines ABCG2 has a secondary role in MDR, more important for these cells seem to be ABCC1 and ABCB1 whose participation in MDR in breast, ovarian and colon cancer cells was excluded by Calcagno and coll. [384].

Preliminary data obtained by our group (Rosso, N. *et* al. *in preparation*) suggest a more clear role of ABC transporters in determine cell survival by exposing the cells with Dox concentrations 1000 folds lower than the LC_{50}. Such low drug doses, even if able to kill sensitive cells, induce a chemoresistance phenotype in surviving cells which overexpress these ABC transporters especially ABCB1 and ABCC1.

The ability of Dox to induce ABCB1, ABCC1 and ABCG2 expression, even at low doses, should be taken into consideration in clinical practice. Dox is one of the widely used antineoplastic drugs and the understanding the role of Dox in the ABCB1, ABCC1 And ABCG2 induced chemoresistance would represent a useful point for ameliorating current therapy in order to increase patients' overall survival.

During tumours treatment, if the first drug dose is not able to kill all the cancer cells, the surviving population undergoes to a selection process that makes those cells more resistant to drugs and at the same time they can recover cancer growth. Indeed Atalay and Coll. [385] found that an ABCB1 increased expression after anthracyclines treatment was associated with a decreased disease free and overall survival in patients with advanced breast cancer. These results suggest a possible role of ABC as negative prognostic markers in some type of cancers such as breast cancer pancreatic cancer [100,126,127] and HCC [132,133]. In particular, in liver tumours ABCC1 expression has been correlated with cancer stage and invasiveness [133].

In our cellular model we showed that Dox is able to modulate ABCB1, ABCC1 and ABCG2 expression within 24h after the treatment suggesting that ABC up-regulation is an early event of cellular adaptation. Although cells are no more able to modulate gene expression, as demonstrated by a general mRNA down-regulation, they are probably able to decrease ABC

turnover determining the ABC protein up-regulation as observed in the tumoral cell lines (HuH7 and JHH6). We are not able to correlate the tumorigenic potential of the two tumoral cell lines with the ABC expression since the different behaviour of ABCs and the different contribution to MDR. Thus in order to better clarify the contribution of each single ABC to MDR our future steps will consider the use specific inhibitors such as: CP 100356 Hydrochloride, Reversan and Ko134, specific ABCB1, ABCC1 and ABCG2 inhibitor respectively.

Although not conclusive this study contributes in elucidating the role of Dox in modulating the pattern of expression of ABCB1, ABCC1 and ABCG2 in different HCC derived cell lines. By considering the contrasting and incomplete information available about the ABC transporters expression in HCC probably due to the different cellular *in vitro* models used, the different criteria of selection among studied patients and the difficulty to reproduce the clinical and patho-/physiological settings with an *in vitro* model, we are not able to strongly support our data with previously reported studies. However we provide more information about Dox influence on ABCB1, ACBC1 and ABCG2 expression in hepatic derived cell lines. The capability of Dox in modulating the ABCs that might induce a MDR phenotype states the limitations of chemotherapy in treating HCC and opens new research fields in alternative therapies.

Task 2: Telomerase silencing effects in HCC

The establishment of MDR limits the therapy options for HCC. Curative treatments, such as OLT or liver resection are not always immediately available and chemotherapy represents an alternative for the arrest of tumoral growth. Unfortunately the MDR phenomena could lead to unsuccessful drug treatments and other downstaging therapies are required. Gene silencing could represent, in a near future, a good option for HCC treatments since it is not subjected to MDR and to side effects that usually characterize chemotherapy.

The best gene candidate for a successful gene therapy applied to cancer should be essential for cancer cell survival, growing and maintenance; over-expressed or exclusively expressed in tumoral cells and involved in as less pathways as possible.

From literature four genes were selected: 1) Aurora kinase A; 2) midkine; 3) surviving; and 4) telomerase. Aurora Kinases are serine/threonine kinases that are essential for cell proliferation. They play a crucial role in cellular division by controlling chromatid segregation, in particular aurora kinase A has well-established but perhaps not yet fully

understood roles in centrosome function and duplication, mitotic entry, and bipolar spindle assembly. Aurora kinase A was found to be overexpressed in 61% HCC and its overexpression was associated with high-grade (grade II-IV), and high-stage (stage IIIB-IV) tumors, p53 mutation, infrequent -catenin mutation, and poor outcome [386]. Among our HCC samples, aurora kinase A results up-regulated in tumoral samples, although not significantly. Moreover it is moderately expressed also in controls. Midkine is an heparin-binding growth and differentiation factor, it appears to enhance the angiogenic and proliferative activities of cancer cells. The expression of midkine (mRNA and protein expression) has been found to be elevated in multiple cancer types, whereas in normal adult tissues is low or undetectable [387-389]. In our case midkine is overexpressed in tumours and its expression decreases progressively from the tumoral to the distal portion of the HCC samples. Although expressed at very low levels midkine is also expressed in non tumoral samples. Surviving is a IAP (inhibitor of apoptosis) family member. It inhibits the caspase's activation [390]. Its overexpression has an oncogenic potential because it may overcome the G2/M phase checkpoint to enforce progression of cells through mitosis, thus promoting proliferation. Survivin is highly expressed tumours, including HCC and is absent in normal cells [391]. There is no survivin differential expression between controls and tumoral samples analyzed in this study. The lack of a definite differential expression among tumoral, peripheral and distal portion of the diseased liver and the expression in controls, lead to discard of aurora kinase A, midkine and survivin as targets for gene therapy.

On the contrary, telomerase reverse transcriptase is overexpressed in tumoral samples with a significant lower expression in peripheral ($p<0.01$) and distal ($p<0.001$) tissues. Moreover, as expected, no telomerase mRNA is detectable in non tumoral samples. For these reasons telomerase is eligible for the gene silencing experiments. hTERT is expressed in 100% of tumoral HCC tissues analysed and this is in accordance with previous studies which found telomerase to be expressed in 80-100% of HCCs [259,261]. Although the evidence that telomerase is expressed in HCC, very little information exist about the effects of its inhibition in this cancer type supporting our goal of silencing hTERT.

RNA interference (RNAi) is a gene silencing process induced by 21–23-nucleotide RNA duplexes called small interfering RNAs (siRNAs) and resulting in sequence-specific messenger RNA degradation post-transcriptionally in the cellular cytoplasmic region.

Among the different purposes for which the silencing techniques have been developed, more and more interest is given to the clinical applications. Recently several siRNAs for clinical use have been developed, most of them are in phase II clinical trial, such as *Excellair,*

targeting SYK kinase, used for asthma treatments and distributed by ZaBeCor; and *QPI-1002,* targeting p53, for acute renal failure and distributed by Quark/Novartis.

Designing a siRNA for *in vivo* applications involves the use of several devices, for example the siRNA should be as much specific as possible to avoid off-target effects. Using the lowest effective concentration helps in preventing the off-target effects. The target secondary structure should also be taken into consideration since it can influence the siRNA efficacy [392]. Moreover the inflammatory response that could derive from the siRNA cellular uptake should be considered [393]. The induction of the proinflammatory cytokines by dsRNAs could lead to a cellular improper response. Kim and coll. [394] showed that direct dsRNA Dicer substrates can prevent inflammation. In our case the accurate design of a direct Dicer dsRNA against hTERT (SirTel 1) has been shown to successfully not induce the TNFα, IL6 and IL8 expression (Fig. 11, results section). In contrast the "standard" 3'overhang dsRNA (SirTel 2, SirTel 3, SirTel 4) trigger an acute response from the innate immune system (Fig. 11, results section).

The silencing efficacy of each designed siRNA was tested in JHH6 cell line, which have the highest hTERT expression among the four cell lines evaluated (Fig. 10, results section). SirTel 1 and SirTel 4 are the most effective siRNAs in reducing hTERT expression in a dose dependent manner after 72h silencing (Fig. 12, results section). To assess the presence of off-target effects, primary cultures of human fibroblasts were included into the experiments. Human fibroblasts are telomerase negative cells and for this reason they have a limited life span. These cells were transfected with SirTel 1 and SirTel 4 and cell viability was evaluated. In SirTel 1 transfected cells, the observed reduction in cell viability was due only to the vehicle. On the contrary the cellular decreased cell viability observed in SirTel 4 transfected cells is not only due to the use of the transfection reagent but also by the SirTel 4 uptake (Fig. 13, results section). These results indicate that, in a telomerase negative setting, SirTel 4 induces an unspecific cell toxicity probably due to off-target effects.

The poor SirTel 2 and SirTel 3 silencing effect, the inflammatory response induced by SirTel 2, SirTel 3 and SirTel 4, and the off-target effects caused by SirTel 4 lead to the election of the only SirTel 1 (the direct Dicer substrate) as siRNA candidate for further silencing experiments in JHH6 cells.

Although there is a dose dependent hTERT silencing effect induced by SirTel 1, we decided to use for most of the experiments the lower effective dose (25nM), even if this concentration induce a 50% decrease in hTERT mRNA expression in spite of the marked silencing efficacy of higher SirTel 1 concentrations (100nM). Important to notice is that hTERT gene as the

other components of Telomerase holoenzyme naturally may be subjected to haploinsufficiency thus a 50% reduction in telomerase expression could be sufficient to obtain the desired effects.

hTERT silencing has not only effect in the mRNA expression but also in the telomerase activity, validating the effectiveness of the silencing system. Indeed, after hTERT silencing there is more than 60% reduction ($p<0.001$) in the total product generated from active telomerase (Fig. 14, results section).

Time course experiments (Fig. 15, results section) evidenced that hTERT reaches the lower mRNA expression at 72h of 25nM SirTel 1 silencing, thus at this time point we expect to observe the silencing effects in JHH6 cells. The first evident consequence of hTERT silencing is a morphological change of treated cells compared to controls. Only the siRNA transfected cells change their morphology from a fibroblast-like shape to an hepatocyte-like shape. The altered phenotype can be appreciated both by optical microscope and by flow cytometry analysis (Fig. 16 and 17, results section). These modifications that makes JHH6 more similar to normal hepatocytes lead us to the assessment of hepatic hallmarks in these cells.

The albumin expression is a typical feature of normal hepatocytes, thus we decided to evaluate its expression in silenced cells. Albumin is not expressed or expressed at very low levels in poor differentiated untreated JHH6 cells but it becomes expressed after hTERT silencing. Although not significant, due to high S.Ds (to notice that in most samples albumin mRNA is detectable only after hTERT silencing), the maximum albumin mRNA expression corresponds to the minimum in hTERT mRNA expression. The link between hTERT inhibition and albumin expression/cellular differentiation is not known, as a consequence these interesting results are eligible for future investigations on the telomerase extratelomeric effects.

Several reports [395-397] showed that a telomerase inhibition results in an impaired cell growth, dependent on telomere length, taking weeks or months to occur. However Cao Y. and Coll. [342] demonstrated that hTERT silencing in human breast cancer cells results in an impaired cell survival and proliferation independently of telomere length. Similar results were obtained by Li S. and Coll. [398] in colon cancer and melanoma cell lines and by Gandellini P. and Coll. [399] in prostate cancer cells. In agreemente with these previously published [342,399] studies we observed a statistically significant G1 cell cycle arrest of silenced cells compared to untreated controls (Fig. 20 and 21, results section). Furthermore after 72h of 25nM siRNA treatment there is a 37% reduction in cell viability and about the 60% of surviving cells are arrested in G1 phase, as mentioned before. This data resulted more

interesting especially when compared with the percentage of viable cells after 72h of 25nM Dox treatment.

Indeed Dox treatment determines only a 15% reduction in cell viability. Thus SirTel 1 resulted more effective in reducing cell viability than Dox, especially at lower concentrations. This has important implications for a potential clinical use of this molecular strategy. The use of the siRNA instead of chemotherapeutic drugs could prevent all the side effects generally associated with chemotherapy.

The rapid cell death and cell cycle arrest caused by hTERT depletion highlight the presence of a novel unknown pathway that links telomerase to cell survival independently of telomere length. This is supported by previous observations in which targeting hTERT or hTERC has similar effect in telomerase activity, but only the down-regulation of hTERT causes a rapid decline in cell growth suggesting an enzymatic activity independent mechanism by which hTERT maintains tumor cell survival and proliferation in human prostate cancer cells DU145 [400].

Other interesting results were obtained when, after an initial silencing, cells were re-exposed to 25nM of SirTel 1. Re-exposure of the cells to additional 25nM of siRNA induces a reduction of mRNA levels by 76% compared to the amount already present after the first treatment (Fig. 22, results section). Thus supposing a possible clinical application for SirTel 1, a re-treatment every 4-5 days could be useful in deplete cancer cell populations.

In summary, hTERT silencing in JHH6 cells with a direct dicer substrate siRNA induces a decreased hTERT mRNA expression and a decreased telomerase activity. This leads to changes in cell morphology, from a fibroblast-like shape to an hepatocyte-like shape combined with an increased albumin expression; to a significant reduction in cell viability with more than 60% of surviving cells arrested in cell cycle phase. All together these results suggest a possible future application for telomerase silencing as anticancer treatment.

In a clinical setting, telomerase inhibition may work as a down-staging therapy in conjunction with surgery or after an initial surgery, telomerase inhibition might be used in an adjuvant setting to limit the recovery of residual cancer cells. Moreover telomerase inhibition might act in a synergistic fashion with existing therapeutic modalities and amplify their effectiveness.

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Reference List

[1] H. B. El-Serag, K. L. Rudolph, *Gastroenterology* **2007**; *132*: 2557-76.

[2] M. Roncalli, *Liver Transpl* **2004**; *10*: S9-15.

[3] W. R. Kim, G. J. Gores, J. T. Benson, T. M. Therneau, L. J. Melton, III, *Gastroenterology* **2005**; *129*: 486-93.

[4] J. Bruix, M. Sherman, J. M. Llovet, M. Beaugrand, R. Lencioni, A. K. Burroughs, E. Christensen, L. Pagliaro, M. Colombo, J. Rodes, *J Hepatol* **2001**; *35*: 421-30.

[5] J. Bruix, M. Sherman, *Hepatology* **2011**; *53*: 1020-2.

[6] M. Omata, L. A. Lesmana, R. Tateishi, P. J. Chen, S. M. Lin, H. Yoshida, M. Kudo, J. M. Lee, B. I. Choi, R. T. Poon, S. Shiina, A. L. Cheng, J. D. Jia, S. Obi, K. H. Han, W. Jafri, P. Chow, S. G. Lim, Y. K. Chawla, U. Budihusodo, R. A. Gani, C. R. Lesmana, T. A. Putranto, Y. F. Liaw, S. K. Sarin, *Hepatol Int* **2010**; *4*: 439-74.

[7] J. M. Llovet, C. Bru, J. Bruix, *Semin Liver Dis* **1999**; *19*: 329-38.

[8] R. T. Poon, S. T. Fan, C. M. Lo, I. O. Ng, C. L. Liu, C. M. Lam, J. Wong, *Ann Surg* **2001**; *234*: 63-70.

[9] W. K. Yun, M. S. Choi, D. Choi, H. C. Rhim, J. W. Joh, K. H. Kim, T. H. Jang, J. H. Lee, K. C. Koh, S. W. Paik, B. C. Yoo, *Hepatol Int* **2011**; *5*: 722-9.

[10] S. N. Hong, S. Y. Lee, M. S. Choi, J. H. Lee, K. C. Koh, S. W. Paik, B. C. Yoo, J. C. Rhee, D. Choi, H. K. Lim, K. W. Lee, J. W. Joh, *J Clin Gastroenterol* **2005**; *39*: 247-52.

[11] J. Huang, L. Yan, Z. Cheng, H. Wu, L. Du, J. Wang, Y. Xu, Y. Zeng, *Ann Surg* **2010**; *252*: 903-12.

[12] M. S. Chen, J. Q. Li, Y. Zheng, R. P. Guo, H. H. Liang, Y. Q. Zhang, X. J. Lin, W. Y. Lau, *Ann Surg* **2006**; *243*: 321-8.

[13] B. J. Trock, F. Leonessa, R. Clarke, *J Natl Cancer Inst* **1997**; *89*: 917-31.

[14] H. B. van der, B. Lowenberg, A. K. Burnett, W. U. Knauf, J. Shepherd, P. P. Piccaluga, G. J. Ossenkoppele, G. E. Verhoef, A. Ferrant, M. Crump, D. Selleslag, M. Theobald, M. F. Fey, E. Vellenga, M. Dugan, P. Sonneveld, *Blood* **2005**; *106*: 2646-54.

[15] G. Szakacs, J. K. Paterson, J. A. Ludwig, C. Booth-Genthe, M. M. Gottesman, *Nat Rev Drug Discov* **2006**; *5*: 219-34.

[16] R. L. Juliano, V. Ling, *Biochim Biophys Acta* **1976**; *455*: 152-62.

[17] K. Ueda, C. Cardarelli, M. M. Gottesman, I. Pastan, *Proc Natl Acad Sci U S A* **1987**; *84*: 3004-8.

[18] L. W. LAW, *Nature* **1952**; *169*: 628-9.

[19] K. Dano, *Biochim Biophys Acta* **1973**; *323*: 466-83.

[20] P. Gros, Y. B. Ben Neriah, J. M. Croop, D. E. Housman, *Nature* **1986**; *323*: 728-31.

[21] C. R. Fairchild, S. P. Ivy, T. Rushmore, G. Lee, P. Koo, M. E. Goldsmith, C. E. Myers, E. Farber, K. H. Cowan, *Proc Natl Acad Sci U S A* **1987**; *84*: 7701-5.

[22] L. D. Teeter, F. F. Becker, F. V. Chisari, D. J. Li, M. T. Kuo, *Mol Cell Biol* **1990**; *10*: 5728-35.

[23] M. Dean, R. Allikmets, *Curr Opin Genet Dev* **1995**; *5*: 779-85.

[24] C. F. Higgins, *Annu Rev Cell Biol* **1992**; *8*: 67-113.

[25] R. G. Deeley, C. Westlake, S. P. Cole, *Physiol Rev* **2006**; *86*: 849-99.

[26] G. Szakacs, J. K. Paterson, J. A. Ludwig, C. Booth-Genthe, M. M. Gottesman, *Nat Rev Drug Discov* **2006**; *5*: 219-34.

[27] B. Sarkadi, L. Homolya, G. Szakacs, A. Varadi, *Physiol Rev* **2006**; *86*: 1179-236.

[28] M. Dean, A. Rzhetsky, R. Allikmets, *Genome Res* **2001**; *11*: 1156-66.

[29] L. A. Doyle, W. Yang, L. V. Abruzzo, T. Krogmann, Y. Gao, A. K. Rishi, D. D. Ross, *Proc Natl Acad Sci U S A* **1998**; *95*: 15665-70.

[30] J. E. Walker, M. Saraste, M. J. Runswick, N. J. Gay, *EMBO J* **1982**; *1*: 945-51.

[31] C. F. Higgins, I. D. Hiles, G. P. Salmond, D. R. Gill, J. A. Downie, I. J. Evans, I. B. Holland, L. Gray, S. D. Buckel, A. W. Bell, ., *Nature* **1986**; *323*: 448-50.

[32] G. Tombline, A. Muharemagic, L. B. White, A. E. Senior, *Biochemistry* **2005**; *44*: 12879-86.

[33] A. E. Senior, M. K. al-Shawi, I. L. Urbatsch, *FEBS Lett* **1995**; *377*: 285-9.

[34] L. Beaudet, P. Gros, *J Biol Chem* **1995**; *270*: 17159-70.

[35] Z. E. Sauna, S. V. Ambudkar, *J Biol Chem* **2001**; *276*: 11653-61.

[36] C. Martin, G. Berridge, C. F. Higgins, P. Mistry, P. Charlton, R. Callaghan, *Mol Pharmacol* **2000**; *58*: 624-32.

[37] C. Martin, C. F. Higgins, R. Callaghan, *Biochemistry* **2001**; *40*: 15733-42.

[38] R. Daoud, J. Desneves, L. W. Deady, L. Tilley, R. J. Scheper, P. Gros, E. Georges, *Biochemistry* **2000**; *39*: 6094-102.

[39] Q. Mao, W. Qiu, K. E. Weigl, P. A. Lander, L. B. Tabas, R. L. Shepard, A. H. Dantzig, R. G. Deeley, S. P. Cole, *J Biol Chem* **2002**; *277*: 28690-9.

[40] G. Conseil, R. G. Deeley, S. P. Cole, *Biochem Pharmacol* **2005**; *69*: 451-61.

[41] S. Conrad, H. M. Kauffmann, K. Ito, E. M. Leslie, R. G. Deeley, D. Schrenk, S. P. Cole, *Pharmacogenetics* **2002**; *12*: 321-30.

[42] K. Koike, C. J. Oleschuk, A. Haimeur, S. L. Olsen, R. G. Deeley, S. P. Cole, *J Biol Chem* **2002**; *277*: 49495-503.

[43] K. Koike, G. Conseil, E. M. Leslie, R. G. Deeley, S. P. Cole, *J Biol Chem* **2004**; *279*: 12325-36.

[44] D. W. Zhang, S. P. Cole, R. G. Deeley, *J Biol Chem* **2001**; *276*: 13231-9.

[45] D. W. Zhang, S. P. Cole, R. G. Deeley, *J Biol Chem* **2001**; *276*: 34966-74.

[46] S. Ryu, T. Kawabe, S. Nada, A. Yamaguchi, *J Biol Chem* **2000**; *275*: 39617-24.

[47] C. J. Oleschuk, R. G. Deeley, S. P. Cole, *Am J Physiol Gastrointest Liver Physiol* **2003**; *284*: G280-G289.

[48] K. Ito, C. J. Oleschuk, C. Westlake, M. Z. Vasa, R. G. Deeley, S. P. Cole, *J Biol Chem* **2001**; *276*: 38108-14.

[49] N. Godinot, P. W. Iversen, L. Tabas, X. Xia, D. C. Williams, A. H. Dantzig, W. L. Perry, III, *Mol Cancer Ther* **2003**; *2*: 307-16.

[50] L. Ma, S. E. Pratt, J. Cao, A. H. Dantzig, R. E. Moore, C. A. Slapak, *Mol Cancer Ther* **2002**; *1*: 1335-42.

[51] K. Nunoya, C. E. Grant, D. Zhang, S. P. Cole, R. G. Deeley, *Drug Metab Dispos* **2003**; *31*: 1016-26.

[52] B. D. Stride, G. Valdimarsson, J. H. Gerlach, G. M. Wilson, S. P. Cole, R. G. Deeley, *Mol Pharmacol* **1996**; *49*: 962-71.

[53] B. D. Stride, C. E. Grant, D. W. Loe, D. R. Hipfner, S. P. Cole, R. G. Deeley, *Mol Pharmacol* **1997**; *52*: 344-53.

[54] G. J. Zaman, N. H. Cnubben, P. J. van Bladeren, R. Evers, P. Borst, *FEBS Lett* **1996**; *391*: 126-30.

[55] M. J. Zamek-Gliszczynski, K. A. Hoffmaster, X. Tian, R. Zhao, J. W. Polli, J. E. Humphreys, L. O. Webster, A. S. Bridges, J. C. Kalvass, K. L. Brouwer, *Drug Metab Dispos* **2005**; *33*: 1158-65.

[56] L. Cui, Y. X. Hou, J. R. Riordan, X. B. Chang, *Arch Biochem Biophys* **2001**; *392*: 153-61.

[57] I. Leier, G. Jedlitschky, U. Buchholz, S. P. Cole, R. G. Deeley, D. Keppler, *J Biol Chem* **1994**; *269*: 27807-10.

[58] T. Kamisako, I. Leier, Y. Cui, J. Konig, U. Buchholz, J. Hummel-Eisenbeiss, D. Keppler, *Hepatology* **1999**; *30*: 485-90.

[59] Y. M. Qian, W. C. Song, H. Cui, S. P. Cole, R. G. Deeley, *J Biol Chem* **2001**; *276*: 6404-11.

[60] G. Jedlitschky, B. Burchell, D. Keppler, *J Biol Chem* **2000**; *275*: 30069-74.

[61] J. D. Adams, Jr., B. H. Lauterburg, J. R. Mitchell, *J Pharmacol Exp Ther* **1983**; *227*: 749-54.

[62] T. Hirohashi, H. Suzuki, H. Takikawa, Y. Sugiyama, *J Biol Chem* **2000**; *275*: 2905-10.

[63] P. A. Di, G. Conseil, J. M. Perez-Victoria, G. Dayan, H. Baubichon-Cortay, D. Trompier, E. Steinfels, J. M. Jault, W. H. de, M. Maitrejean, G. Comte, A. Boumendjel, A. M. Mariotte, C. Dumontet, D. B. McIntosh, A. Goffeau, S. Castanys, F. Gamarro, D. Barron, *Cell Mol Life Sci* **2002**; *59*: 307-22.

[64] J. H. Hooijberg, H. J. Broxterman, G. L. Scheffer, C. Vrasdonk, M. Heijn, M. C. de Jong, R. J. Scheper, J. Lankelma, H. M. Pinedo, *Br J Cancer* **1999**; *81*: 269-76.

[65] E. M. Leslie, Q. Mao, C. J. Oleschuk, R. G. Deeley, S. P. Cole, *Mol Pharmacol* **2001**; *59*: 1171-80.

[66] A. Boumendjel, H. Baubichon-Cortay, D. Trompier, T. Perrotton, P. A. Di, *Med Res Rev* **2005**; *25*: 453-72.

[67] F. Narasaki, M. Oka, M. Fukuda, R. Nakano, K. Ikeda, H. Takatani, K. Terashi, H. Soda, O. Yano, T. Nakamura, L. A. Doyle, T. Tsuruo, S. Kohno, *Cancer Chemother Pharmacol* **1997**; *40*: 425-32.

[68] U. A. Germann, P. J. Ford, D. Shlyakhter, V. S. Mason, M. W. Harding, *Anticancer Drugs* **1997**; *8*: 141-55.

[69] S. Wang, A. Folkes, I. Chuckowree, X. Cockcroft, S. Sohal, W. Miller, J. Milton, S. P. Wren, N. Vicker, P. Depledge, J. Scott, L. Smith, H. Jones, P. Mistry, R. Faint, D. Thompson, S. Cocks, *J Med Chem* **2004**; *47*: 1329-38.

[70] D. M. Bradshaw, R. J. Arceci, *J Clin Oncol* **1998**; *16*: 3674-90.

[71] D. R. Ferry, H. Traunecker, D. J. Kerr, *Eur J Cancer* **1996**; *32A*: 1070-81.

[72] G. A. Fisher, B. I. Sikic, *Hematol Oncol Clin North Am* **1995**; *9*: 363-82.

[73] G. A. Fisher, B. L. Lum, J. Hausdorff, B. I. Sikic, *Eur J Cancer* **1996**; *32A*: 1082-8.

[74] G. D. Leonard, T. Fojo, S. E. Bates, *Oncologist* **2003**; *8*: 411-24.

[75] P. R. Twentyman, *Biochem Pharmacol* **1992**; *43*: 109-17.

[76] P. M. Fracasso, L. J. Goldstein, D. P. De Alwis, J. S. Rader, M. A. Arquette, S. A. Goodner, L. P. Wright, C. L. Fears, R. J. Gazak, V. A. Andre, M. F. Burgess, C. A. Slapak, J. H. Schellens, *Clin Cancer Res* **2004**; *10*: 7220-8.

[77] G. Gerrard, E. Payne, R. J. Baker, D. T. Jones, M. Potter, H. G. Prentice, M. Ethell, H. McCullough, M. Burgess, A. B. Mehta, K. Ganeshaguru, *Haematologica* **2004**; *89*: 782-90.

[78] A. Sandler, M. Gordon, D. P. De Alwis, I. Pouliquen, L. Green, P. Marder, A. Chaudhary, K. Fife, L. Battiato, C. Sweeney, C. Jordan, M. Burgess, C. A. Slapak, *Clin Cancer Res* **2004**; *10*: 3265-72.

[79] P. Ruff, D. A. Vorobiof, J. P. Jordaan, G. S. Demetriou, S. D. Moodley, A. L. Nosworthy, I. D. Werner, J. Raats, L. J. Burgess, *Cancer Chemother Pharmacol* **2009**; *64*: 763-8.

[80] F. J. Sharom, X. Yu, P. Lu, R. Liu, J. W. Chu, K. Szabo, M. Muller, C. D. Hose, A. Monks, A. Varadi, J. Seprodi, B. Sarkadi, *Biochem Pharmacol* **1999**; *58*: 571-86.

[81] N. I. Tarasova, R. Seth, S. G. Tarasov, T. Kosakowska-Cholody, C. A. Hrycyna, M. M. Gottesman, C. J. Michejda, *J Med Chem* **2005**; *48*: 3768-75.

[82] E. B. Mechetner, I. B. Roninson, *Proc Natl Acad Sci U S A* **1992**; *89*: 5824-8.

[83] C. Pawlak-Roblin, P. F. Tosi, L. Perrin, J. Devy, L. Venteo, P. Albert, C. Nicolau, C. Madoulet, *Eur J Cancer* **2004**; *40*: 606-13.

[84] M. J. Edelman, M. Shvartsbeyn, *Clin Lung Cancer* **2011**.

[85] V. V. Bartsevich, R. L. Juliano, *Mol Pharmacol* **2000**; *58*: 1-10.

[86] D. Xu, D. Ye, M. Fisher, R. L. Juliano, *J Pharmacol Exp Ther* **2002**; *302*: 963-71.

[87] D. Xu, H. Kang, M. Fisher, R. L. Juliano, *Mol Pharmacol* **2004**; *66*: 268-75.

[88] A. Pichler, N. Zelcer, J. L. Prior, A. J. Kuil, D. Piwnica-Worms, *Clin Cancer Res* **2005**; *11*: 4487-94.

[89] I. B. Roninson, J. E. Chin, K. G. Choi, P. Gros, D. E. Housman, A. Fojo, D. W. Shen, M. M. Gottesman, I. Pastan, *Proc Natl Acad Sci U S A* **1986**; *83*: 4538-42.

[90] J. R. Riordan, K. Deuchars, N. Kartner, N. Alon, J. Trent, V. Ling, *Nature* **1985**; *316*: 817-9.

[91] N. Kartner, D. Evernden-Porelle, G. Bradley, V. Ling, *Nature* **1985**; *316*: 820-3.

[92] S. V. Ambudkar, S. Dey, C. A. Hrycyna, M. Ramachandra, I. Pastan, M. M. Gottesman, *Annu Rev Pharmacol Toxicol* **1999**; *39*: 361-98.

[93] M. M. Gottesman, T. Fojo, S. E. Bates, *Nat Rev Cancer* **2002**; *2*: 48-58.

[94] G. Fricker, D. S. Miller, *Pharmacology* **2004**; *70*: 169-76.

[95] S. Gazzin, N. Strazielle, C. Schmitt, M. Fevre-Montange, J. D. Ostrow, C. Tiribelli, J. F. Ghersi-Egea, *J Comp Neurol* **2008**; *510*: 497-507.

[96] C. Cordon-Cardo, J. P. O'Brien, J. Boccia, D. Casals, J. R. Bertino, M. R. Melamed, *J Histochem Cytochem* **1990**; *38*: 1277-87.

[97] F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan, M. C. Willingham, *J Histochem Cytochem* **1989**; *37*: 159-64.

[98] A. H. Schinkel, *Semin Cancer Biol* **1997**; *8*: 161-70.

[99] A. H. Schinkel, U. Mayer, E. Wagenaar, C. A. Mol, D. L. van, J. J. Smit, M. A. van der Valk, A. C. Voordouw, H. Spits, T. O. van, J. M. Zijlmans, W. E. Fibbe, P. Borst, *Proc Natl Acad Sci U S A* **1997**; *94*: 4028-33.

[100] B. J. Trock, F. Leonessa, R. Clarke, *J Natl Cancer Inst* **1997**; *89*: 917-31.

[101] A. Abolhoda, A. E. Wilson, H. Ross, P. V. Danenberg, M. Burt, K. W. Scotto, *Clin Cancer Res* **1999**; *5*: 3352-6.

[102] Y. Soini, N. Virkajarvi, H. Raunio, P. Paakko, *J Clin Pathol* **1996**; *49*: 470-3.

[103] Y. Y. Chou, A. L. Cheng, H. C. Hsu, *J Gastroenterol Hepatol* **1997**; *12*: 569-75.

[104] I. O. Ng, C. L. Liu, S. T. Fan, M. Ng, *Am J Clin Pathol* **2000**; *113*: 355-63.

[105] E. Marthinet, G. Divita, J. Bernaud, D. Rigal, L. G. Baggetto, *Gene Ther* **2000**; *7*: 1224-33.

[106] K. V. Chin, K. Ueda, I. Pastan, M. M. Gottesman, *Science* **1992**; *255*: 459-62.

[107] J. A. Bush, G. Li, *Int J Cancer* **2002**; *98*: 323-30.

[108] R. A. Johnson, T. A. Ince, K. W. Scotto, *J Biol Chem* **2001**; *276*: 27716-20.

[109] R. A. Johnson, E. M. Shepard, K. W. Scotto, *J Biol Chem* **2005**; *280*: 13213-9.

[110] T. Tsuruo, H. Iida, S. Tsukagoshi, Y. Sakurai, *Cancer Res* **1981**; *41*: 1967-72.

[111] S. P. Cole, G. Bhardwaj, J. H. Gerlach, J. E. Mackie, C. E. Grant, K. C. Almquist, A. J. Stewart, E. U. Kurz, A. M. Duncan, R. G. Deeley, *Science* **1992**; *258*: 1650-4.

[112] S. E. Mirski, J. H. Gerlach, S. P. Cole, *Cancer Res* **1987**; *47*: 2594-8.

[113] E. Bakos, T. Hegedus, Z. Hollo, E. Welker, G. E. Tusnady, G. J. Zaman, M. J. Flens, A. Varadi, B. Sarkadi, *J Biol Chem* **1996**; *271*: 12322-6.

[114] D. R. Hipfner, K. C. Almquist, E. M. Leslie, J. H. Gerlach, C. E. Grant, R. G. Deeley, S. P. Cole, *J Biol Chem* **1997**; *272*: 23623-30.

[115] S. P. Cole, R. G. Deeley, *Bioessays* **1998**; *20*: 931-40.

[116] M. Kuwano, S. Toh, T. Uchiumi, H. Takano, K. Kohno, M. Wada, *Anticancer Drug Des* **1999**; *14*: 123-31.

[117] P. Borst, R. Evers, M. Kool, J. Wijnholds, *J Natl Cancer Inst* **2000**; *92*: 1295-302.

[118] G. J. Zaman, M. J. Flens, M. R. van Leusden, H. M. de, H. S. Mulder, J. Lankelma, H. M. Pinedo, R. J. Scheper, F. Baas, H. J. Broxterman, ., *Proc Natl Acad Sci U S A* **1994**; *91*: 8822-6.

[119] M. J. Flens, G. J. Zaman, d. van, V, M. A. Izquierdo, A. B. Schroeijers, G. L. Scheffer, P. van der Groep, H. M. de, C. J. Meijer, R. J. Scheper, *Am J Pathol* **1996**; *148*: 1237-47.

[120] K. Nooter, F. T. Bosman, H. Burger, K. E. van Wingerden, M. J. Flens, R. J. Scheper, R. G. Oostrum, A. W. Boersma, A. van der Gaast, G. Stoter, *Ann Oncol* **1996**; *7*: 75-81.

[121] I. Sugawara, H. Yamada, H. Nakamura, T. Sumizawa, S. Akiyama, A. Masunaga, S. Itoyama, *Int J Cancer* **1995**; *64*: 322-5.

[122] S. R. Wright, A. H. Boag, G. Valdimarsson, D. R. Hipfner, B. G. Campling, S. P. Cole, R. G. Deeley, *Clin Cancer Res* **1998**; *4*: 2279-89.

[123] W. Berger, U. Setinek, P. Hollaus, T. Zidek, E. Steiner, L. Elbling, H. Cantonati, J. Attems, A. Gsur, M. Micksche, *J Cancer Res Clin Oncol* **2005**; *131*: 355-63.

[124] Y. Oshika, M. Nakamura, T. Tokunaga, Y. Fukushima, Y. Abe, Y. Ozeki, H. Yamazaki, N. Tamaoki, Y. Ueyama, *Mod Pathol* **1998**; *11*: 1059-63.

[125] E. Ota, Y. Abe, Y. Oshika, Y. Ozeki, M. Iwasaki, H. Inoue, H. Yamazaki, Y. Ueyama, K. Takagi, T. Ogata, ., *Br J Cancer* **1995**; *72*: 550-4.

[126] M. Rudas, M. Filipits, S. Taucher, T. Stranzl, G. G. Steger, R. Jakesz, R. Pirker, G. Pohl, *Breast Cancer Res Treat* **2003**; *81*: 149-57.

[127] M. Filipits, G. Pohl, M. Rudas, O. Dietze, S. Lax, R. Grill, R. Pirker, C. C. Zielinski, H. Hausmaninger, E. Kubista, H. Samonigg, R. Jakesz, *J Clin Oncol* **2005**; *23*: 1161-8.

[128] G. F. Sullivan, P. S. Amenta, J. D. Villanueva, C. J. Alvarez, J. M. Yang, W. N. Hait, *Clin Cancer Res* **1998**; *4*: 1393-403.

[129] G. F. Sullivan, J. M. Yang, A. Vassil, J. Yang, J. Bash-Babula, W. N. Hait, *J Clin Invest* **2000**; *105*: 1261-7.

[130] G. Wang, R. Pincheira, J. T. Zhang, *Eur J Biochem* **1998**; *255*: 383-90.

[131] J. E. Ros, L. Libbrecht, M. Geuken, P. L. Jansen, T. A. Roskams, *J Pathol* **2003**; *200*: 553-60.

[132] S. Bonin, L. Pascolo, L. S. Croce, G. Stanta, C. Tiribelli, *Mol Med* **2002**; *8*: 318-25.

[133] B. S. Vander, M. Komuta, L. Libbrecht, A. Katoonizadeh, R. Aerts, S. Dymarkowski, C. Verslype, F. Nevens, T. Roskams, *Liver Int* **2008**; *28*: 1370-80.

[134] A. T. Nies, J. Konig, M. Pfannschmidt, E. Klar, W. J. Hofmann, D. Keppler, *Int J Cancer* **2001**; *94*: 492-9.

[135] S. E. Bates, R. Robey, K. Miyake, K. Rao, D. D. Ross, T. Litman, *J Bioenerg Biomembr* **2001**; *33*: 503-11.

[136] K. Miyake, L. Mickley, T. Litman, Z. Zhan, R. Robey, B. Cristensen, M. Brangi, L. Greenberger, M. Dean, T. Fojo, S. E. Bates, *Cancer Res* **1999**; *59*: 8-13.

[137] R. Allikmets, L. M. Schriml, A. Hutchinson, V. Romano-Spica, M. Dean, *Cancer Res* **1998**; *58*: 5337-9.

[138] G. L. Scheffer, A. C. Pijnenborg, E. F. Smit, M. Muller, D. S. Postma, W. Timens, d. van, V, E. G. De Vries, R. J. Scheper, *J Clin Pathol* **2002**; *55*: 332-9.

[139] F. Thiebaut, T. Tsuruo, H. Hamada, M. M. Gottesman, I. Pastan, M. C. Willingham, *Proc Natl Acad Sci U S A* **1987**; *84*: 7735-8.

[140] A. D. Mottino, T. Hoffman, L. Jennes, M. Vore, *J Pharmacol Exp Ther* **2000**; *293*: 717-23.

[141] M. Maliepaard, G. L. Scheffer, I. F. Faneyte, M. A. van Gastelen, A. C. Pijnenborg, A. H. Schinkel, M. J. van De Vijver, R. J. Scheper, J. H. Schellens, *Cancer Res* **2001**; *61*: 3458-64.

[142] S. Cisternino, C. Mercier, F. Bourasset, F. Roux, J. M. Scherrmann, *Cancer Res* **2004**; *64*: 3296-301.

[143] H. C. Cooray, C. G. Blackmore, L. Maskell, M. A. Barrand, *Neuroreport* **2002**; *13*: 2059-63.

[144] E. Rocchi, A. Khodjakov, E. L. Volk, C. H. Yang, T. Litman, S. E. Bates, E. Schneider, *Biochem Biophys Res Commun* **2000**; *271*: 42-6.

[145] J. W. Jonker, J. W. Smit, R. F. Brinkhuis, M. Maliepaard, J. H. Beijnen, J. H. Schellens, A. H. Schinkel, *J Natl Cancer Inst* **2000**; *92*: 1651-6.

[146] A. H. Schinkel, J. W. Jonker, *Adv Drug Deliv Rev* **2003**; *55*: 3-29.

[147] J. W. Jonker, M. Buitelaar, E. Wagenaar, M. A. van der Valk, G. L. Scheffer, R. J. Scheper, T. Plosch, F. Kuipers, R. P. Elferink, H. Rosing, J. H. Beijnen, A. H. Schinkel, *Proc Natl Acad Sci U S A* **2002**; *99*: 15649-54.

[148] A. E. van Herwaarden, J. W. Jonker, E. Wagenaar, R. F. Brinkhuis, J. H. Schellens, J. H. Beijnen, A. H. Schinkel, *Cancer Res* **2003**; *63*: 6447-52.

[149] J. E. Diestra, G. L. Scheffer, I. Catala, M. Maliepaard, J. H. Schellens, R. J. Scheper, J. R. Germa-Lluch, M. A. Izquierdo, *J Pathol* **2002**; *198*: 213-9.

[150] B. Uggla, E. Stahl, D. Wagsater, C. Paul, M. G. Karlsson, A. Sirsjo, U. Tidefelt, *Leuk Res* **2005**; *29*: 141-6.

[151] K. Yoh, G. Ishii, T. Yokose, Y. Minegishi, K. Tsuta, K. Goto, Y. Nishiwaki, T. Kodama, M. Suga, A. Ochiai, *Clin Cancer Res* **2004**; *10*: 1691-7.

[152] Z. Sun, Z. Zhao, G. Li, S. Dong, Z. Huang, L. Ye, H. Liang, J. Qu, X. Ai, W. Zhang, X. Chen, *Tumori* **2010**; *96*: 90-6.

[153] J. D. Allen, A. H. Schinkel, *Mol Cancer Ther* **2002**; *1*: 427-34.

[154] C. Ozvegy-Laczka, J. Cserepes, N. B. Elkind, B. Sarkadi, *Drug Resist Updat* **2005**; *8*: 15-26.

[155] K. Collins, *Curr Opin Cell Biol* **2000**; *12*: 378-83.

[156] L. T. De, *Genes Dev* **2005**; *19*: 2100-10.

[157] J. D. Griffith, L. Comeau, S. Rosenfield, R. M. Stansel, A. Bianchi, H. Moss, L. T. De, *Cell* **1999**; *97*: 503-14.

[158] L. T. De, *Nat Rev Mol Cell Biol* **2004**; *5*: 323-9.

[159] P. M. Lansdorp, N. P. Verwoerd, F. M. van de Rijke, V. Dragowska, M. T. Little, R. W. Dirks, A. K. Raap, H. J. Tanke, *Hum Mol Genet* **1996**; *5*: 685-91.

[160] J. M. Zijlmans, U. M. Martens, S. S. Poon, A. K. Raap, H. J. Tanke, R. K. Ward, P. M. Lansdorp, *Proc Natl Acad Sci U S A* **1997**; *94*: 7423-8.

[161] D. Broccoli, A. Smogorzewska, L. Chong, L. T. de, *Nat Genet* **1997**; *17*: 231-5.

[162] S. B. van, L. T. de, *Nature* **1997**; *385*: 740-3.

[163] S. B. van, A. Smogorzewska, L. T. De, *Cell* **1998**; *92*: 401-13.

[164] K. A. Mattern, S. J. Swiggers, A. L. Nigg, B. Lowenberg, A. B. Houtsmuller, J. M. Zijlmans, *Mol Cell Biol* **2004**; *24*: 5587-94.

[165] C. B. Harley, *Mutat Res* **1991**; *256*: 271-82.

[166] W. E. Wright, J. W. Shay, *Nat Med* **2000**; *6*: 849-51.

[167] S. R. Chan, E. H. Blackburn, *Philos Trans R Soc Lond B Biol Sci* **2004**; *359*: 109-21.

[168] J. W. Shay, *Clin Cancer Res* **2003**; *9*: 3521-5.

[169] C. J. Sherr, F. McCormick, *Cancer Cell* **2002**; *2*: 103-12.

[170] A. Smogorzewska, L. T. de, *EMBO J* **2002**; *21*: 4338-48.

[171] J. W. Shay, O. M. Pereira-Smith, W. E. Wright, *Exp Cell Res* **1991**; *196*: 33-9.

[172] J. W. Shay, W. E. Wright, H. Werbin, *Biochim Biophys Acta* **1991**; *1072*: 1-7.

[173] L. HAYFLICK, *Exp Cell Res* **1965**; *37*: 614-36.

[174] C. W. Greider, *Annu Rev Biochem* **1996**; *65*: 337-65.

[175] C. M. Counter, A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, S. Bacchetti, *EMBO J* **1992**; *11*: 1921-9.

[176] H. W. Lee, M. A. Blasco, G. J. Gottlieb, J. W. Horner, C. W. Greider, R. A. DePinho, *Nature* **1998**; *392*: 569-74.

[177] K. L. Rudolph, S. Chang, H. W. Lee, M. Blasco, G. J. Gottlieb, C. Greider, R. A. DePinho, *Cell* **1999**; *96*: 701-12.

[178] L. Chin, S. E. Artandi, Q. Shen, A. Tam, S. L. Lee, G. J. Gottlieb, C. W. Greider, R. A. DePinho, *Cell* **1999**; *97*: 527-38.

[179] M. L. Pardue, P. G. DeBaryshe, *Genetica* **1999**; *107*: 189-96.

[180] S. Le, J. K. Moore, J. E. Haber, C. W. Greider, *Genetics* **1999**; *152*: 143-52.

[181] V. Lundblad, E. H. Blackburn, *Cell* **1993**; *73*: 347-60.

[182] M. J. McEachern, E. H. Blackburn, *Genes Dev* **1996**; *10*: 1822-34.

[183] S. C. Teng, V. A. Zakian, *Mol Cell Biol* **1999**; *19*: 8083-93.

[184] T. M. Bryan, A. Englezou, L. Dalla-Pozza, M. A. Dunham, R. R. Reddel, *Nat Med* **1997**; *3*: 1271-4.

[185] S. Chang, C. M. Khoo, M. L. Naylor, R. S. Maser, R. A. DePinho, *Genes Dev* **2003**; *17*: 88-100.

[186] L. Harrington, T. McPhail, V. Mar, W. Zhou, R. Oulton, M. B. Bass, I. Arruda, M. O. Robinson, *Science* **1997**; *275*: 973-7.

[187] J. R. Mitchell, E. Wood, K. Collins, *Nature* **1999**; *402*: 551-5.

[188] S. Le, R. Sternglanz, C. W. Greider, *Mol Biol Cell* **2000**; *11*: 999-1010.

[189] F. Dragon, V. Pogacic, W. Filipowicz, *Mol Cell Biol* **2000**; *20*: 3037-48.

[190] V. Pogacic, F. Dragon, W. Filipowicz, *Mol Cell Biol* **2000**; *20*: 9028-40.

[191] L. P. Ford, J. M. Suh, W. E. Wright, J. W. Shay, *Mol Cell Biol* **2000**; *20*: 9084-91.

[192] L. P. Ford, J. W. Shay, W. E. Wright, *RNA* **2001**; *7*: 1068-75.

[193] N. S. Ting, Y. Yu, B. Pohorelic, S. P. Lees-Miller, T. L. Beattie, *Nucleic Acids Res* **2005**; *33*: 2090-8.

[194] D. Fu, K. Collins, *Genes Dev* **2006**; *20*: 531-6.

[195] C. Hoareau-Aveilla, M. Bonoli, M. Caizergues-Ferrer, Y. Henry, *RNA* **2006**; *12*: 832-40.

[196] H. Li, L. L. Zhao, J. W. Funder, J. P. Liu, *J Biol Chem* **1997**; *272*: 16729-32.

[197] S. E. Holt, D. L. Aisner, J. Baur, V. M. Tesmer, M. Dy, M. Ouellette, J. B. Trager, G. B. Morin, D. O. Toft, J. W. Shay, W. E. Wright, M. A. White, *Genes Dev* **1999**; *13*: 817-26.

[198] H. Li, Y. Cao, M. C. Berndt, J. W. Funder, J. P. Liu, *Oncogene* **1999**; *18*: 6785-94.

[199] S. Kharbanda, V. Kumar, S. Dhar, P. Pandey, C. Chen, P. Majumder, Z. M. Yuan, Y. Whang, W. Strauss, T. K. Pandita, D. Weaver, D. Kufe, *Curr Biol* **2000**; *10*: 568-75.

[200] S. S. Banik, C. M. Counter, *J Biol Chem* **2004**; *279*: 51745-8.

[201] F. Bachand, F. M. Boisvert, J. Cote, S. Richard, C. Autexier, *Mol Biol Cell* **2002**; *13*: 3192-202.

[202] W. Chai, L. P. Ford, L. Lenertz, W. E. Wright, J. W. Shay, *J Biol Chem* **2002**; *277*: 47242-7.

[203] J. Haendeler, J. Hoffmann, R. P. Brandes, A. M. Zeiher, S. Dimmeler, *Mol Cell Biol* **2003**; *23*: 4598-610.

[204] G. E. Lee, E. Y. Yu, C. H. Cho, J. Lee, M. T. Muller, I. K. Chung, *J Biol Chem* **2004**; *279*: 34750-5.

[205] S. Khurts, K. Masutomi, L. Delgermaa, K. Arai, N. Oishi, H. Mizuno, N. Hayashi, W. C. Hahn, S. Murakami, *J Biol Chem* **2004**; *279*: 51508-15.

[206] J. H. Kim, S. M. Park, M. R. Kang, S. Y. Oh, T. H. Lee, M. T. Muller, I. K. Chung, *Genes Dev* **2005**; *19*: 776-81.

[207] M. K. Mateyak, V. A. Zakian, *Cell Cycle* **2006**; *5*: 2796-804.

[208] P. Reichenbach, M. Hoss, C. M. Azzalin, M. Nabholz, P. Bucher, J. Lingner, *Curr Biol* **2003**; *13*: 568-74.

[209] Q. S. Zhang, L. Manche, R. M. Xu, A. R. Krainer, *RNA* **2006**; *12*: 1116-28.

[210] C. W. Greider, E. H. Blackburn, *Cell* **1985**; *43*: 405-13.

[211] S. B. Cohen, M. E. Graham, G. O. Lovrecz, N. Bache, P. J. Robinson, R. R. Reddel, *Science* **2007**; *315*: 1850-3.

[212] N. S. Heiss, S. W. Knight, T. J. Vulliamy, S. M. Klauck, S. Wiemann, P. J. Mason, A. Poustka, I. Dokal, *Nat Genet* **1998**; *19*: 32-8.

[213] J. M. Wong, K. Collins, *Genes Dev* **2006**; *20*: 2848-58.

[214] T. M. Nakamura, G. B. Morin, K. B. Chapman, S. L. Weinrich, W. H. Andrews, J. Lingner, C. B. Harley, T. R. Cech, *Science* **1997**; *277*: 955-9.

[215] C. W. Greider, E. H. Blackburn, *Nature* **1989**; *337*: 331-7.

[216] J. Feng, W. D. Funk, S. S. Wang, S. L. Weinrich, A. A. Avilion, C. P. Chiu, R. R. Adams, E. Chang, R. C. Allsopp, J. Yu, ., *Science* **1995**; *269*: 1236-41.

[217] K. Collins, J. R. Mitchell, *Oncogene* **2002**; *21*: 564-79.

[218] C. Wang, U. T. Meier, *EMBO J* **2004**; *23*: 1857-67.

[219] T. M. Bryan, K. J. Goodrich, T. R. Cech, *Mol Biol Cell* **2003**; *14*: 4794-804.

[220] J. Prescott, E. H. Blackburn, *Genes Dev* **1997**; *11*: 528-40.

[221] H. Ly, L. Xu, M. A. Rivera, T. G. Parslow, E. H. Blackburn, *Genes Dev* **2003**; *17*: 1078-83.

[222] M. A. Rivera, E. H. Blackburn, *J Biol Chem* **2004**; *279*: 53770-81.

[223] J. Prescott, E. H. Blackburn, *Genes Dev* **1997**; *11*: 2790-800.

[224] J. L. Osterhage, J. M. Talley, K. L. Friedman, *Nat Struct Mol Biol* **2006**; *13*: 720-8.

[225] M. Hsu, E. Y. Yu, S. M. Singh, N. F. Lue, *Eukaryot Cell* **2007**; *6*: 1330-8.

[226] K. Collins, *Nat Rev Mol Cell Biol* **2006**; *7*: 484-94.

[227] J. M. Wong, L. Kusdra, K. Collins, *Nat Cell Biol* **2002**; *4*: 731-6.

[228] A. A. Avilion, M. A. Piatyszek, J. Gupta, J. W. Shay, S. Bacchetti, C. W. Greider, *Cancer Res* **1996**; *56*: 645-50.

[229] C. Kelleher, M. T. Teixeira, K. Forstemann, J. Lingner, *Trends Biochem Sci* **2002**; *27*: 572-9.

[230] J. R. Vermeesch, D. Williams, C. M. Price, *Nucleic Acids Res* **1993**; *21*: 5366-71.

[231] A. Bianchi, D. Shore, *Genes Dev* **2007**; *21*: 1726-30.

[232] M. Sabourin, C. T. Tuzon, V. A. Zakian, *Mol Cell* **2007**; *27*: 550-61.

[233] S. Marcand, V. Brevet, C. Mann, E. Gilson, *Curr Biol* **2000**; *10*: 487-90.

[234] E. H. Blackburn, *Nature* **2000**; *408*: 53-6.

[235] G. Cristofari, J. Lingner, *EMBO J* **2006**; *25*: 565-74.

[236] J. Lin, E. H. Blackburn, *Genes Dev* **2004**; *18*: 387-96.

[237] X. Z. Zhou, K. P. Lu, *Cell* **2001**; *107*: 347-59.

[238] W. E. Wright, M. A. Piatyszek, W. E. Rainey, W. Byrd, J. W. Shay, *Dev Genet* **1996**; *18*: 173-9.

[239] G. A. Ulaner, L. C. Giudice, *Mol Hum Reprod* **1997**; *3*: 769-73.

[240] G. A. Ulaner, J. F. Hu, T. H. Vu, L. C. Giudice, A. R. Hoffman, *Cancer Res* **1998**; *58*: 4168-72.

[241] N. W. Kim, M. A. Piatyszek, K. R. Prowse, C. B. Harley, M. D. West, P. L. Ho, G. M. Coviello, W. E. Wright, S. L. Weinrich, J. W. Shay, *Science* **1994**; *266*: 2011-5.

[242] K. Masutomi, E. Y. Yu, S. Khurts, I. Ben-Porath, J. L. Currier, G. B. Metz, M. W. Brooks, S. Kaneko, S. Murakami, J. A. DeCaprio, R. A. Weinberg, S. A. Stewart, W. C. Hahn, *Cell* **2003**; *114*: 241-53.

[243] C. D. Belair, T. R. Yeager, P. M. Lopez, C. A. Reznikoff, *Proc Natl Acad Sci U S A* **1997**; *94*: 13677-82.

[244] E. Hiyama, N. Tatsumoto, T. Kodama, K. Hiyama, J. Shay, T. Yokoyama, *Int J Oncol* **1996**; *9*: 453-8.

[245] K. Takubo, K. Nakamura, N. Izumiyama, K. Mafune, Y. Tanaka, M. Miyashita, K. Sasajima, M. Kato, M. Oshimura, *J Surg Oncol* **1997**; *66*: 88-92.

[246] G. Bonatz, W. Klapper, A. Barthe, K. Heidorn, W. Jonat, G. Krupp, R. Parwaresch, *Biochem Biophys Res Commun* **1998**; *253*: 214-21.

[247] C. Harle-Bachor, P. Boukamp, *Proc Natl Acad Sci U S A* **1996**; *93*: 6476-81.

[248] S. Yasumoto, C. Kunimura, K. Kikuchi, H. Tahara, H. Ohji, H. Yamamoto, T. Ide, T. Utakoji, *Oncogene* **1996**; *13*: 433-9.

[249] J. Yui, C. P. Chiu, P. M. Lansdorp, *Blood* **1998**; *91*: 3255-62.

[250] A. G. Bodnar, N. W. Kim, R. B. Effros, C. P. Chiu, *Exp Cell Res* **1996**; *228*: 58-64.

[251] H. Stoppler, D. P. Hartmann, L. Sherman, R. Schlegel, *J Biol Chem* **1997**; *272*: 13332-7.

[252] N. P. Weng, L. Granger, R. J. Hodes, *Proc Natl Acad Sci U S A* **1997**; *94*: 10827-32.

[253] E. Hiyama, T. Yokoyama, N. Tatsumoto, K. Hiyama, Y. Imamura, Y. Murakami, T. Kodama, M. A. Piatyszek, J. W. Shay, Y. Matsuura, *Cancer Res* **1995**; *55*: 3258-62.

[254] C. P. Chiu, W. Dragowska, N. W. Kim, H. Vaziri, J. Yui, T. E. Thomas, C. B. Harley, P. M. Lansdorp, *Stem Cells* **1996**; *14*: 239-48.

[255] J. W. Shay, S. Bacchetti, *Eur J Cancer* **1997**; *33*: 787-91.

[256] D. Januszkiewicz, J. Wysoki, K. Lewandowski, M. Pernak, K. Nowicka, J. Rembowska, J. Nowak, *Int J Mol Med* **2003**; *12*: 935-8.

[257] J. W. Shay, A. F. Gazdar, *J Clin Pathol* **1997**; *50*: 106-9.

[258] S. E. Artandi, *Breast Cancer Res* **2003**; *5*: 37-41.

[259] N. Toshikuni, K. Nouso, T. Higashi, H. Nakatsukasa, T. Onishi, T. Kaneyoshi, Y. Kobayashi, K. Kariyama, K. Yamamoto, T. Tsuji, *Br J Cancer* **2000**; *82*: 833-7.

[260] Y. M. Park, J. Y. Choi, B. H. Byun, C. H. Cho, H. S. Kim, B. S. Kim, *Exp Mol Med* **1998**; *30*: 35-40.

[261] Y. Kawakami, M. Kitamoto, T. Nakanishi, W. Yasui, E. Tahara, J. Nakayama, F. Ishikawa, H. Tahara, T. Ide, G. Kajiyama, *Oncogene* **2000**; *19*: 3888-93.

[262] K. Nouso, Y. Urabe, T. Higashi, H. Nakatsukasa, N. Hino, K. Ashida, N. Kinugasa, K. Yoshida, S. Uematsu, T. Tsuji, *Cancer* **1996**; *78*: 232-6.

[263] H. Tahara, T. Nakanishi, M. Kitamoto, R. Nakashio, J. W. Shay, E. Tahara, G. Kajiyama, T. Ide, *Cancer Res* **1995**; *55*: 2734-6.

[264] K. Ohta, T. Kanamaru, Y. Morita, Y. Hayashi, H. Ito, M. Yamamoto, *J Gastroenterol* **1997**; *32*: 791-6.

[265] K. Kishimoto, J. Fujimoto, M. Takeuchi, H. Yamamoto, T. Ueki, E. Okamoto, *J Surg Oncol* **1998**; *69*: 119-24.

[266] T. S. Yeh, T. C. Chen, M. F. Chen, *Arch Surg* **2000**; *135*: 1334-9.

[267] R. Nakashio, M. Kitamoto, H. Tahara, T. Nakanishi, T. Ide, G. Kajiyama, *Int J Cancer* **1997**; *74*: 141-7.

[268] A. G. Bodnar, M. Ouellette, M. Frolkis, S. E. Holt, C. P. Chiu, G. B. Morin, C. B. Harley, J. W. Shay, S. Lichtsteiner, W. E. Wright, *Science* **1998**; *279*: 349-52.

[269] C. M. Counter, W. C. Hahn, W. Wei, S. D. Caddle, R. L. Beijersbergen, P. M. Lansdorp, J. M. Sedivy, R. A. Weinberg, *Proc Natl Acad Sci U S A* **1998**; *95*: 14723-8.

[270] C. P. Morales, S. E. Holt, M. Ouellette, K. J. Kaur, Y. Yan, K. S. Wilson, M. A. White, W. E. Wright, J. W. Shay, *Nat Genet* **1999**; *21*: 115-8.

[271] H. Vaziri, J. A. Squire, T. K. Pandita, G. Bradley, R. M. Kuba, H. Zhang, S. Gulyas, R. P. Hill, G. P. Nolan, S. Benchimol, *Mol Cell Biol* **1999**; *19*: 2373-9.

[272] L. HAYFLICK, P. S. MOORHEAD, *Exp Cell Res* **1961**; *25*: 585-621.

[273] L. I. Huschtscha, R. Holliday, *J Cell Sci* **1983**; *63*: 77-99.

[274] C. M. Counter, A. A. Avilion, C. E. LeFeuvre, N. G. Stewart, C. W. Greider, C. B. Harley, S. Bacchetti, *EMBO J* **1992**; *11*: 1921-9.

[275] T. Kiyono, S. A. Foster, J. I. Koop, J. K. McDougall, D. A. Galloway, A. J. Klingelhutz, *Nature* **1998**; *396*: 84-8.

[276] W. C. Hahn, C. M. Counter, A. S. Lundberg, R. L. Beijersbergen, M. W. Brooks, R. A. Weinberg, *Nature* **1999**; *400*: 464-8.

[277] S. A. Stewart, R. A. Weinberg, *Semin Cancer Biol* **2000**; *10*: 399-406.

[278] S. T. Yu, L. Chen, H. J. Wang, X. D. Tang, D. C. Fang, S. M. Yang, *Int J Oncol* **2009**; *35*: 329-36.

[279] K. J. Wu, C. Grandori, M. Amacker, N. Simon-Vermot, A. Polack, J. Lingner, R. Dalla-Favera, *Nat Genet* **1999**; *21*: 220-4.

[280] S. Kyo, M. Takakura, T. Taira, T. Kanaya, H. Itoh, M. Yutsudo, H. Ariga, M. Inoue, *Nucleic Acids Res* **2000**; *28*: 669-77.

[281] M. Takakura, S. Kyo, T. Kanaya, H. Hirano, J. Takeda, M. Yutsudo, M. Inoue, *Cancer Res* **1999**; *59*: 551-7.

[282] S. Kyo, M. Takakura, T. Kanaya, W. Zhuo, K. Fujimoto, Y. Nishio, A. Orimo, M. Inoue, *Cancer Res* **1999**; *59*: 5917-21.

[283] B. S. Goueli, R. Janknecht, *Oncogene* **2003**; *22*: 8042-7.

[284] T. Kanaya, S. Kyo, K. Hamada, M. Takakura, Y. Kitagawa, H. Harada, M. Inoue, *Clin Cancer Res* **2000**; *6*: 1239-47.

[285] D. Xu, Q. Wang, A. Gruber, M. Bjorkholm, Z. Chen, A. Zaid, G. Selivanova, C. Peterson, K. G. Wiman, P. Pisa, *Oncogene* **2000**; *19*: 5123-33.

[286] K. Fujimoto, S. Kyo, M. Takakura, T. Kanaya, Y. Kitagawa, H. Itoh, M. Takahashi, M. Inoue, *Nucleic Acids Res* **2000**; *28*: 2557-62.

[287] S. Oh, Y. Song, J. Yim, T. K. Kim, *J Biol Chem* **1999**; *274*: 37473-8.

[288] L. Liu, S. Lai, L. G. Andrews, T. O. Tollefsbol, *Gene* **2004**; *340*: 1-10.

[289] S. Y. Lin, S. J. Elledge, *Cell* **2003**; *113*: 881-9.

[290] A. Wu, M. Ichihashi, M. Ueda, *Cancer* **1999**; *86*: 2038-44.

[291] C. Gunes, S. Lichtsteiner, A. P. Vasserot, C. Englert, *Cancer Res* **2000**; *60*: 2116-21.

[292] E. M. Blackwood, R. N. Eisenman, *Science* **1991**; *251*: 1211-7.

[293] M. A. Casillas, Jr., N. Lopatina, L. G. Andrews, T. O. Tollefsbol, *Mol Cell Biochem* **2003**; *252*: 33-43.

[294] I. Horikawa, P. L. Cable, C. Afshari, J. C. Barrett, *Cancer Res* **1999**; *59*: 826-30.

[295] O. E. Bechter, W. Eisterer, M. Dlaska, T. Kuhr, J. Thaler, *Exp Hematol* **2002**; *30*: 26-33.

[296] N. G. Lopatina, J. C. Poole, S. N. Saldanha, N. J. Hansen, J. S. Key, M. A. Pita, L. G. Andrews, T. O. Tollefsbol, *Biochem Biophys Res Commun* **2003**; *306*: 650-9.

[297] K. H. Shin, M. K. Kang, E. Dicterow, N. H. Park, *Br J Cancer* **2003**; *89*: 1473-8.

[298] A. Kilian, D. D. Bowtell, H. E. Abud, G. R. Hime, D. J. Venter, P. K. Keese, E. L. Duncan, R. R. Reddel, R. A. Jefferson, *Hum Mol Genet* **1997**; *6*: 2011-9.

[299] M. Wick, D. Zubov, G. Hagen, *Gene* **1999**; *232*: 97-106.

[300] H. Hisatomi, K. Ohyashiki, J. H. Ohyashiki, K. Nagao, T. Kanamaru, H. Hirata, N. Hibi, Y. Tsukada, *Neoplasia* **2003**; *5*: 193-7.

[301] L. F. Lincz, L. M. Mudge, F. E. Scorgie, J. A. Sakoff, C. S. Hamilton, M. Seldon, *Neoplasia* **2008**; *10*: 1131-7.

[302] L. M. Colgin, C. Wilkinson, A. Englezou, A. Kilian, M. O. Robinson, R. R. Reddel, *Neoplasia* **2000**; *2*: 426-32.

[303] B. N. Nguyen, L. W. Elmore, S. E. Holt, *Cell Cycle* **2009**; *8*: 3227-33.

[304] S. Mitomo, C. Maesawa, S. Ogasawara, T. Iwaya, M. Shibazaki, A. Yashima-Abo, K. Kotani, H. Oikawa, E. Sakurai, N. Izutsu, K. Kato, H. Komatsu, K. Ikeda, G. Wakabayashi, T. Masuda, *Cancer Sci* **2008**; *99*: 280-6.

[305] S. A. Jacobs, E. R. Podell, T. R. Cech, *Nat Struct Mol Biol* **2006**; *13*: 218-25.

[306] E. Romi, N. Baran, M. Gantman, M. Shmoish, B. Min, K. Collins, H. Manor, *Proc Natl Acad Sci U S A* **2007**; *104*: 8791-6.

[307] S. Richards, R. A. Gibbs, G. M. Weinstock, S. J. Brown, R. Denell, R. W. Beeman, R. Gibbs, R. W. Beeman, S. J. Brown, G. Bucher, M. Friedrich, C. J. Grimmelikhuijzen, M. Klingler, M. Lorenzen, S. Richards, S. Roth, R. Schroder, D. Tautz, E. M. Zdobnov, D. Muzny, R. A. Gibbs, G. M. Weinstock, T. Attaway, S. Bell, C. J. Buhay, M. N. Chandrabose, D. Chavez, K. P. Clerk-Blankenburg, A. Cree, M. Dao, C. Davis, J. Chacko, H. Dinh, S. Dugan-Rocha, G. Fowler, T. T. Garner, J. Garnes, A. Gnirke, A. Hawes, J. Hernandez, S. Hines, M. Holder, J. Hume, S. N. Jhangiani, V. Joshi, Z. M. Khan, L. Jackson, C. Kovar, A. Kowis, S. Lee, L. R. Lewis, J. Margolis, M. Morgan, L. V. Nazareth, N. Nguyen, G. Okwuonu, D. Parker, S. Richards, S. J. Ruiz, J. Santibanez, J. Savard, S. E. Scherer, B. Schneider, E. Sodergren, D. Tautz, S. Vattahil, D. Villasana, C. S. White, R. Wright, Y. Park, R. W. Beeman, J. Lord, B. Oppert, M. Lorenzen, S. Brown, L. Wang, J. Savard, D. Tautz, S. Richards, G. Weinstock, R. A. Gibbs, Y. Liu, K. Worley, G. Weinstock, C. G. Elsik, J. T. Reese, E. Elhaik, G. Landan, D. Graur, P. Arensburger, P. Atkinson, R. W. Beeman, J. Beidler, S. J. Brown, J. P. Demuth, D. W. Drury, Y. Z. Du, H. Fujiwara, M. Lorenzen, V. Maselli, M. Osanai, Y. Park, H. M. Robertson, Z. Tu, J. J. Wang, S. Wang, S. Richards, H. Song, L. Zhang, E. Sodergren, D. Werner, M. Stanke, B. Morgenstern, V. Solovyev, P. Kosarev, G. Brown, H. C. Chen, O. Ermolaeva, W. Hlavina, Y. Kapustin, B. Kiryutin, P. Kitts, D. Maglott, K. Pruitt, V. Sapojnikov, A. Souvorov, A. J. Mackey, R. M. Waterhouse, S. Wyder, E. M. Zdobnov, E. M. Zdobnov, S. Wyder, E. V. Kriventseva, T. Kadowaki, P. Bork, M. Aranda, R. Bao, A. Beermann, N. Berns, R. Bolognesi, F. Bonneton, D. Bopp, S. J. Brown, G. Bucher, T. Butts, A. Chaumot, R. E. Denell, D. E. Ferrier, M. Friedrich, C. M. Gordon, M. Jindra, M. Klingler, Q. Lan, H. M. Lattorff, V. Laudet, L. C. von, Z. Liu, R. Lutz, J. A. Lynch, R. N. da Fonseca, N. Posnien, R. Reuter, S. Roth, J. Savard, J. B. Schinko, C. Schmitt, M. Schoppmeier, R. Schroder, T. D. Shippy, F. Simonnet, H. Marques-Souza, D. Tautz, Y. Tomoyasu, J. Trauner, M. Van der Zee, M. Vervoort, N. Wittkopp, E. A. Wimmer, X. Yang, A. K. Jones, D. B. Sattelle, P. R. Ebert, D. Nelson, J. G. Scott, R. W. Beeman, S. Muthukrishnan, K. J. Kramer, Y. Arakane, R. W. Beeman, Q. Zhu, D. Hogenkamp, R. Dixit, B. Oppert, H. Jiang, Z. Zou, J. Marshall, E. Elpidina, K. Vinokurov, C. Oppert, Z. Zou, J. Evans, Z. Lu, P. Zhao, N. Sumathipala, B. Altincicek, A. Vilcinskas, M. Williams, D. Hultmark, C. Hetru, H. Jiang, C. J. Grimmelikhuijzen, F. Hauser, G. Cazzamali, M. Williamson, Y. Park, B. Li, Y. Tanaka, R. Predel, S. Neupert, J. Schachtner, P. Verleyen, F. Raible, P. Bork, M. Friedrich, K. K. Walden, H. M. Robertson, S. Angeli, S. Foret, G. Bucher, S. Schuetz, R. Maleszka, E. A. Wimmer, R. W. Beeman, M. Lorenzen, Y. Tomoyasu, S. C. Miller, D. Grossmann, G. Bucher, *Nature* **2008**; *452*: 949-55.

[308] C. Autexier, N. F. Lue, *Annu Rev Biochem* **2006**; *75*: 493-517.

[309] Q. Zhang, N. K. Kim, J. Feigon, *Proc Natl Acad Sci U S A* **2011**; *108*: 20325-32.

[310] J. R. Mitchell, K. Collins, *Mol Cell* **2000**; *6*: 361-71.

[311] L. Martin-Rivera, M. A. Blasco, *J Biol Chem* **2001**; *276*: 5856-65.

[312] H. L. Forsythe, J. L. Jarvis, J. W. Turner, L. W. Elmore, S. E. Holt, *J Biol Chem* **2001**; *276*: 15571-4.

[313] K. Collins, *Annu Rev Biochem* **1999**; *68*: 187-218.

[314] N. F. Lue, *Bioessays* **2004**; *26*: 955-62.

[315] G. B. Morin, *Cell* **1989**; *59*: 521-9.

[316] C. Wei, M. Price, *Cell Mol Life Sci* **2003**; *60*: 2283-94.

[317] S. Marcand, E. Gilson, D. Shore, *Science* **1997**; *275*: 986-90.

[318] A. Bianchi, D. Shore, *Mol Cell* **2008**; *31*: 153-65.

[319] A. K. Taggart, S. C. Teng, V. A. Zakian, *Science* **2002**; *297*: 1023-6.

[320] S. F. Tseng, J. J. Lin, S. C. Teng, *Nucleic Acids Res* **2006**; *34*: 6327-36.

[321] K. B. Ritchie, J. C. Mallory, T. D. Petes, *Mol Cell Biol* **1999**; *19*: 6065-75.

[322] E. Pennock, K. Buckley, V. Lundblad, *Cell* **2001**; *104*: 387-96.

[323] S. Amiard, M. Doudeau, S. Pinte, A. Poulet, C. Lenain, C. Faivre-Moskalenko, D. Angelov, N. Hug, A. Vindigni, P. Bouvet, J. Paoletti, E. Gilson, M. J. Giraud-Panis, *Nat Struct Mol Biol* **2007**; *14*: 147-54.

[324] D. Hockemeyer, W. Palm, T. Else, J. P. Daniels, K. K. Takai, J. Z. Ye, C. E. Keegan, L. T. De, G. D. Hammer, *Nat Struct Mol Biol* **2007**; *14*: 754-61.

[325] D. Loayza, L. T. De, *Nature* **2003**; *423*: 1013-8.

[326] F. Wang, E. R. Podell, A. J. Zaug, Y. Yang, P. Baciu, T. R. Cech, M. Lei, *Nature* **2007**; *445*: 506-10.

[327] A. Smogorzewska, S. B. van, A. Bianchi, S. Oelmann, M. R. Schaefer, G. Schnapp, L. T. De, *Mol Cell Biol* **2000**; *20*: 1659-68.

[328] B. Li, L. T. De, *Mol Biol Cell* **2003**; *14*: 5060-8.

[329] B. Li, S. Oestreich, L. T. De, *Cell* **2000**; *101*: 471-83.

[330] M. S. O'Connor, A. Safari, D. Liu, J. Qin, Z. Songyang, *J Biol Chem* **2004**; *279*: 28585-91.

[331] B. R. Houghtaling, L. Cuttonaro, W. Chang, S. Smith, *Curr Biol* **2004**; *14*: 1621-31.

[332] J. Z. Ye, L. T. De, *Nat Genet* **2004**; *36*: 618-23.

[333] D. Liu, A. Safari, M. S. O'Connor, D. W. Chan, A. Laegeler, J. Qin, Z. Songyang, *Nat Cell Biol* **2004**; *6*: 673-80.

[334] T. Veldman, K. T. Etheridge, C. M. Counter, *Curr Biol* **2004**; *14*: 2264-70.

[335] J. Z. Ye, D. Hockemeyer, A. N. Krutchinsky, D. Loayza, S. M. Hooper, B. T. Chait, L. T. De, *Genes Dev* **2004**; *18*: 1649-54.

[336] Y. Wu, S. Xiao, X. D. Zhu, *Nat Struct Mol Biol* **2007**; *14*: 832-40.

[337] J. Karlseder, K. Hoke, O. K. Mirzoeva, C. Bakkenist, M. B. Kastan, J. H. Petrini, L. T. De, *PLoS Biol* **2004**; *2*: E240.

[338] H. Li, L. Zhao, Z. Yang, J. W. Funder, J. P. Liu, *J Biol Chem* **1998**; *273*: 33436-42.

[339] M. Folini, R. Bandiera, E. Millo, P. Gandellini, G. Sozzi, P. Gasparini, N. Longoni, M. Binda, M. G. Daidone, K. Berg, N. Zaffaroni, *Cell Prolif* **2007**; *40*: 905-20.

[340] L. Xi, G. Chen, J. Zhou, G. Xu, S. Wang, P. Wu, T. Zhu, A. Zhang, W. Yang, Q. Xu, Y. Lu, D. Ma, *Apoptosis* **2006**; *11*: 789-98.

[341] C. Dudognon, F. Pendino, J. Hillion, A. Saumet, M. Lanotte, E. Segal-Bendirdjian, *Oncogene* **2004**; *23*: 7469-74.

[342] Y. Cao, H. Li, S. Deb, J. P. Liu, *Oncogene* **2002**; *21*: 3130-8.

[343] B. D. Del, A. Rizzo, D. Trisciuoglio, G. Cardinali, M. R. Torrisi, U. Zangemeister-Wittke, G. Zupi, A. Biroccio, *Cell Death Differ* **2005**; *12*: 1429-38.

[344] R. Rahman, L. Latonen, K. G. Wiman, *Oncogene* **2005**; *24*: 1320-7.

[345] L. L. Smith, H. A. Coller, J. M. Roberts, *Nat Cell Biol* **2003**; *5*: 474-9.

[346] C. Geserick, A. Tejera, E. Gonzalez-Suarez, P. Klatt, M. A. Blasco, *Oncogene* **2006**; *25*: 4310-9.

[347] K. Masutomi, R. Possemato, J. M. Wong, J. L. Currier, Z. Tothova, J. B. Manola, S. Ganesan, P. M. Lansdorp, K. Collins, W. C. Hahn, *Proc Natl Acad Sci U S A* **2005**; *102*: 8222-7.

[348] J. H. Santos, J. N. Meyer, M. Skorvaga, L. A. Annab, H. B. Van, *Aging Cell* **2004**; *3*: 399-411.

[349] S. Ahmed, J. F. Passos, M. J. Birket, T. Beckmann, S. Brings, H. Peters, M. A. Birch-Machin, Z. T. von, G. Saretzki, *J Cell Sci* **2008**; *121*: 1046-53.

[350] M. Kedde, S. C. le, A. Duursma, E. Zlotorynski, L. B. van, W. Nijkamp, R. Beijersbergen, R. Agami, *J Biol Chem* **2006**; *281*: 40503-14.

[351] M. A. Blasco, M. Rizen, C. W. Greider, D. Hanahan, *Nat Genet* **1996**; *12*: 200-4.

[352] M. M. Haddad, W. Xu, D. J. Schwahn, F. Liao, E. E. Medrano, *Exp Cell Res* **1999**; *253*: 561-72.

[353] A. J. Walne, A. Marrone, I. Dokal, *Int J Hematol* **2005**; *82*: 184-9.

[354] P. J. Mason, D. B. Wilson, M. Bessler, *Curr Mol Med* **2005**; *5*: 159-70.

[355] I. Dokal, *Br J Haematol* **2000**; *110*: 768-79.

[356] S. R. Romanov, B. K. Kozakiewicz, C. R. Holst, M. R. Stampfer, L. M. Haupt, T. D. Tlsty, *Nature* **2001**; *409*: 633-7.

[357] J. R. Mitchell, J. Cheng, K. Collins, *Mol Cell Biol* **1999**; *19*: 567-76.

[358] T. J. Vulliamy, A. Marrone, S. W. Knight, A. Walne, P. J. Mason, I. Dokal, *Blood* **2006**; *107*: 2680-5.

[359] T. H. Nguyen, G. Mai, P. Villiger, J. Oberholzer, P. Salmon, P. Morel, L. Buhler, D. Trono, *J Hepatol* **2005**; *43*: 1031-7.

[360] S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele, C. T. Wittwer, *Clin Chem* **2009**; *55*: 611-22.

[361] P. K. Smith, R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, D. C. Klenk, *Anal Biochem* **1985**; *150*: 76-85.

[362] W. Chearwae, S. Anuchapreeda, K. Nandigama, S. V. Ambudkar, P. Limtrakul, *Biochem Pharmacol* **2004**; *68*: 2043-52.

[363] W. Chearwae, C. P. Wu, H. Y. Chu, T. R. Lee, S. V. Ambudkar, P. Limtrakul, *Cancer Chemother Pharmacol* **2006**; *57*: 376-88.

[364] W. Chearwae, S. Anuchapreeda, K. Nandigama, S. V. Ambudkar, P. Limtrakul, *Biochem Pharmacol* **2004**; *68*: 2043-52.

[365] W. Chearwae, C. P. Wu, H. Y. Chu, T. R. Lee, S. V. Ambudkar, P. Limtrakul, *Cancer Chemother Pharmacol* **2006**; *57*: 376-88.

[366] D. Kornbrot, *Br J Math Stat Psychol* **2000**; *53 (Pt 2)*: 335-7.

[367] M. Zuker, *Nucleic Acids Res* **2003**; *31*: 3406-15.

[368] N. R. Markham, M. Zuker, *Methods Mol Biol* **2008**; *453*: 3-31.

[369] J. Santoyo, J. M. Vaquerizas, J. Dopazo, *Bioinformatics* **2005**; *21*: 1376-82.

[370] L. Wang, F. Y. Mu, *Bioinformatics* **2004**; *20*: 1818-20.

[371] A. Henschel, F. Buchholz, B. Habermann, *Nucleic Acids Res* **2004**; *32*: W113-W120.

[372] A. M. Chalk, C. Wahlestedt, E. L. Sonnhammer, *Biochem Biophys Res Commun* **2004**; *319*: 264-74.

[373] W. Li, L. Cha, *Cell Mol Life Sci* **2007**; *64*: 1785-92.

[374] K. Ui-Tei, Y. Naito, F. Takahashi, T. Haraguchi, H. Ohki-Hamazaki, A. Juni, R. Ueda, K. Saigo, *Nucleic Acids Res* **2004**; *32*: 936-48.

[375] K. Ui-Tei, Y. Naito, K. Saigo, *J Biomed Biotechnol* **2006**; *2006*: 65052.

[376] J. A. Gredell, A. K. Berger, S. P. Walton, *Biotechnol Bioeng* **2008**; *100*: 744-55.

[377] S. Schubert, A. Grunweller, V. A. Erdmann, J. Kurreck, *J Mol Biol* **2005**; *348*: 883-93.

[378] E. Bernstein, A. A. Caudy, S. M. Hammond, G. J. Hannon, *Nature* **2001**; *409*: 363-6.

[379] J. F. Rual, N. Klitgord, G. Achaz, *BMC Genomics* **2007**; *8*: 106.

[380] T. H. Nguyen, G. Mai, P. Villiger, J. Oberholzer, P. Salmon, P. Morel, L. Buhler, D. Trono, *J Hepatol* **2005**; *43*: 1031-7.

[381] J. Saffi, M. H. Agnoletto, T. N. Guecheva, L. F. Batista, H. Carvalho, J. A. Henriques, A. Stary, C. F. Menck, A. Sarasin, *DNA Repair (Amst)* **2010**; *9*: 40-7.

[382] M. Agostinho, V. Santos, F. Ferreira, R. Costa, J. Cardoso, I. Pinheiro, J. Rino, E. Jaffray, R. T. Hay, J. Ferreira, *Cancer Res* **2008**; *68*: 2409-18.

[383] S. Wang, E. A. Konorev, S. Kotamraju, J. Joseph, S. Kalivendi, B. Kalyanaraman, *J Biol Chem* **2004**; *279*: 25535-43.

[384] A. M. Calcagno, J. M. Fostel, K. K. To, C. D. Salcido, S. E. Martin, K. J. Chewning, C. P. Wu, L. Varticovski, S. E. Bates, N. J. Caplen, S. V. Ambudkar, *Br J Cancer* **2008**; *98*: 1515-24.

[385] C. Atalay, A. Demirkazik, U. Gunduz, *J Chemother* **2008**; *20*: 734-9.

[386] Y. M. Jeng, S. Y. Peng, C. Y. Lin, H. C. Hsu, *Clin Cancer Res* **2004**; *10*: 2065-71.

[387] M. Kato, H. Maeta, S. Kato, T. Shinozawa, T. Terada, *Mod Pathol* **2000**; *13*: 1060-5.

[388] L. C. Dai, *World J Gastroenterol* **2009**; *15*: 412-6.

[389] S. Ohhashi, K. Ohuchida, K. Mizumoto, T. Egami, J. Yu, L. Cui, H. Toma, S. Takahata, T. Nabae, M. Tanaka, *Dig Dis Sci* **2009**; *54*: 811-5.

[390] I. Tamm, Y. Wang, E. Sausville, D. A. Scudiero, N. Vigna, T. Oltersdorf, J. C. Reed, *Cancer Res* **1998**; *58*: 5315-20.

[391] T. Ito, K. Shiraki, K. Sugimoto, T. Yamanaka, K. Fujikawa, M. Ito, K. Takase, M. Moriyama, H. Kawano, M. Hayashida, T. Nakano, A. Suzuki, *Hepatology* **2000**; *31*: 1080-5.

[392] M. Overhoff, M. Alken, R. K. Far, M. Lemaitre, B. Lebleu, G. Sczakiel, I. Robbins, *J Mol Biol* **2005**; *348*: 871-81.

[393] A. D. Judge, V. Sood, J. R. Shaw, D. Fang, K. McClintock, I. MacLachlan, *Nat Biotechnol* **2005**; *23*: 457-62.

[394] D. H. Kim, M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi, J. J. Rossi, *Nat Biotechnol* **2005**; *23*: 222-6.

[395] Z. Chen, B. P. Monia, D. R. Corey, *J Med Chem* **2002**; *45*: 5423-5.

[396] H. Seimiya, T. Oh-hara, T. Suzuki, I. Naasani, T. Shimazaki, K. Tsuchiya, T. Tsuruo, *Mol Cancer Ther* **2002**; *1*: 657-65.

[397] J. Beisner, M. Dong, S. Taetz, N. Nafee, E. U. Griese, U. Schaefer, C. M. Lehr, U. Klotz, T. E. Murdter, *Lung Cancer* **2010**; *68*: 346-54.

[398] S. Li, J. Crothers, C. M. Haqq, E. H. Blackburn, *J Biol Chem* **2005**; *280*: 23709-17.

[399] P. Gandellini, M. Folini, R. Bandiera, C. M. De, M. Binda, S. Veronese, M. G. Daidone, F. Zunino, N. Zaffaroni, *Biochem Pharmacol* **2007**; *73*: 1703-14.

[400] M. Folini, C. Brambilla, R. Villa, P. Gandellini, S. Vignati, F. Paduano, M. G. Daidone, N. Zaffaroni, *Eur J Cancer* **2005**; *41*: 624-34.