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Down-regulation of Transcobalamin Receptor TCblR/*CD320* by siRNA Inhibits Cobalamin uptake and Proliferation of Cells in Culture

Shao-Chiang Lai[2], **Yasumi Nakayama**[1], **Jeffrey M. Sequeira**[1], and **Edward V. Quadros**[1,2]

[1] Departments of Medicine, Cell Biology, SUNY-Downstate Medical Center, Brooklyn, NY 11203

[2] School of Graduate Studies, SUNY-Downstate Medical Center, Brooklyn, NY 11203

Abstract

The clinical phenotype of cobalamin (Cbl) deficiency is dictated by the essential role of this vitamin in two key enzymatic reactions. Multiple proteins and receptors participate in the absorption, transport and delivery of this vitamin to tissue cells. Cellular uptake of Cbl is mediated by transcobalamin (TC), a plasma protein and a transmembrane receptor (TCblR) with high affinity for TC saturated with Cbl. Knockdown of TCblR with siRNA results in decreased TC-Cbl uptake. The ensuing Cbl deficiency leads to an increase in doubling time and decreased proliferation of these cells. The study confirms the seminal role of this receptor in the cellular uptake of Cbl and its down-regulation as a potential strategy to inhibit proliferation of cancer cells.

Introduction

Vitamin B12 (Cobalamin;Cbl) is an essential water soluble micronutrient. The two coenzyme forms, 5′deoxyadenosyl (ado-Cbl) and methyl-Cbl, are involved in metabolic reactions. Ado-Cbl is a cofactor of methylmalonyl-CoA mutase that converts methylmalonyl-CoA to succinyl-CoA, an intermediate in the TCA cycle [1]. In the *de novo* synthesis of methionine catalyzed by methionine synthase (MS), methyl-Cbl acts as coenzyme to transfer methyl group from N^5methyltetrahydrofolate (methyl-FH4) to homocysteine [2]. Thus, consequences of Cbl deficiency would be decreased Ado-Cbl and Methyl-Cbl contributing to decreased activity of the two enzymes which leads to elevated methylmalonic acid (MMA) and homocysteine (HCY), respectively [3]. Moreover, methyl-FH4 is one of the intermediates in folate pathways involved in single carbon exchange reactions and DNA synthesis. Thus, Cbl plays an important role in recycling of methylFH_4 to maintain adequate supply of reduced folates [4].

The cellular uptake of Cbl requires transcobalamin (TC), a plasma protein secreted by the vascular endothelial cells [5], and a receptor (TCblR/CD320) that mediates binding and internalization of TC saturated with Cbl [6]. Human TCblR, a transmembrane glycosylated protein, is encoded by the CD320 gene located at p13.2 on chromosome 19. The predicted TCblR protein structure contains a cytoplasmic tail, a transmembrane domain and an

Address correspondence to: Edward Quadros, Box 20, SUNY Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, NY 11203. Fax: 718-270-3316. Edward.Quadros@downstate.edu.

extracellular domain with two LDL receptor type A domains [7]. The expression of TCblR is associated with the cell cycle and reaches highest expression of about 2000–6000 receptors per cell when cells are actively proliferating during early log phase of growth. During the resting and fully differentiated phase, TCblR expression is down regulated to fewer than 300 receptors [8–10]. The increased expression of TCblR may be due to the demand for Cbl during DNA synthesis. Blocking Cbl uptake by monoclonal antibodies to TC has shown to inhibit proliferation of cancer cells [11]. Depletion of Cbl by sequestering Cbl in the cytoplasm using stable over-expression of TC linked with a plant protein, oleosin, produces B12 deficiency in cells, impairs B12 metabolism and reduces the proliferation of neuroblastoma cells [12]. Furthermore, monoclonal antibody (mAb) 8D6, now identified as mAb against TCblR, inhibited the proliferation of lymphoma cell line, L3055, in combination with mAb, 4G10 [13]. Here, we provide evidence using siRNA to TCblR, that blocking TCblR expression can decrease Cbl uptake in SW48 and HEK293 cells and this affects propagation of cells in culture.

Materials and Methods

Cell culture

A human colorectal adenocarcinoma SW48 (ATCC #CCL-231) and a human embryonic kidney cell line HEK293 (ATCC #CRL-1573) were cultured in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM) with 10% Fetal Bovine Serum (Hyclone), 2mM Glutamax (Gibco) and 50units/ml penicillin/50ug/ml streptomycin (Invitrogen). HEK293 cells over expressing TCblR were produced by transfecting with the cDNA in pcDNA 3.1 plasmid and stable clones expressing high levels of TCblR were selected by culturing cells in medium containing G418 (Geneticin, Invitrogen).

Binding and uptake of TC-Cbl

[^{57}Co]Cbl was obtained from (MP Biomedical) and recombinant human TC (rhTC) was collected from the medium of HEK293 cells that stably express human TC. The concentration and binding capacity of TC were determined by a competitive binding assay [14]. The TC[^{57}Co]Cbl for the uptake studies was prepared by incubating [^{57}Co]Cbl with a 3X molar excess of rhTC for 1 hour at room temperature. Binding of radiolabeled Cbl to TC and saturation was confirmed by mixing an aliquot of the sample with hemoglobin coated charcoal that adsorbs free Cbl and provides a measure of protein bound and free [^{57}Co]Cbl.

Functional receptor assay

The TCblR functional assay was performed by measuring TC[^{57}Co]Cbl uptake in cells seeded at a density of $0.2x10^6$ in 6-well plates as previously described [5, 15]. At each time point, medium was removed and cells were washed once with 1ml of Ca^{++}-free Hank's balanced salt solution (HBSS, Sigma). TC[^{57}Co]Cbl (10,000 CPM) in 1ml DMEM was added to each well and incubated at 37°C in a humidified 5% CO_2 incubator for 1 hour. Cells were harvested by using 0.05% trypsin/0.02% EDTA (Sigma) after washing cells twice with 1ml of \ HBSS. Cells were collected with 0.8ml of HBSS and TC[^{57}Co]Cbl uptake in cells was measured using a gamma counter. Cell number was determined by counting in a hemocytometer. Cell viability was determined by trypan blue dye (Gibco) exclusion for both TCblR expression and cell proliferation experiments.

siRNA transfection

Three CD320 siRNAs (No.88 [5′-GCGAUGAGGAGGAGUGCAGGAUUGA-3′], No.89 [5′-GAACUGACAAGAAACUGCGC AACUG-3′] and No.90 [5′-CGAGCUCGGCUGUGGAACCAAUGAG-3′]) and a scrambled siRNA for negative

control were purchased from Invitrogen. Cells were seeded in 6 well plates at a density of $0.1x10^6$ cells/ml in 2ml complete medium without antibiotics for 24 hours prior to transfection. The siRNA was mixed with transfection reagent Lipofectamine 2000 (Invitrogen) and DMEM according to the manufacturer's instructions. The transfected cells were incubated at 37°C in a humidified 5% CO_2 incubator for the indicated time period without changing medium.

qRT-PCR analysis

Total RNA was isolated using Trizol reagent (Invitrogen). Before total RNA isolation, cells were washed with 1ml HBSS twice followed by harvesting cells with 0.2ml of 1X trypsin/EDTA and 0.8ml Hank's balanced salt solution. Centrifugation of cells was done by spinning at 3000 rpm and 4°C for 5 minutes. Trizol reagent was added to cell pellet and total RNA was extracted using the protocol provided by the manufacturer. Total RNA (1ug) was used for first-strand cDNA synthesis with SuperScript II reverse transcriptase and random primers (Invitrogen). The first-strand cDNA synthesis product was further diluted 1:10 and 2ul of the diluted first-strand cDNA was used for qRT-PCR with SYBR GreenER supermix for ABI PRISM (Invitrogen), Human TCblR primers [Forward 5′-AAGTTCCAGTGCCGCACCAGT-3′; Reverse 5′-AGTCACTGACGCCGGTGCAGG-3′] and human beta actin [Forward 5′-GGATGCAGAAGGAGATCACTG-3′; Reverse 5′-CGATCCACACGGAGTACTTG-3′] as internal control were used for PCR amplification and fluorescence detection in a ABI StepOne instrument (Applied Biosystem) under the following conditions: (95°C, 10 min, followed by 40 cycles of 95 °C, 15 sec and 60°C, 1 min). The fold change was calculated by relative comparison method, ΔCt.

Cell proliferation assay

Cells (2000/well) were plated in 100 ul complete DMEM medium without antibiotics in 96-well plate and siRNA transfection was done with 10 pmoles siRNA and 0.25 ul Lipofectamine 2000 per well according to the manufacturer's instructions. Cells were incubated at 37°C in a humidified 5% CO_2 incubator for 96 hours before assay. The proliferation assay was performed using the CellTiter96 Aqueous One solution cell proliferation assay (Promega). Optical density at 495nm was read in a microplate reader (Bio-Rad). Statistical analysis was done using GraphPad software Prism 3.03.

Results

The expression of functional receptors in SW48 and HEK293 cells, as indicated by the binding of TC-Cbl during 120 hours in culture showed highest TC-Cbl binding 24 – 48h after seeding when most of the cells were actively dividing. During the subsequent 4 days in culture, the receptor expression gradually decreased to that typically found in resting cells (Figure 1a). The mRNA level, as determined by qRT-PCR showed a similar profile with a peak level between 24 and 48h (Figure 1b). In HEK293 cells, the level of functional receptor expression appeared to coincide with the level of mRNA; however, in SW48 cells peak TC-Cbl binding was observed at 24h, even though the mRNA level peaked at 48h. At peak expression, functional receptor was 4.2 fold higher in SW48 compared to HEK293 cells. However, this difference was virtually eliminated after 120 hours in culture. In contrast, the mRNA level was only 1.1 fold higher in SW48 cells (Figure 1, A and B insets).

The three commercially available siRNA oligonucleotides were effective in blocking the synthesis of TCblR as indicated by the decrease in TC-Cbl binding to cells 72 hours after transfecting HEK293 cells with the siRNA. As little as 2 nM siRNA inhibited receptor expression by about 80% (Figure 2). Based on these results, a concentration of 20nM was chosen for subsequent experiments which produced a greater than 90% decrease in

functional receptor at 72h in both cell lines but not in HEK293 cells engineered to over express TCblR (Figure 2 inset). The decrease in functional receptor expression was accompanied by a proportionate decrease in mRNA level. In cells exposed to siRNA, the decrease in functional receptor and mRNA expression was accompanied by a decrease in cell number, which at 144 hours accounted for a 72% decrease for HEK 293 cells (Figure 3A) and a 59% decrease for SW48 cells (Figure 4A). The concentration of siRNA used resulted in sustained suppression of TCblR mRNA throughout the 144 hour culture period in SW48 cells but increased slightly in HEK293 cells likely due to differences in transfection efficiency (Figs 3B & 4B). The decrease in cell numbers when SW48 and normal HEK293 cells were transfected with siRNA was confirmed by a MTS based cell proliferation assay which also showed inhibition of cell proliferation in both cell lines and lack of inhibition in HEK293 cells over expressing TCblR (Figure 5). The decrease in proliferation could not be corrected by adding 100uM free B12 to the culture medium. This inhibition appeared to be due to an increase in the doubling time of cells specifically due to decreased TCblR expression contributing to decreased cellular uptake of TC-Cbl, since both SW48 and normal HEK293 cells showed an increase in doubling time. However, HEK293 cells over expressing TCblR showed no change in doubling time (Figure 6).

Discussion

Cbl deficiency in humans results in megaloblastic anemia that is indistinguishable from the anemia of folate deficiency due to the fact that defective DNA synthesis in Cbl deficiency, is a consequence of entrapment of folate in the form of methylfolate contributing to intracellular deficiency of folate cofactors [4]. Thus intracellular Cbl deficiency should affect DNA synthesis and replication. The expression of TCblR, the receptor for cellular uptake of TC-Cbl is cell cycle associated with peak expression during the S phase of the cell cycle [8–10]. Due to the proliferative nature of neoplastic cells, TCblR expression is 5 to 10 fold up regulated and sustained in certain cancer cells to meet the increased demand for Cbl and folate as evident from the accumulation of these compounds in tumors [16]. Blocking folate metabolism has proven to be an effective strategy in cancer therapy [17, 18, 19]. Therefore in Cbl deficiency, the reliance on Cbl for generating the much needed folate pool is also likely to be effective in inhibiting the replication of cancer cells. The higher expression of the receptor in sync with sustained proliferation of cancer cells is likely to provide preferential targeting of cancer cells. Our previous study on the characterization of mAbs to human TC had identified Cbl blocking and receptor blocking antibodies to TC [20]. These properties of the mAbs have been exploited to deplete cells of Cbl and inhibit Cbl dependent proliferation of cancer cells. Using mAbs to TC that block the binding of Cbl or block the binding of TC-Cbl to TCblR have proven to be effective in blocking Cbl uptake in cells and inhibiting proliferation of these cells in culture [11]. A similar approach using monoclonal antibodies to TCblR, the receptor for holo-TC also blocks cellular uptake of Cbl as we have shown with the blocking anti TCblR antibody [21]. Selective targeting of antibody-Saporin toxin to cancer cells has been achieved with monoclonal antibody to TCblR [22]. Inhibition of TCblR expression by specific siRNA results in decreased mRNA and loss of functional receptor. The resulting decrease in cellular uptake of TC-Cbl confirms the existence of a singular pathway for cellular uptake of Cbl in most cancer cell types. A single publication has previously described the uptake of free B12 in human skin fibroblasts in culture (23). The failure to rescue our cell lines with a fairly large amount of free B12 suggests that free B12 is not taken up adequately in the two cell lines tested. However, response to high dose B12 therapy in congenital TC deficiency suggests that other mechanisms may operate *in vivo* for Cbl uptake in some tissues. The decrease in proliferation is unlikely to be due to secondary nonspecific effects of siRNA and transfection since over expression of TCblR to a level that cannot be suppressed by siRNA, corrects the effect. The increase in doubling time and decrease in cell number resulting from

decreased uptake of TC-Cbl provides proof of the essential role of Cbl in cellular proliferation. Identifying target genes with selectivity for siRNA based knockdown in cancer therapy would provide an alternate strategy to control neoplastic growth, especially when conventional therapy fails [24]. The feasibility of such an approach has been tested where a target gene is over expressed and suppression of this gene by siRNA inhibits proliferation of cancer cells. For example blocking of CDC25 oncogene in hepatocellular carcinoma [25] or vascular endothelial growth factors in metastatic breast cancer [26] inhibits proliferation of these tumors. Based on the clinical presentation of Cbl deficiency, TCblR knockdown is unlikely to produce the severe systemic and off-target effects seen in most convention chemotherapy protocols. It is also less likely to culminate in resistance due to the fact that the strategy involves depleting an essential nutrient. Identifying novel targets and strategies provides the opportunity to expand the multi pronged attack on cancer cells that become resistant to conventional therapies.

Acknowledgments

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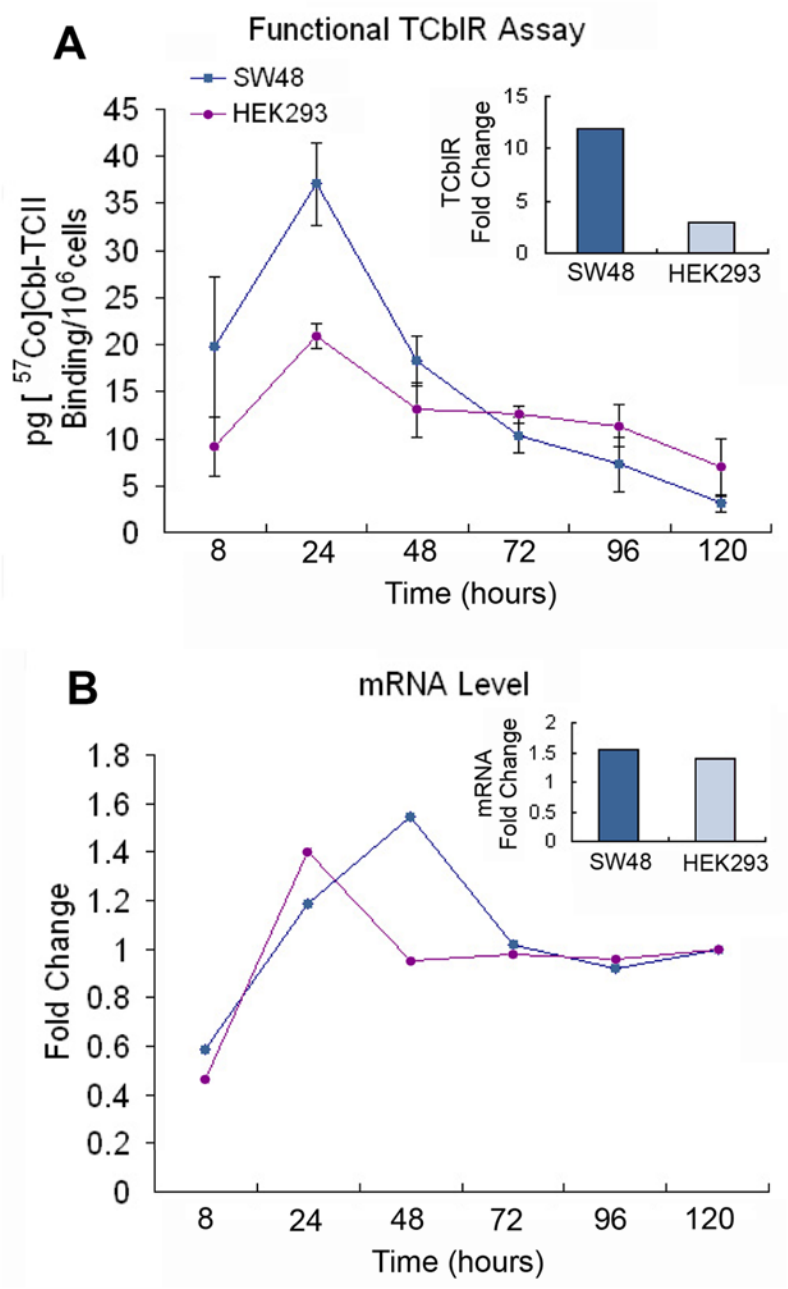

Fig. 1.

TC-Cbl binding activity and TCblR mRNA expression as a function of time in culture: A) $0.2x10^6$ cells were seeded with 2 ml complete medium in 6 well plates. At various time points the medium was removed and cells were incubated with TC[^{57}Co]Cbl in 1ml DMEM for 1 hour at 37°C. Labeled TC binding was calculated from the specific activity of [^{57}Co]Cbl. The data represents the mean±SD of two experiments done in triplicates. Inset: each bar represents the ratio of TC[^{57}Co]Cbl uptake at peak time point to that at 120 hours. (B) Total RNA was isolated from cells pooled at each time point to synthesize first strand cDNA and mRNA level was determined by RTqPCR. Data represent the mean relative fold

change compared to mRNA level at 120 hours. Inset: Ratio of relative fold change of TCblR mRNA level at peak to that at 120 hours in SW48 and HEK293 cells.

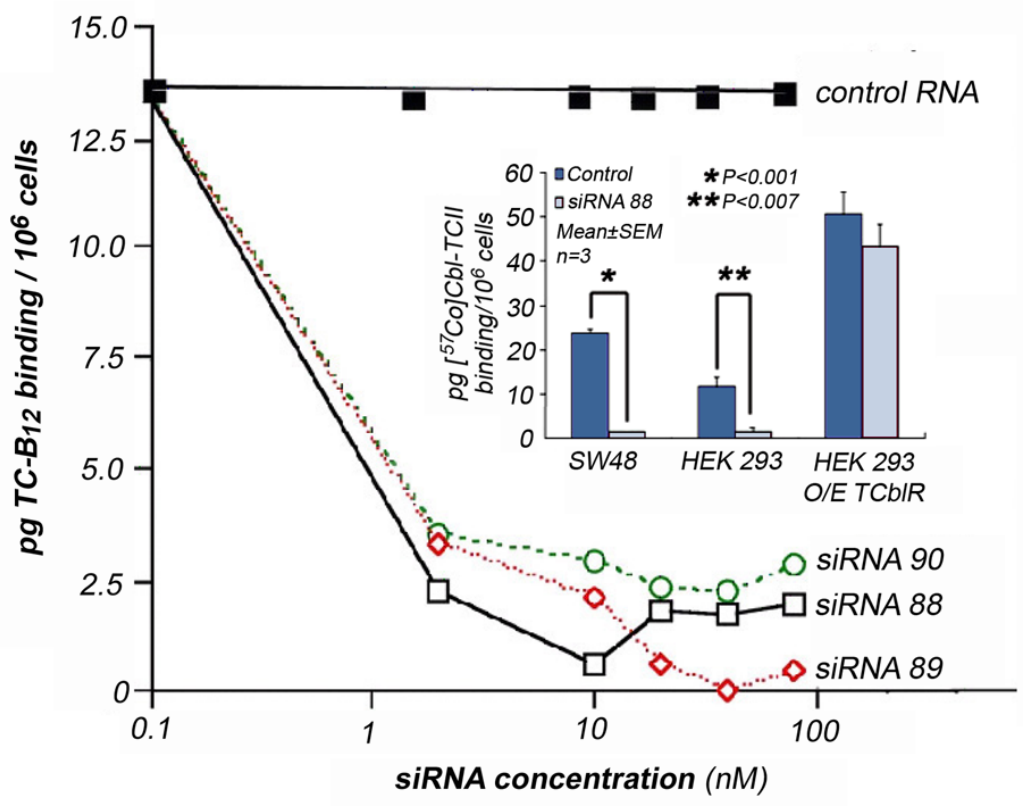

Fig. 2.

The effect of siRNA on TCblR expression as determined by TC-Cbl binding in HEK293 cells. HEK293 cells were seeded in a 6 well plate overnight and then transfected with siRNA 88 or control scrambled siRNA. Binding of TC[^{57}Co]Cbl was determined after 72 hours by incubating cells in fresh DMEM with TC[^{57}Co]Cbl for 1 hour at 37°C. Inset shows significant decrease in functional receptor in SW48 and HEK293 cells transfected with 20nM siRNA and lack of effect in HEK293 cells constitutively over expressing TCblR.

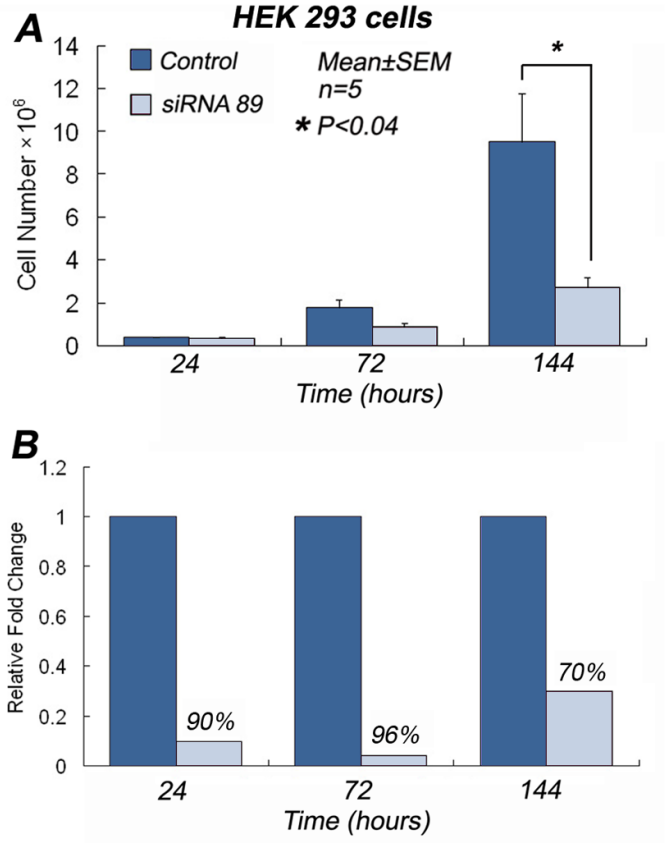

Fig. 3.

Effect of siRNA on TCblR mRNA expression and proliferation of HEK293 cells. A) cell number and B) mRNA level at three time points. In each experiment, the effect of siRNA (20nM) is compared to an equivalent amount of control scrambled siRNA. The number above each bar in panel B represents the inhibition induced by the siRNA.

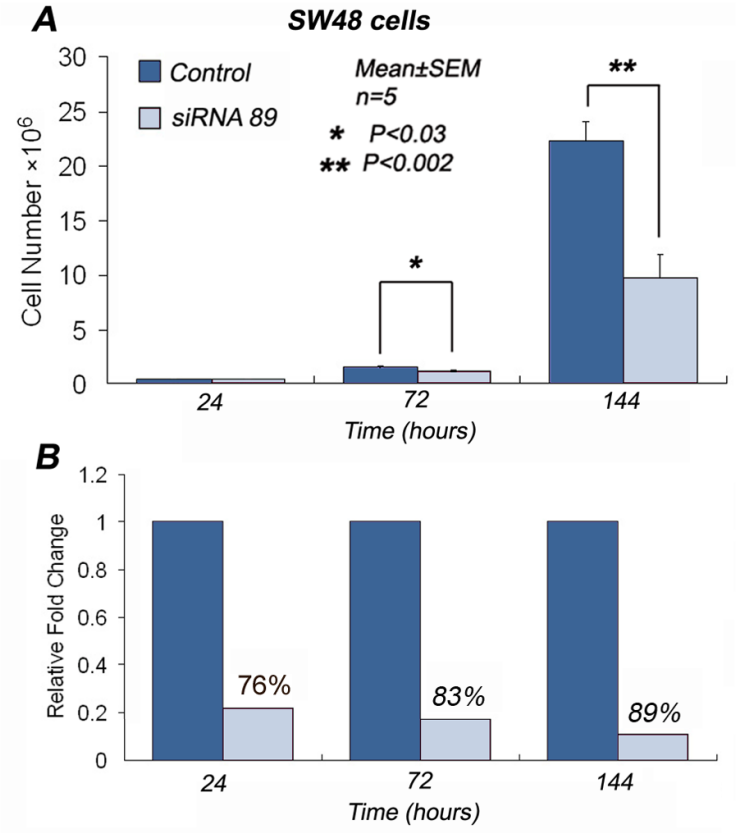

Fig. 4.

Effect of siRNA on TCblR mRNA expression and proliferation of SW48 cells. A. Cell number and B. mRNA level at three time points. In each experiment, the effect of isRNA (20nM) is compared to an equivalent amount of control scrambled siRNA. The number above each bar in panel B represents the inhibition induced by the siRNA.

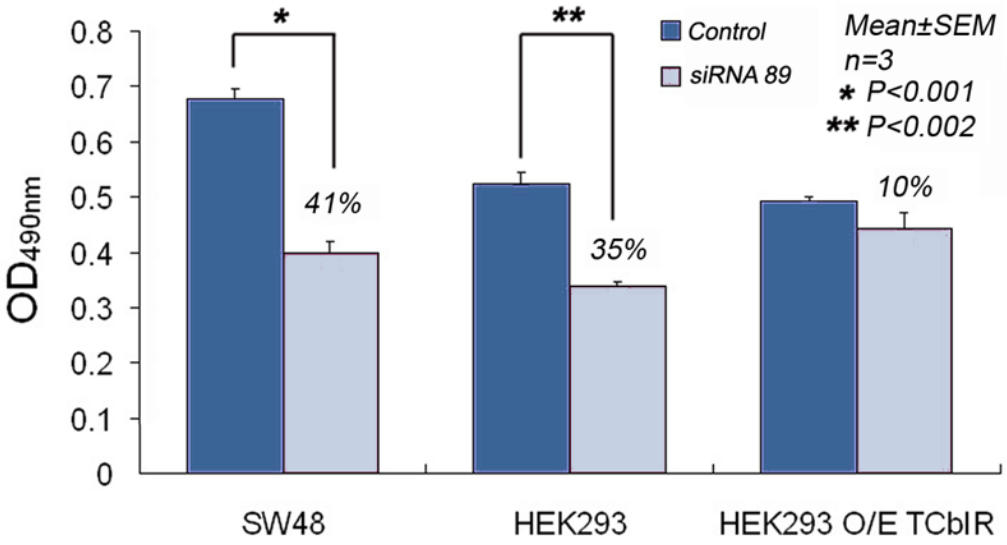

Fig. 5.

Effect of receptor specific siRNA on cell proliferation as determined by MTS assay. SW48, HEK293 and HEK293 O/E TCblR cells were seeded in a 96 well plate overnight and transfected with 10 pmoles siRNA. Cell proliferation was determined at 96 hours using MTS assay. The number above each bar represents the inhibition of cell proliferation.

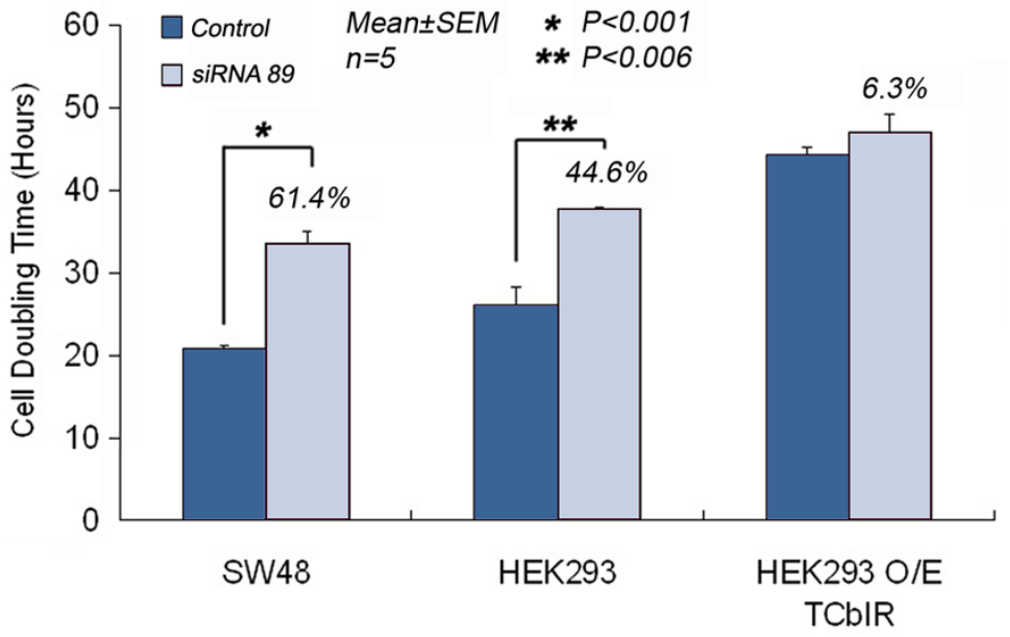

Fig. 6.

The inhibition of cell proliferation is specifically due to TCblR siRNA gene silencing. The experiment setup is the same as described in Fig.4 and Fig.5. The cell doubling time was calculated using the equation ln 2/ln (Cf/Ci) x hours; Ci: cell number at 24 hours time point; Cf: cell number at 144 hour time point. Data shows Mean ± SEM for doubling time and the value above the bar represents the percent increase in doubling time.