


A marginal anticancer effect of regorafenib on pancreatic carcinoma cells in vitro, ex vivo, and in vivo

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Abstract Activation of receptor tyrosine kinases is recognized as a hallmark of cancer. Vascular endothelial growth factor (VEGF) and its receptor VEGFR are the prominent players in the induction of tumor neoangiogenesis. Strategies to inhibit VEGF and VEGFR are under intensive investigation in preclinical and clinical settings. Regorafenib is a multikinase inhibitor targeting some VEGFR and other receptor kinases. Preclinical results led to the FDA approval of regorafenib for treatment of metastatic colorectal cancer patients. Effects of this drug in pancreatic ductal adenocarcinoma (PDAC) have not been investigated yet. Gene expression was assessed with real-time PCR analysis. In vitro cell viability, proliferation, apoptosis, necrosis, migration, and invasion of the PDAC cells were assessed after regorafenib treatment.

Ex vivo anti-tumor effects of regorafenib were investigated in a spheroid model of PDAC. In vivo anti-tumor effects of the drug were evaluated in a fertilized chicken egg model. In this work, we have demonstrated only a marginal anticancer effect of regorafenib in PDAC in vitro and ex vivo. However, in the egg model of PDAC, this drug reduced tumor volume. Besides, regorafenib is capable of modulating the expression of cancer stem cell (CSC) markers and epithelial-to-mesenchymal transition (EMT) markers on PDAC cells. We found out that effects of regorafenib on the expression of CSC and EMT markers are very heterogeneous and depend obviously on original expression of these markers. We concluded that regorafenib might be a potential drug for PDAC and it should be investigated in future clinical trials.

Barbara Mayer and Svetlana Karakhanova have equal contribution

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Keywords Regorafenib · PDAC · Cancer stem cells · Epithelial-to-mesenchymal transition · Spheroid tumor model

Abbreviations

CSC Cancer stem cells
EMT Epithelial-to-mesenchymal transition
PDAC Pancreatic ductal adenocarcinoma
VEGF Vascular endothelial growth factor
ECAD E-cadherin

Introduction

Activation of receptor tyrosine kinases is recognized meanwhile as a hallmark of cancer (Hanahan and Weinberg 2011). Such activation can be followed by the induction of tumor neoangiogenesis through the activation of the vascular endothelial growth factor (VEGF) signaling (Jitawatanarat and Wee 2013). Therefore, strategies to inhibit VEGF and its receptors

(VEGFR) attract a high attention to create inhibitory small molecules and/or antibodies. Regorafenib (BAY 73-4506) is a multikinase inhibitor targeting VEGFR-1, -3, RAF, TIE-2, BRAF, and other receptor kinases (Wilhelm et al. 2011). In vitro and in vivo preclinical studies showed an anti-tumor activity of regorafenib in renal cell cancer, hepatocellular carcinoma, colorectal (CRC), and gastric cancer (Abou-Elkacem et al. 2013; Carr et al. 2013; Huynh et al. 2015; Schmieder et al. 2014; Wei et al. 2015; Wilhelm et al. 2011). Anticancer effect of regorafenib in vivo is ascribed to the antiangiogenic property of this drug (Huynh et al. 2015; Schmieder et al. 2014; Wilhelm et al. 2011). There is evidence that regorafenib could be cytotoxic for cancer stem cells (CSC) in soft tissue sarcoma (Canter et al. 2014) and can target epithelial-to-mesenchymal transition (EMT) in CRC (Fan et al. 2016).

Earlier phase I clinical studies proved that regorafenib possesses an acceptable safety profile (Mross et al. 2012; Strumberg et al. 2012). In a further phase III clinical trial, regorafenib demonstrated survival benefits in patients with metastatic CRC (Grothey et al. 2013). This result led to the FDA approval of this drug for treatment of metastatic CRC patients. Also, in clinical trials, regorafenib improved the survival of patients with gastrointestinal stromal tumors (Demetri et al. 2013; George et al. 2012), which resulted in the approval of the drug for treatment of such patients. Presently, this drug is under investigation in clinical trials with renal cell and hepatocellular carcinoma patients.

Patients suffering from pancreatic ductal adenocarcinoma (PDAC) have an especially poor prognosis, with 5-year survival rates of ~ 1% and median survival of 4–6 months (Werner et al. 2013). Upon tumor resection, 5-year survival rates increase to approximately 15%, while a 25% survival rate is attained in the context of adjuvant chemotherapy. Currently, regimens based on gemcitabine constitute the standard therapeutic approach to advanced PDAC (Neoptolemos et al. 2010). However, the clinical benefit from these adjuvant therapies is very modest. Therefore, new therapeutic approaches are needed to combat this tremendous disease.

One of the reasons for highly therapeutic resistance of PDAC could be the presence of CSC in this tumor (Abel and Simeone 2013). Our studies also showed the importance of CSC for PDAC development and progression (Isayev et al. 2014; Zhu et al. 2014). Another important molecular feature of PDAC progression is represented by the EMT (Brabletz 2012), which is strongly associated with the systemic aggressiveness of PDAC (Beuran et al. 2015). Therefore, therapeutic strategies for PDAC should recognize the targeting of CSC and inhibition of EMT in this tumor as an important treatment option.

In this work, we have demonstrated that regorafenib might be recognized as a potential drug for PDAC. Besides, this drug is capable to modulate expression of CSC and EMT markers on PDAC cells.

Materials and methods

Materials and cells

Regorafenib (BAY 73-4506) was purchased from Selleck as a supplier of Pfizer's bioactive compounds (Germany). Human and murine real-time PCR primers for CD24, CD44, CD133, vimentin, E-Cadherin, β -actin and GAPDH, and SYBR-Green system were purchased from Qiagen (Germany). Human PDAC cell lines Panc1, Capan1, and Dan-G were purchased from CLS (Germany); MiaPaca and BxPC3 came from ATCC (USA).

Patients

Fresh cancer tissue samples were obtained from surgical resections of nine patients newly diagnosed with primary pancreatic cancer. An informed consent was obtained from all patients in accordance with the guidelines of the Biobank under the administration of the Human Tissue and Cell Research Foundation, Department of General, Visceral and Transplantation Surgery, University Hospital of the LMU, Munich, Germany. Patients 18 years or older were eligible if a confirmed case of PDAC had been diagnosed and a treatment of surgery followed by adjuvant standard chemotherapy was performed. Patients with a previous diagnosis and/or treatment of a malignant disease were excluded.

Cell cultures

Cells were cultivated in RPMI-1640 medium with 10% fetal calf serum (whole medium), 100 U/ml penicillin, and 100 μ g/ml streptomycin obtained from PAA Laboratories (Germany). Cell lines were cultivated at 37 °C and 5% CO₂. Cells were routinely checked for mycoplasma contamination and commercially authenticated by Multiplexon GmbH (Germany).

RNA isolation and real-time RT-PCR analysis

Total RNA from cell lines was isolated using a RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instruction. RNA concentrations were determined using a NanoPhotometer™ Pearl (SERVA Electrophoresis, Germany). Real-time RT-PCR analysis was performed as described elsewhere (Zhu et al. 2014), using the SYBR-Green system, and measured using a Light-Cycler (Roche, Germany). For each experiment, a melting curve analysis and a gel electrophoresis of PCR products were performed to exclude primer dimers. The data were analyzed using the comparative C_t method (Schmittgen and Livak 2008). Each measurement was performed in a technical duplicate.

Human PDAC spheroid model

Fresh tumor samples were collected in DMEM/F12 culture medium, containing 10% fetal calf serum and a mixture of antibiotic/antifungal compounds (all PAN, Germany). Spheroids directly derived from PDAC tissue were generated as described previously (Halfter et al. 2016; Hoffmann et al. 2015). Briefly, tumor samples underwent mechanical and enzymatic digestion using an enzyme cocktail containing Liberase™, which consisted of a mixture of collagenases and neutral protease enzymes (Roche, Germany). For spheroid formation, 5×10^4 vital suspension cells were seeded in 50- μ l cell culture medium per well in 96-well plate and cultured for 48 h at 37 °C and 5% CO₂. A single spheroid was obtained in each well.

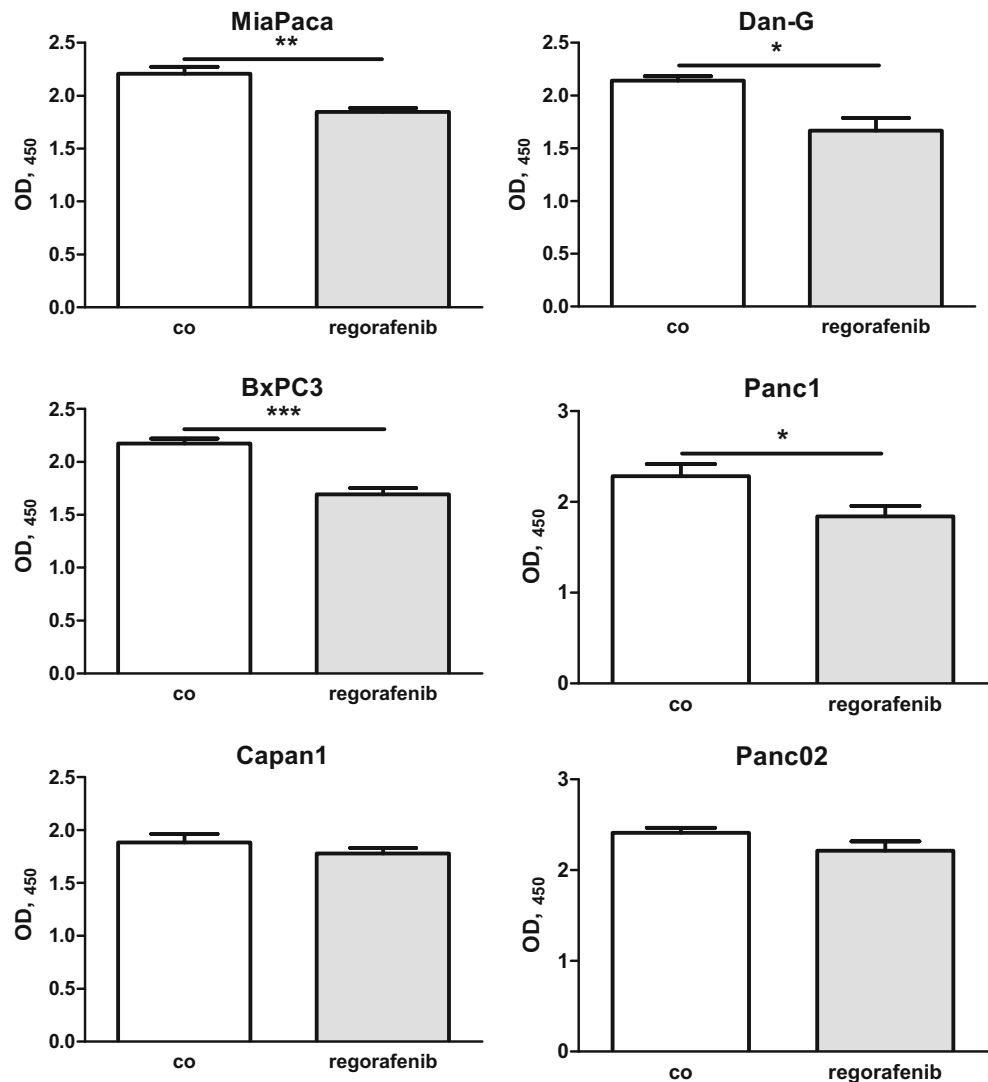
After spheroid formation, drugs were administered to the spheroids for 72 h at the peak plasma concentration, respectively. Regorafenib was applied in a concentration of 2.5 μ g/ml (Rey et al. 2015) and 5 μ g/ml in some cases, and gemcitabine (Fogli et al. 2002) was used in a concentration of 22.3 μ g/ml.

In addition, regorafenib was combined with gemcitabine. Solvent controls were 0.05% DMSO for regorafenib, 0.06% NaCl for gemcitabine, and 0.05% DMSO plus 0.06% NaCl for the combination therapy. Each treatment and control was performed in five replicates.

Transplantation of human tumor cells on fertilized chicken eggs

This assay was performed as described previously (Labsch et al. 2014). Fertilized chicken eggs (Geflügelzucht Hockenberg, Eppingen, Germany) were incubated at 37.8 °C and a humidity of 45–55%. At day 4 of embryonic development (EDD), a cave was cut into the eggshell. At EDD9, the rings from Thermanox™ cover disks were placed on the chorioallantoic membrane (CAM), and MiaPaca cells mixed with Matrigel at a ratio of 1:1 were transplanted into the rings. For the treatment of xenografts, a Whatman paper was placed next to the tumors and sopped with regorafenib or solvent control solution (5 μ g, 10 μ l)

Fig. 1 Cytotoxic effects of regorafenib in vitro on PDAC cell lines. Analysis of cell viability (high cell viability corresponds to high OD measured photometrically) after 72-h incubation with 2 μ M regorafenib or with a vehicle control (0.2% DMSO) (co). The data of five independent experiments are presented with SE and analyzed with the unpaired two-tailed *t* test, **p* < 0.05, ***p* < 0.01, and ****p* < 0.001



at EDD12 and EDD15. The xenografts were resected at EDD18, after euthanasia of the chicken embryo with ketanest, and the tumor volume was determined. All the embryos that died before day 18 were excluded from analyses. The tumor volumes are calculated by the following formula: $\text{volume} = 4/3 \times \pi \times r^3$ ($r = 1/2 \times \text{square root of diameter} \times \text{diameter} 2$).

Cell viability assay

Cell viability after regorafenib treatment was measured with an EZ4U Kit (Biomedica, Austria) as described elsewhere (Karakhanova et al. 2014). Briefly, for the EZ4U assay, 20,000 cells were seeded in 96-well plates and incubated overnight. Afterwards, regorafenib to the end concentration of 2 μM was added (Wilhelm et al. 2011). After 72-h incubation, a substrate compound from the kit was added, and the cells were incubated for 5 h at 37 °C to convert the yellow colored tetrazolium to its red formazan deriviate by living cells.

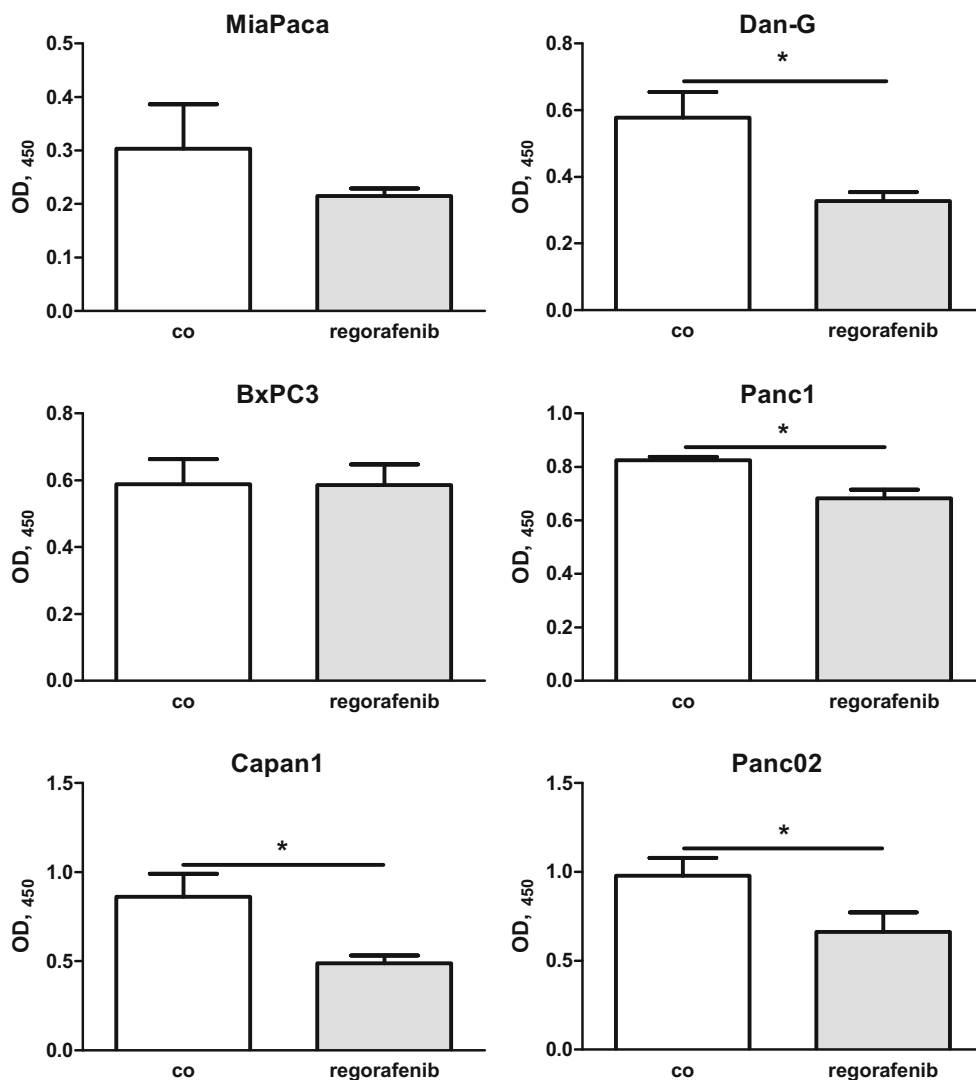
Finally, the absorbance was measured at 450 nm. Cell viability assay was performed for five independent experiments.

Treatment efficacy in the human PDAC spheroid model was assessed using CellTiter-Glo® Luminescence Cell Viability Assay (Promega, Germany).

Cell proliferation assay

The proliferation of cell lines after drug treatment was analyzed using a BrdU cell Proliferation Assay kit (Millipore, USA) as described elsewhere (Karakhanova et al. 2014). Twenty thousand cells were seeded in 96-well plates and incubated overnight. Afterwards, 24-h drug incubation was performed, then the BrdU reagent was added, and the cells were incubated for 12 h at 37 °C to allow the BrdU incorporation into proliferating cells. After the incubation, the cells were fixed and washed, then detector antibody was added, and the plates were incubated for 1 h at room temperature. After

Fig. 2 Analysis of cell migration (high level of the tested parameter corresponds to high optical density (OD) measured photometrically) with 2 μM regorafenib or with a vehicle control (0.2% DMSO) (co). The data of three independent experiments are presented with SE and analyzed with the unpaired two-tailed *t* test, **p* < 0.05



washing, a goat anti-mouse IgG peroxidase conjugate from the kit was added, and the plates were incubated for 30 min at room temperature. After washing, the cells were incubated for the next 30 min at room temperature in the dark with the TMB peroxidase substrate. The reaction was stopped by adding the acid stop solution from the kit. Finally, the absorbance was measured at 450 nm. Cell proliferation assay was performed for five independent experiments.

Cell migration and invasion assays

Cell migration and invasion assays were performed with a CytoSelect™ Cell Migration and Invasion Assay kit (Cell Biolabs, Inc., USA) as described elsewhere (Karakhanova et al. 2014). For the cell migration assay, the polycarbonate inserts (8 μ m pore size) from the kit were used. For the cell invasion assay, the polycarbonate inserts (8 μ m pore size) coated with a uniform layer of dried basement membrane matrix solution from the kit were used. For both assays, 300,000 cells were seeded per well in a serum-free medium. Plates were incubated for 24 h at 37 °C, and the migrated or invaded cells were stained and washed. Afterwards, the extraction solution from the kit was added, and the intensity of color was measured at 560 nm using a TECAN.SPECTRAFluor Plus. Absorbance intensity was proportional to the number of migrated or invaded cells. Cell migration and invasion assays were performed for three independent experiments.

Statistical analysis

All statistical analyses were performed using GraphPad Prism Version 5.01. Distributions of continuous variables were described by means, SD, SE, medians, 25 and 75% percentiles, and were presented as column bar graphs and box-and-whisker plots. The null hypothesis (mean values were equal) versus the alternative hypothesis (mean values were not equal) was tested using unpaired two-tailed *t* test (for two groups) and by one-way ANOVA with the Bonferroni's post hoc test (for more than two groups) or with Mann-Whitney test for tumor growth. A *p* value < 0.05 was considered significant.

Results

Anticancer effects of regorafenib are heterogenic in the PDAC cell lines

First of all, we analyzed a cytotoxic potential of regorafenib in vitro using five human and one murine PDAC cell lines. The cytotoxic effect of 2 μ M (Wilhelm et al. 2011) regorafenib after 72-h treatment was observed only in the human cell lines MiaPaca, Dan-G, BxPC3, and Panc1, whereas no cytotoxicity was found in Capan1 or in the murine PDAC cell line Panc02

(Fig. 1). It should be noted that the cytotoxic effect found was rather moderate and did not exceed 20%. These data were supported by a precise analysis of cell proliferation based on BrdU incorporation after regorafenib treatment (Fig. S1). Besides, regorafenib reduced the migration capacity of Dan-G, Panc1, Capan1, and Panc02 cells, but not of MiaPaca and BxPC3 (Fig. 2). At the same time, no influence of regorafenib treatment on the cancer cell invasion was detected (Fig. S2).

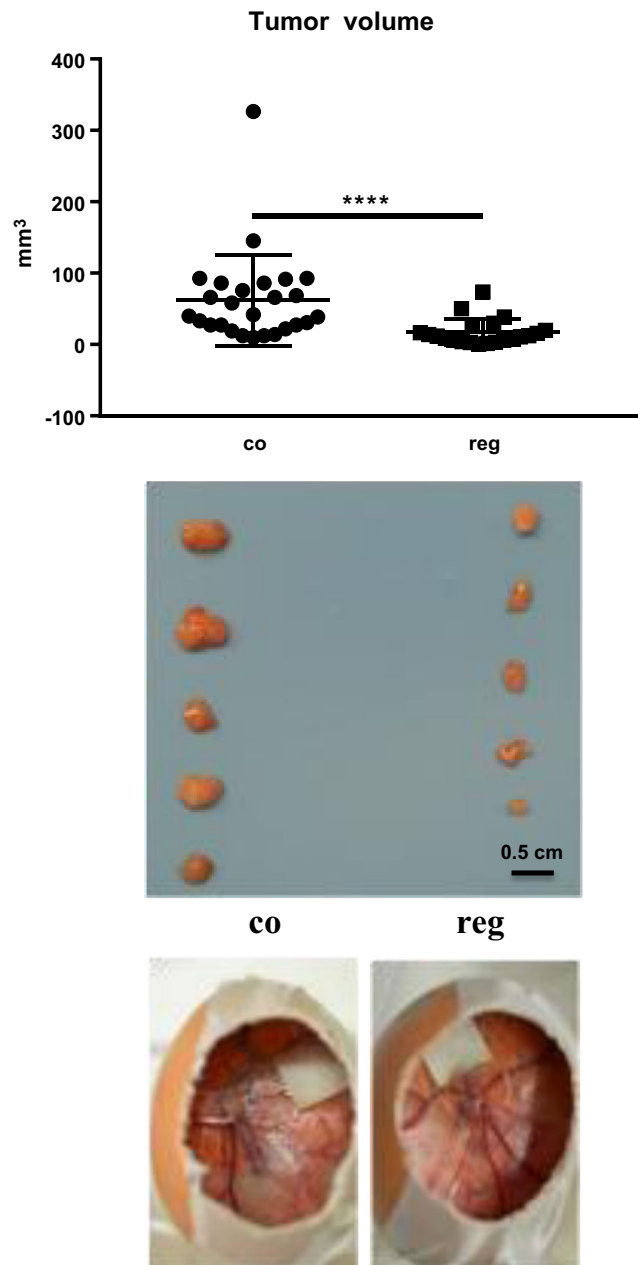


Fig. 3 Analysis of anticancer effect of regorafenib in the human PDAC model of fertilized chicken eggs. MiaPaCa cells were transplanted onto the CAM of fertilized chicken eggs at EDD9 and treated two times with regorafenib (reg) or vehicle control (co) solution. At day 18, the developed xenograft tumors were resected, and the tumor volumes were analyzed (a). b Images of the resected tumors and of CAM vascularization

Regorafenib reduces tumor volume of PDAC in the human model of fertilized chicken eggs

At the next step, we investigated anticancer effect of regorafenib on PDAC in our fertilized chicken egg model (Isayev et al. 2014; Zhang et al. 2015). Since Capan1 cells, which did not show cytotoxicity towards regorafenib, are not applicable for this model, only MiaPaca cells were used for xenotransplantation *in ovo*. In line with our *in vitro* data, regorafenib indeed reduces the volume of human PDAC tumors *in ovo* (Fig. 3a). However, no visible effects on vascularization were detected (Fig. 3b). We have also seen no side effects of regorafenib particularly on the weight of embryos (data not shown).

Regorafenib does not show anticancer effect in a human PDAC spheroid model

Established tumor cell lines, which we used in our *in vitro* and *in vivo* experiments, do not generally reflect tumor biology precisely. However, Panc02 cells were not affected by regorafenib, that is why we decided not to test this drug *in vivo* in our Panc02 model of PDAC. Therefore, we tested the effect of

regorafenib on cancer cells in a human PDAC spheroid model. As a control, the gemcitabine (gem) treatment of PDAC spheroids has been performed. While gem treatment led to the cytotoxicity of tumor cells of some individual patients (data not shown), regorafenib did not show obvious effects on the spheroids by 2.5 $\mu\text{g/ml}$ regorafenib (Fig. 4a), as well as by 5 $\mu\text{g/ml}$ of the drug tested only by patients 1 and 2 (Fig. 4b). In the cohort of all patients tested, gem demonstrated a trend to be cytotoxic, while regorafenib had no effect (Fig. 4c). Besides, a combination of both drugs did not improve the cytotoxicity (Fig. 4c).

Regorafenib diminishes expression of the CSC markers and modulates expression of EMT markers

As mentioned in “Introduction,” CSC and EMT are important features of PDAC. Therefore, we checked gene expression of CSC markers (CD24, CD44, and CD133) and of EMT markers (vimentin and e-cadherin (ECAD)) in the PDAC cells. This analysis revealed a heterogenic expression of these markers among PDAC cell lines tested (Fig. 5). Whereat the MiaPaca cells seem to have a low amount of CSC and to be of

Fig. 4 **a** Analysis of cell viability in the human PDAC spheroid model treated with regorafenib (2.5 $\mu\text{g/ml}$ (a) or 5 $\mu\text{g/ml}$ (b)) (reg) or with a vehicle control (0.2% DMSO) (co) for 72 h. The data of individual patients are presented. *Cps* counts per second. **c** Analysis of cell viability in the human PDAC spheroid model treated with regorafenib (2.5 $\mu\text{g/ml}$) (reg) as single-agent, gemcitabine (22.3 $\mu\text{g/ml}$) (gem) as single-agent, and both drugs in combination (reg + gem) for 72 h. Solvent controls were 0.05% DMSO for regorafenib (co-1), 0.06% NaCl for gemcitabine (co-2), and 0.05% DMSO plus 0.06% NaCl for the combination (co-3) therapy. *Cps* counts per second. The data of all patients are presented with SE and analyzed with the unpaired two-tailed *t* test

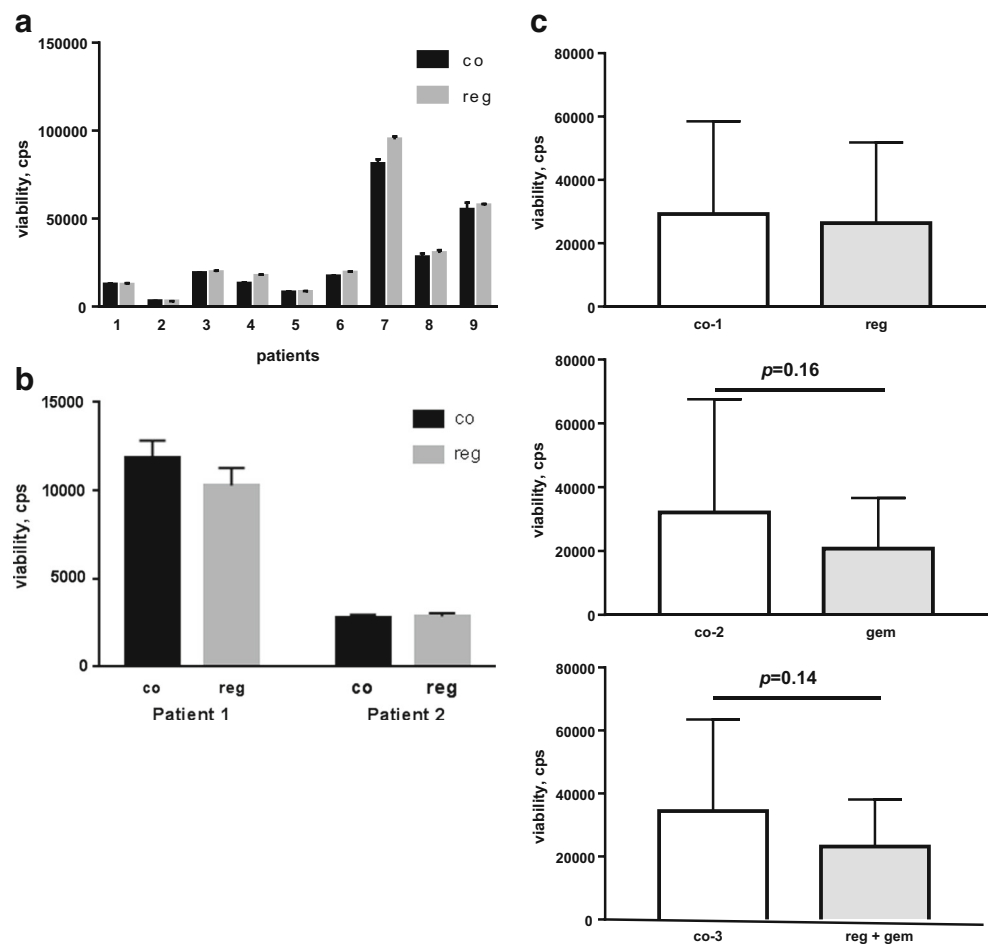
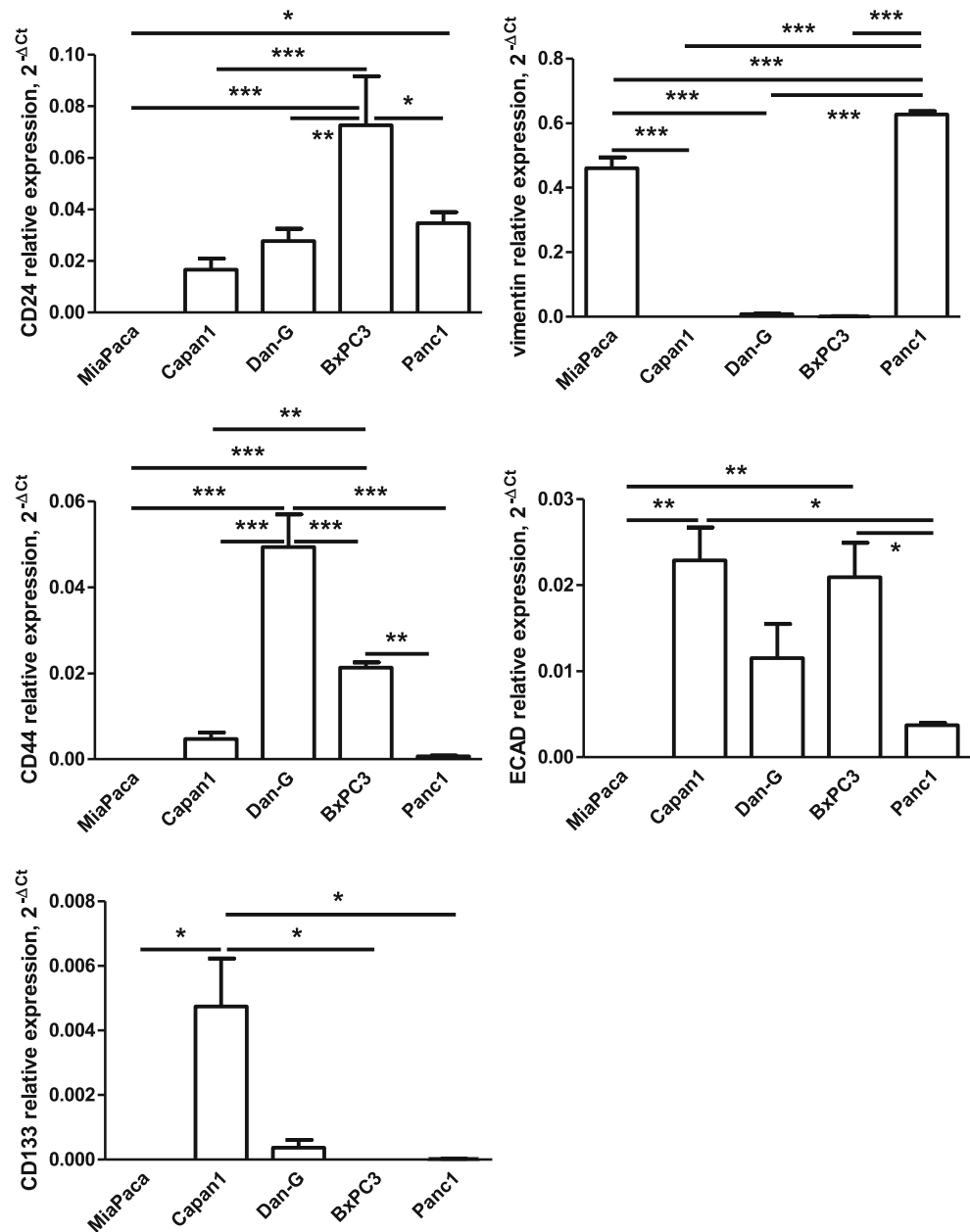


Fig. 5 Real-time PCR analysis of the CD24, CD44, CD133, vimentin, and ECAD gene expression in PDAC cell lines. The data from cell culture passages for each cell line are presented with SE and analyzed using one-way ANOVA with the Bonferroni's post hoc test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$



more mesenchymal phenotype, the Capan1 cells possess obviously more of CSC and represent rather epithelial features. It should be stressed that MiaPaca cells were sensitive for regorafenib toxicity, while Capan1 cells were regorafenib resistant. Therefore, we decide to use these two cell lines in our further experiments.

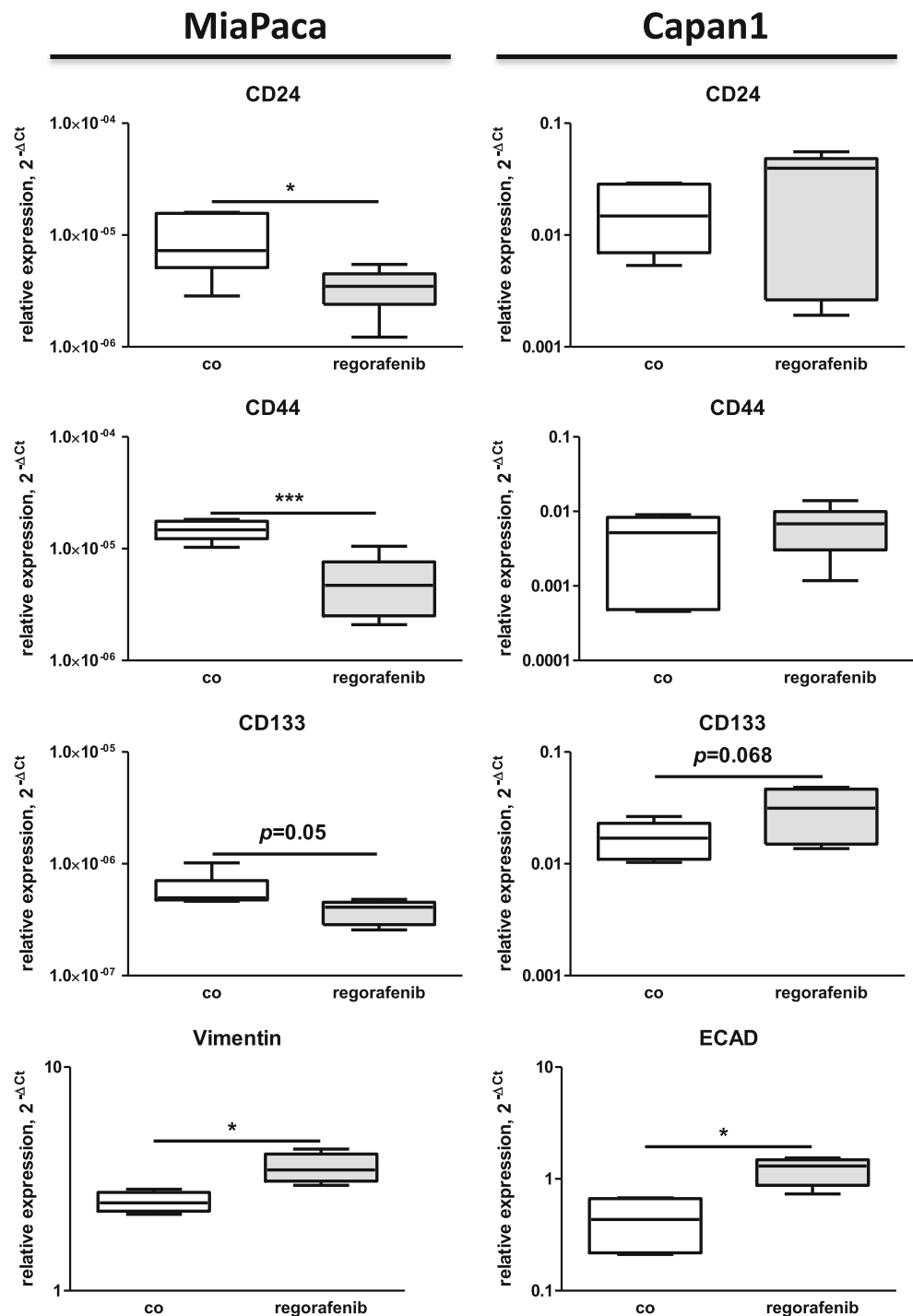
Does regorafenib have an influence on the CSC and EMT marker expression? To answer this question, we assessed the CD24, CD44, CD133, vimentin, and ECAD gene expression with real-time PCR analysis in MiaPaca and Capan1 cells, with and without regorafenib treatment. MiaPaca cells, which originally had a low level of the CSC marker expression, showed a down-regulation of their mRNA expression upon regorafenib treatment (Fig. 6). At the same time, the drug

treatment produced no obvious effect on the CD24, CD44, and CD133 gene expression in Capan1 cells (Fig. 6). Regorafenib did not affect expression of EMT markers virtually absent in the cells (ECAD in MiaPaca and vimentin in Capan1, data not shown). However, this drug up-regulated the vimentin and ECAD expression in MiaPaca and Capan1, respectively (Fig. 6 and Table S1).

Discussion

As mentioned in “Introduction,” attempt to inhibit VEGF and VEGFR could be a possibility to treat patients with some types of cancer. Regorafenib as a multikinase inhibitor

Fig. 6 Real-time PCR analysis of the CD24, CD44, CD133, vimentin, and EACD gene expression in the PDAC cell lines MiaPaca and Capan1 treated 72 h with 2 μ M regorafenib or with a vehicle control (0.2% DMSO) (co). The data of four independent experiments are presented with SE and analyzed with the unpaired two-tailed *t* test, **p* < 0.05 and ****p* < 0.001



possesses an anti-tumor activity against renal cell cancer, hepatocellular carcinoma, CRC, and gastric cancer in preclinical experimental settings (Abou-Elkacem et al. 2013; Carr et al. 2013; Huynh et al. 2015; Schmieder et al. 2014; Wei et al. 2015; Wilhelm et al. 2011). So far, FDA approved this drug for treatment of metastatic CRC patients, and currently, this drug is under investigation in clinical trials with renal cell and hepatocellular carcinoma patients. What about PDAC? While preclinical data for regorafenib in PDAC is absent, two

clinical trials are undergone and one is completed (<https://clinicaltrials.gov/ct2/results?term=regorafenib+pancreatic+cancer&Search=Search>) which are summarized in the Table S2. We are waiting for the results of the completed trial in about 2 years.

In this work, we first analyzed anticancer properties of regorafenib in vitro and in vivo using PDAC cell lines as well as the human model of fertilized chicken eggs and ex vivo using a human PDAC spheroid model. While some anticancer

properties were detected after treatment of PDAC cells in vitro and in vivo, no cytotoxicity was shown in a PDAC spheroid model. In vivo xenotransplantation model of PDAC in fertilized chicken eggs represents a rather new, but convenient approach for tumor establishing and investigation. This model is time-saving and may help to replace many costly mouse experiments (Labsch et al. 2014). In this PDAC model, we indeed saw reduction of tumor volume after regorafenib treatment. The impact of immune system cannot be recognized in this model. However, vascularization which is important for drug treatment is gut established in the PDAC model of fertilized chicken eggs. Based on the fact that we did not see any obvious effects of regorafenib on the egg CAM vascularization, we supposed that that anticancer effect of regorafenib can be referred to its cytotoxicity, which we also observe in the same PDAC cells in vitro. Therefore, our work could be recognized as a preclinical hint to investigate this drug in clinical studies with PDAC patients, especially in a combination with established chemotherapeutics.

Apart from cytotoxicity, we investigated effects of regorafenib on expression of CSC and EMT markers. So, a down-regulation of the CD24, CD44, and CD133 expression was detected after regorafenib treatment in a PDAC cell line with an originally low level of expression of these markers. Thus, we may suggest that regorafenib could moderately eliminate the CSC cells from PDAC, especially if the amount of these cells is originally low. This data provides an indirect link to the work of Takigawa et al. (2016) showing an inhibition of growth and metastasis of CRC cells which were injected in mice together with bone marrow-derived mesenchymal stem cells. However, another recent work from Canter et al. (2014) demonstrated an enrichment of CSC in soft tissue sarcoma after regorafenib treatment. Therefore, more studies should be done to clarify the link between regorafenib and CSC, which might be cancer type dependent.

Concerning EMT, there is a report from Fan et al. (2016) which clearly shows that regorafenib indeed targets EMT in CRC via SHP-1 enhancement and consequently activates TGF- β 1-induced EMT. In our work, only Capan1 PDAC cell line, which originally has a high amount of ECAD, was enriched with ECAD after regorafenib treatment. MiaPaca cell line with a high amount of vimentin showed an up-regulation of this gene after regorafenib medication.

Thus, the effects of regorafenib on the expression of CSC and EMT markers are very heterogeneous and depend obviously on the original expression of these markers. Molecular mechanism of these differences should be clarified in future experiments.

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