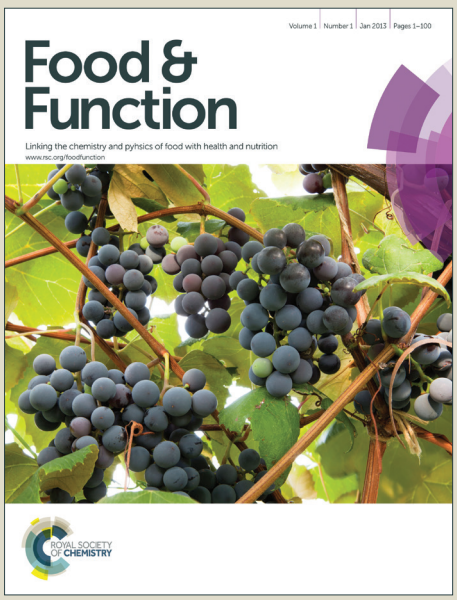
Food & Function

Accepted Manuscript



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Response to reviewers' comments:
We apologize for the inconvenience caused by the incorrect reference list.

Reviewers' Comments to Author:
Referee: 1

Comments to the Author
Mojsin et al. performed a comprehensive study of quercetin anti-cancer mechanism in human teratocarcinoma cell line NT2/D1. The results showed quercetin as promising anti-cancer agent with antimetastatic potential. The modulating activity towards cancer stem phenotype was analyzed. The role of quercetin in Wnt/beta-catenin signaling inhibition was also assessed. The results are very interesting. However, minor corrections are needed:

1. The order of references in the body of manuscript does not correspond to the list of references.

OUR RESPONSE: The reference list is corrected and citations in the manuscript correspond to the bibliography

2. Please, avoid discussion (with quotations) in the Results section. Plainly explain the reason why such experiments were performed.

OUR RESPONSE: According to the reviewer's comment, the following text was removed from the Results:
From section **Quercetin inhibits expression of stemness factors SOX2, Oct4 and Nanog in NT2/D1 cells**

"Embryonal carcinoma cells isolated from invasive non-seminomaous tumors were the first cancer stem cells to be isolated and characterized [13]. Self-renewal and multi-lineage differentiation capacity and expression of associated factors SOX2, Oct4 and Nanog, are hallmarks of cancer stem cells . Since cancer stem cells represent subpopulation of cancer cells responsible for tumorigenesis and metastasis we investigated the effect of quercetin treatment on the expression of pluripotency maintenance factors SOX2, Oct4 and Nanog in NT2/D1 cells"
and

"Among stemness factors SOX2 is of particular interest since it represents diagnostic marker for embryonal carcinoma [9]. It is also consider as a discriminatory marker between embryonal carcinoma and seminoma [9]."

From section **Quercetin down-regulates β-catenin dependent transcriptional activity in NT2/D1 cells**

"It has been shown that β-catenin up-regulates pluripotency maintenance genes in mouse model of testicular carcinoma. Previously, we have analyzed the effect of quercetin on the expression of Wnt target genes and showed that quercetin down-regulates Wnt/β-catenin signaling in NT2/D1 cells [1]."

and

”It has been previously shown that quercetin inhibits β-catenin translocation from cytosol into the nucleus in colon cancer cell line SW400 [1].”

3. Discuss the connection between inhibition of stem characteristics and Wnt signaling. These two parts of quercetin action are given separately. Please, explain how antimetastatic potential of quercetin could be the related with beta-catenin inhibition.

OUR RESPONSE: According to the reviewer’s comment the following text is added in the Discussion:

“Previous studies have shown that activation of Wnt/β-catenin signaling is involved in maintenance of self-renewal and pluripotency of human and mouse ES cells[47]. Wnt signaling activation by specific GSK3β inhibitor, 6-bromoindirubin-3’-oxime (BIO), sustain pluripotency in mouse and human ES cells[48, 49]. *c-myc* gene, Wnt/β catenin target gene, has a critical role in maintenance of pluripotency of mouse ES cells[50, 51]. In addition, previous results emphasized functional importance of c-myc in the maintenance of pluripotency in human and mouse EC cells[52, 53, 54, 55]. In NT2/D1 cells *c-myc* expression is high in the undifferentiated state but significantly decreased during differentiation[53, 54]. Activation of Wnt/β-catenin signaling using lithium as an GSK3β inhibitor up-regulates c-myc expression in NT2/D1 cells, while β-catenin inhibition significantly decreased c-myc level[19]. In addition, in P19 cells, mouse model of teratocarcinoma, activation of Wnt/β-catenin signaling promoted proliferation by inducing expression of *c-myc* and maintained pluripotency by supporting high expression of Sox2, Oct4, and Nanog[55].”

and

“Various studies have demonstrated the role of constitutively active Wnt/β-catenin signaling and nuclear β-catenin accumulation in tumor progression and metastasis[32]. This loss of β-catenin regulation is accompanied by activation of genes involved in cell proliferation and migration[33]. Antimetastic effect of quercetin in NT2/D1 cells is probably caused by several co-existing molecular mechanisms. Inhibition of cell migration induced by β-catenin inactivation has been previously associated with down-regulation of β-catenin downstream targets involved in tumor invasion and metastasis, such as Membrane type 1 matrix metalloproteinase (MT1-MMP)[34, 35] matrix metalloproteinase (MMP)-2[32], MMP-7[34], MMP-9[32], laminin-5 γ2 chain[35], etc. Also, quercetin affects some of the key targets in multiple cellular signaling pathways related to the cell migration and exerts different, more complex effects. For example, quercetin suppresses invasion and migration of MCF10A human epithelial cell by inhibiting phosphatidylinositol 3-kinase (PI3K)/Akt signaling[30]. At the same time, AKT1 kinase, key mediator of the PI3K/AKT pathway, regulates β-catenin directly by increasing the activating phosphorylation of β-catenin[36], or indirectly as a result of the inhibition of GSK3β[37].”

Referee: 2

Comments to the Author
Authors have done systematic studies on quercetin as anti-cancer agent. All experiments were elaborately done and tried to understand the molecular mechanism of

Wnt/β-catenin signalling pathway in NT2/D1 cells in the presence of quercetin.
Following modification is required:

1. References are not in order.

OUR RESPONSE: The reference list is corrected and citations in the manuscript correspond to the bibliography

2. In introduction, page 1, line 25-26, 'It also possess wide range of therapeutic effects including antiviral, antidiabetic, anti-inflammatory and neuroprotective, (reviewed in)', reference is required.

OUR RESPONSE: The reference is added in the text:
"It also possess wide range of therapeutic effects including antiviral, antidiabetic, anti-inflammatory and neuro-protective, (reviewed in Jain, A. K. et al.[1])."

3. Author has repeatedly said in introduction, results, discussion section that 'we have previously shown that ….and quoted the reference no 1' seems that the work has been done by these authors. But reference showed the work is done by different authors.

OUR RESPONSE: Reference no. 1 was incorrect citation. It is replaced with reference no. 19 "M. Mojsin, V. Topalovic, J. Marjanovic and M. Stevanovic, *Arch. Biol. Sci.,*, 2013, **65**, 201-209." which is correct citation.

4. In results line 25, author has mentioned about some previous result in NT2/D1 (ref 1), but in ref 1 NT2/D1 cell line was not included in their studies.

OUR RESPONSE: Reference no. 1 was incorrect citation. It is replaced with reference no. 19 "M. Mojsin, V. Topalovic, J. Marjanovic and M. Stevanovic, *Arch. Biol. Sci.,*, 2013, **65**, 201-209." which is correct citation with NT2/D1 cells as model system in the study.

5. In results, page 2, line 70, 'Quercetin inhibits adhesion……..NT2/D1 cells' (line no 72) it should be 'metastatic' not 'matastatic'.

OUR RESPONSE: According to the reviewer's comment misspelling was corrected.

6. As mentioned in result the condensation of nuclear chromatin, nuclear fragmentation and apoptotic bodies in the presence of quercetin, however in figure 1B it is not very clear to see.

OUR RESPONSE: This comment is well pointed. Poor quality of previous Fig1B was due to low resolution of inserted JPEG image and size of the panels. In revised version of the manuscript TIF image is inserted, panel size is enlarged and two different viewing fields from quercetin treated samples were introduced. Figure 1B is improved by indicating condensation of nuclear chromatin, nuclear fragmentation and apoptotic bodies with arrows.

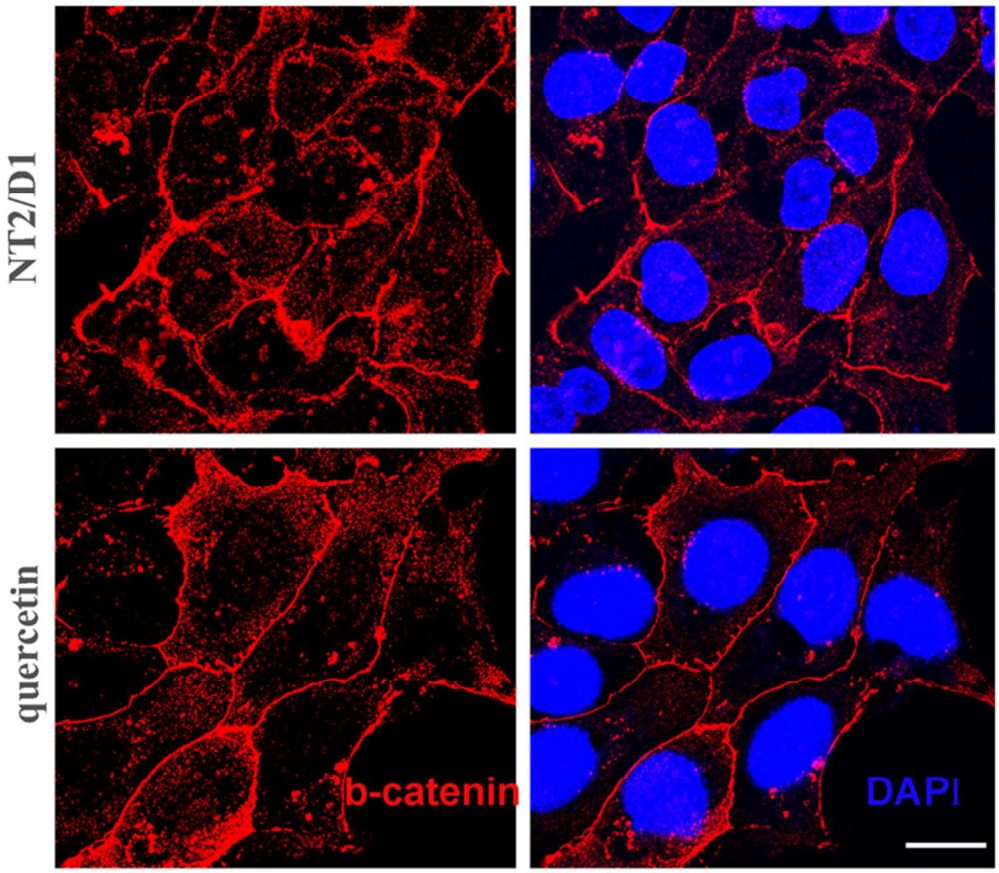
7. Any explanation for inhibition of the cell migration in the presence of quercetin.

OUR RESPONSE: According to the reviewer's comment the following text is added in the Discussion:

"Various studies have demonstrated the role of constitutively active Wnt/β-catenin signaling and nuclear β-catenin accumulation in tumor progression and metastasis[32]. This loss of β-catenin regulation is accompanied by activation of genes involved in cell proliferation and migration[33]. Antimetastic effect of quercetin in NT2/D1 cells is probably caused by several co-existing molecular mechanisms. Inhibition of cell migration induced by β-catenin inactivation has been previously associated with down-regulation of β-catenin downstream targets involved in tumor invasion and metastasis, such as Membrane type 1 matrix metalloproteinase (MT1-MMP)[34, 35] matrix metalloproteinase (MMP)-2[32], MMP-7[34], MMP-9[32], laminin-5 γ2 chain[35], etc. Also, quercetin affects some of the key targets in multiple cellular signaling pathways related to the cell migration and exerts different, more complex effects. For example, quercetin suppresses invasion and migration of MCF10A human epithelial cell by inhibiting phosphatidylinositol 3-kinase (PI3K)/Akt signaling[30]. At the same time, AKT1 kinase, key mediator of the PI3K/AKT pathway, regulates β-catenin directly by increasing the activating phosphorylation of β-catenin[36], or indirectly as a result of the inhibition of GSK3β[37]. Another possible mechanism by which quercetin might suppress migration of NT2/D1 cells is via down-regulation of pluripotency factors. It has been shown previously that SOX2[38], Oct4[39] and Nanog[40] play important role in tumor cell migration. For example, ectopic expression of Sox2 in malignant gliomas was sufficient to induce invasion and migration of these cells, while downregulation of Sox2 reduced migratory and invasive potential[38]. This reduction was the result of the decreased expression of migration and invasion markers EphA2, Rac, RHO, Rock1, Rock2, VEGF, TGFβ, MMP9, MMP2, Notch1, Notch2 and Notch3[38]. Although the basic mechanisms governing all these effects are not fully understood, the existing data support potential use of quercetin as an anticancer agent or an addition to cancer therapies."

8. Page 1, line number 73, repetition of ref no 1 and page 5, line number 49, repetition of ref no 2.

OUR RESPONSE: The corrections are made as suggested.



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QUERCETIN REDUCES PLURIPOTENCY, MIGRATION AND ADHESION OF HUMAN TERATOCARCINOMA CELL LINE NT2/D1 BY INHIBITING WNT/β-CATENIN SIGNALING

MARIJA MOJSIN*, JELENA MARJANOVIC VICENTIC, MARIJA SCHWIRTLICH, VLADANKA TOPALOVIC and MILENA STEVANOVIC

Abstract

Quercetin, bioflavonoid found in plant foods, has a wide range of therapeutic effects. In order to examine therapeutic potential of quercetin in teratocarcinoma, we used human teratocarcinoma cell line NT2/D1 as *in vitro* model. We have shown that quercetin inhibits proliferation, adhesion and migration of NT2/D1 cells and downregulates the expression of pluripotency factors SOX2, Oct4 and Nanog. Our results further suggest that anticancer effect of quercetin against human teratocarcinoma cells is mediated by targeting canonical Wnt signaling pathway. Quercetin antagonized Wnt/β-catenin signaling pathway in NT2/D1 cells by inhibition of β-catenin nuclear translocation and consequent downregulation of β-catenin dependent transcription. Presented data suggest that quercetin as potent inhibitor of Wnt signaling might be effective therapeutic agent in cancers with aberrant activation of Wnt pathway.

Introduction

Use of natural products as a source of active therapeutic agents is the growing field of interest in chemoprevention and anticancer therapy. Phytochemicals found in plant foods represent promising strategy for cancer prevention and treatment. In the search for new agents quercetin shows great potential due to its unique features. This bioflavonoid is widely distributed in fruits and vegetables and plant based food products such as olive oil, tea and red wine. It also possess wide range of therapeutic effects including antiviral, antidiabetic, anti-inflammatory and neuro-protective (reviewed in Jain, A. K. et al.[1]). Dependent of the concentration, quercetin exerts its effect from free radical scavenger to pro-oxidant activity[2]. Quercetin also shows strong anti-proliferative and anti-angiogenic properties[2]. Its anti-cancer activity has been well documented in wide variety of animal cancer models and cancer cell lines[2]. It has been shown that quercetin affects multiple cell signaling pathways. It targets AKT/mTOR/P70S6K signaling by inhibition of vascular endothelial growth factor receptors (VEGFR)[3]. Furthermore, quercetin decreases the expression of ErbB2 and ErbB3 proteins thus affecting ErbB/HER signaling pathway[4].

It has also been reported that quercetin inhibits Wnt signaling pathway[5]. Deregulation of this signaling pathway underlies a wide range of cancer pathologies in humans, including pathogenesis of testicular carcinoma[6]. β-catenin is a central effector of the canonical Wnt signaling pathway[7]. The activity of Wnt downstream targets is regulated by posttranslational modifications of β-catenin and substantial changes in its stability and cellular compartmentalization[7]. In the active state Wnt ligands bind cell-surface Frizzled receptors and inhibit β-catenin ubiquitination and subsequent proteasomal degradation[7]. Elevated cytoplasmatic β-catenin level causes its nuclear translocation and transcriptional modulation of Wnt downstream targets[8]. In the absence of active Wnt signaling, β-catenin is regulated by a multiprotein "destruction complex" which tags it for ubiquitination and proteasomal degradation leading to low levels of cytoplasmic β-catenin[8].

Testicular germ cell tumors (TGCTs) can be divided in two categories: the seminomas and non-seminomas[9]. Nonseminomas TGCTs comprise embryonal carcinoma, yolk sac tumor, immature or mature teratoma, choriocarcinoma, and other

rare trophoblastic tumors[9]. Embryonal carcinoma (EC) cells are the undifferentiated and pluripotent component of nonseminoma TGCT[10]. NT2/D1 cell line is one of several well established human TGCT cell lines that retain the pathogenomic and cellular features of this malignancy[11, 12]. Since these cells retained sensitivity to cytotoxic agents they are adequate model system for studying molecular mechanisms involved in exceptional response of testicular tumors to cytoreductive therapy[13].

Transcription factors SOX2, Oct4 and Nanog are master regulators of pluripotency and self-renewal involved in the regulation of stem cell fate during embryogenesis[14]. SOX2 and Oct4 are also capable of inducing pluripotency in somatic cells along with Klf4 and c-Myc[15]. Factors associated with self-renewal capacity are hallmarks of cancer stem cells, a particular subset of cancer cells responsible for tumorigenesis, chemoresistance and metastasis[16]. SOX2, Oct4 and Nanog are highly expressed in embryonal carcinomas[10, 17, 18]. All three transcription factors have been detected in carcinoma *in situ*, the precursor lesions of testicular germ cell tumours[10]. Recent studies have shown deregulation of SOX2 expression in different human cancer types where it impairs cell growth and tumorigenicity[16]. SOX2 has been reported as a diagnostic marker for embryonal carcinoma exploited as a discriminatory marker between embryonal carcinoma and seminoma[9, 10].

In order to examine therapeutic potential of quercetin in teratocarcinoma, we used human teratocarcinoma cell line NT2/D1 as *in vitro* model system. We have previously shown that quercetin decreased viability of NT2/D1 cells and downregulated Wnt target genes[19]. In the present study we show results of more comprehensive analysis of the mechanism underlying quercetin action in NT2/D1 cells. We found that quercetin inhibits proliferation, adhesion and migration of NT2/D1 cells and downregulates the expression of pluripotency maintenance factors SOX2, Oct4 and Nanog. We also showed that quercetin induced blockage of Wnt signaling pathway in NT2/D1 cells is based on inhibition of β-catenin nuclear translocation and subsequent suppression of β-catenin dependent transcription. Our results confirmed that quercetin, as a potent antagonist of Wnt signaling pathway, possess promising chemopreventive and chemotherapeutic properties for cancer treatment, especially in cancers controlled by Wnt/β-catenin signaling activity such as nonseminoma TGCT.

Materials and methods

Cell culture

NT2/D1 cells, kindly provided by Prof. P.W. Andrews (University of Sheffield, UK) were maintained in high glucose Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L L-glutamine and penicillin/streptomycine (all from Invitrogen™, NY, USA), at 37°C in 10% CO_2 as previously described [11, 20]. For the quercetin treatment, NT2/D1 cells were grown for 24 h either with vehicle control DMSO (Sigma-Aldrich) or 70 µM quercetin (Sigma-Aldrih).

Immunocytochemistry

24 hours following the treatments, cells were fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature (RT) and permeabilized in 0.1% Triton X-100. For β-catenin detection cells were fixed and permeabilized in methanol for 5 minutes at -20°C. Blocking was performed in 10% normal goat serum and 1% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hour at RT. Primary antibodies, diluted in 1% BSA, 0.05% Tween® 20 (Sigma-Aldrich) in PBS were applied overnight at 4°C as follows: anti-β-catenin (BD Transduction Laboratories™, diluted 1:500) and anti-SOX2, (R&D System, diluted 1:50). Coverslips were then incubated with biotinylated secondary antibodies (Vector, Burlingame, CA, USA) for 1 hour at RT in 1% BSA, followed by Cy3-streptavidin (Jackson ImmunoResearch, West Grove, PA, USA, 1:5000) or streptavidine-conjugated FITC (Jackson ImmunoResearch, 1:1000) in PBS for 1 h at RT. Cell staining with anti-Ki67 (Novocastra, 1:500) was performed overnight at 4°C in PBS containing 0.5% Triton and 10% normal goat serum. Alexa Fluor 488 anti-rabbit antibody, 1:500 dilution in PBS, containing 0.5% Triton and 10% normal goat serum was used as the secondary antibody. Nuclei were stained with 0.1 mg/ml diamino phenylindole (DAPI; Sigma-Aldrich). Samples were viewed and images were taken by Leica TCS SP8 confocal microscope and Leica Microsystems LAS AF-TCS SP8 software (Leica Microsystems).

PI (propidium iodide) staining

For PI staining NT2/D1 cells were seeded on coverslips in 6-well plates and treated with 70 μM quercetin or vehicle control (DMSO) for 24 h. After treatment, cells were stained with 1 μg/ml of DAPI and 5 μg/ml of PI in PBS for 10 min at RT. After staining cells were fixed with 4% paraformaldehyde for 15 min. Morphological analysis was performed under fluorescence microscopy (Olympus C-5050).

Western blot

Whole cell lysates were prepared from either vehicle control (DMSO) or quercetin treated NT2/D1 cells using NP-40 buffer [20 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA, 150 mmol/L NaCl, 1% NonidetP-40, 10% glycerol and Protease inhibitor cocktail (Roche Diagnostics GmbH, Germany)].

For the isolation of nuclear and cytoplasmic protein fractions total cell extracts were obtained by resuspending cells in lysis buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, and Protease inhibitor cocktail (Roche Diagnostics GmbH). The nuclear fraction was separated from the first, low-speed pellet by high-speed sedimentation; supernatant of the low-speed centrifugation represented cytoplasmic fraction[21].

Western blots were performed using anti-SOX2 (R&D Systems), anti-β-catenin (BD Biosciences), anti-PARP (Santa Cruz), anti-Ki67 (Novocastra) and anti-α-tubulin (Calbiochem) antibodies.

Cell Adhesion Assay

After 24 h of treatment with 70 μM quercetin NT2/D1 cells were seeded in matrigel coated 96-well plates at a density of $0{,}5\times10^5$ cells/well in DMEM supplemented with 0,1% BSA. After 15 minutes NT2/D1 cells were washed with PBS and number of attached cells was determined using MTT [3-*(*4,5-dimethylthiazol-2yl*)*-2,5-diphenyltetrazolium bromide] assay.

Wound-healing migration assay

NT2/D1 cells were grown into gelatin coated dishes to near confluence, wounded by scraping away with 200 µl pipette tips, rinsed with medium and treated with 70 µM quercetin or vehicle control (DMSO). Cell migration was monitored using confocal time-lapse imaging, Leica TCS SP8 confocal microscope and CO_2/O_2 and temperature cage controllers (OKOLAB). The number of cells migrating in the "wound space" (denuded area) was counted in three independent experiments and results for quercetin treatment were presented as a percentage of values obtained for vehicle control–treated NT2/D1 cells.

Real time PCR analysis

Total RNA were isolated from quercetin treated and vehicle control–treated NT2/D1 cells using TRI-Reagent kit (Ambion, Invitrogen, USA). RNA was treated using a DNA-Free™ kit (Ambion) and subjected to cDNA synthesis. Total RNA (1 µg) was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems®, Invitrogen, USA).

The synthesized cDNAs were subjected to real time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems®) in 7500 Real Time PCR Systems (Applied Biosystems®).

Primer sequences used for amplifications of *SOX2*, *Oct4*, *Nanog* and *GAPDH* were as follows:

SOX2: 5'-TCGGCGCCGGGGAGATACAT-3' (forward)
5'-CCCCTGGCATGGCTCTTGGC-3' (reverse)
Oct4: 5'-TCTCCAGGTTGCCTCTCACT-3' (forward)
5'-GCTTTGAGGCTCTGCAGCTT-3' (reverse)
Nanog: 5'-GGTCCCGGTCAAGAAACAGA-3' (forward)
5'-TCTGGAACCAGGTCTTCACC-3' (reverse)
GAPDH: 5'-GCCTCAAGATCATCAGCAATGC-3' (forward)
5'-CCACGATACCAAAGTTGTCATGG-3' (reverse)

All samples were measured in triplicate and the mean value was considered. The relative levels of *SOX2, Oct4* and *Nanog* expression was determined using a comparative

quantification algorithm where the resulting ΔΔCt value was incorporated to determine the fold difference in expression ($2^{-\Delta\Delta Ct}$). Relative *SOX2, Oct4* and *Nanog* mRNAs levels after quercetin treatment were presented as a percentage of mRNAs expression in vehicle control–treated NT2/D1 cells.

Transfections and luciferase assay

β-catenin/Tcf transcriptional activity was measured using TOPFlash/FOPFlash reporter system as previously described[22, 23]. Transient transfection was performed using Lipofectamine™ 2000 reagent (Invitrogen™). Briefly, NT2/D1 cells were seeded at a density of $0{,}2x10^6$ cells per well in 24-well plates. 24 hours after seeding, cells were transfected with 0,6 μg TOPFlash or FOPFlash and 0,2 μg of pRL-null plasmid, to normalize for transfection efficiency. In cotransfection experiments 0,2 μg of β-catenin pcDNA3.1 was used. 4 hours post-transfection, medium containing quercetin (70 μM) or vehicle control (DMSO) was added and cells were grown for additional 24 hours. Luciferase assay was performed using the Dual Luciferase Assay System kit (Promega).

Results

Quercetin inhibits proliferation and promote apoptosis of NT2/D1 cells

We have previously shown that quercetin decreased viability of human testicular embryonic carcinoma cell line NT2/D1 in dose dependent manner[19]. In order to investigate mechanisms involved in quercetin effect on NT2/D1 cells turnover in more details, we analyzed the proliferation activity and apoptotic status of NT2/D1 after 24 h treatment with quercetin (Figure 1). Immunocytochemical staining for Ki67, nuclear antigen present during active phases of cell cycle (G1, S, G2 and mitosis)[24] showed near 90% actively proliferating cells in vehicle control (DMSO) treated cells. Treatment with quercetin reduced NT2/D1 proliferating rate to approximately 60% (Figure 1, Panel A). This significant decrease in proliferation activity of NT2/D1 cells revealed profound antiproliferative effect of quercetin on NT2/D1 cells.

Next we analyzed apoptotic status of NT2/D1 cells using propidium iodide (PI) staining and PARP (poly(ADP-ribose) polymerase) cleavage detection. PI staining has shown changes characteristic for apoptosis/necrosis in nuclear morphology of NT2/D1 cells treated with quercetin (Figure 1, Panel B). We have detected condensation of nuclear chromatin, nuclear fragmentation and apoptotic bodies (Figure 1, Panel B). In addition, Western blot analysis has shown that PARP cleavage, one of the hallmarks of apoptosis, is induced by quercetin (Figure 1, Panel C). These results suggest that quercetin shows strong antiproliferative and pro-apoptotic effect on NT2/D1 cells.

Quercetin inhibits adhesion and decreases migratory potential of NT2/D1 cells

Since cell adhesion and migratory potential are the crucial steps of the metastatic cascade of tumor progression our next goal was to investigate if quercetin treatment has anti-metastatic effect on NT2/D1 cells. First, we examined whether 24 h quercetin treatment compromise the ability of NT2/D1 cells to attach on matrigel-coated cell culture dishes. As shown in Figure 2, Panel A, treatment with quercetin resulted in 50% inhibition of attachment of NT2/D1 cells to matrigel.

Next, we analyzed potential effect of quercetin on NT2/D1 cells migratory potential using wound healing assay (Figure 2, Panel B). NT2/D1 cells were grown on gelatin coated plate to confluency, wounded and subsequently treated with quercetin or vehicle control (DMSO). Cell migration from the wound edge was monitored using time-lapse confocal microscopy. Live-cell imaging enables evaluation of quercetin effect on single cell level (the distance, the velocity, the direction of single cell migration) and the quantitative analyses of the cell motility. Figure 2, Panel B presents cell migration at 10 h postwounding. In contrast to control NT2/D1 cells, quercetin treated cells do not migrate as single cells to heal the wound, but migrate by colony expansion from the wound edges (Figure 2, Panel B). Quercetin reduced migratory potential of human teratocarcinoma NT2/D1 cells to approximately 35% (Figure 2, Panel B). Taken together, cell adhesion assay and wound healing assay demonstrated profound antimetastatic potential of quercetin on NT2/D1 cells.

Quercetin inhibits expression of stemness factors SOX2, Oct4 and Nanog in NT2/D1 cells

In order to investigate whether quercetin affects self-renewal and pluripotency of NT2/D1 cells we analyzed the effect of quercetin treatment on the expression of stemness factors SOX2, Oct4 and Nanog. As shown in Figure 3, Panel A, real-time PCR analyses revealed significant decrease in mRNA levels of *SOX2*, *Oct4* and *Nanog* in NT2/D1 cells after quercetin treatment (75%, 60% and 85% respectively).

Since SOX2 is diagnostic marker for embryonal carcinoma we further investigated if the inhibitory effect of quercetin observed at the mRNA level persist at the level of SOX2 protein in treated NT2/D1 cells. Figure 3, Panel B and C shows the results of immunocytochemistry and Western blot analysis of the SOX2 protein expression in NT2/D1 cells upon quercetin treatment. Immunocytochemical analysis showed that the expression of SOX2 was significantly decreased in nuclei of NT2/D1 cells after treatment with quercetin (Figure 3, Panel B). The same effect was observed in Western blot assay (Figure 3, Panel C). The SOX2 protein expression was reduced by approximately 60% in whole cell lysate obtained from NT2/D1 cells treated with quercetin (Figure 3, Panel C) compared with the vehicle treated control.

All together these results point to quercetin as a potent inhibitor of pluripotency maintenance factors in NT2/D1 cells. We speculate that observed inhibition of pluripotency may be the mechanism underlying the antitumor and antimetastatic effects of quercetin in NT2/D1 cells.

Quercetin downregulates β-catenin dependent transcriptional activity in NT2/D1 cells

Deregulation of β-catenin and subsequent Wnt/β-catenin signaling activation underlies a pathogenesis of testicular carcinoma[6]. In order to examine the mechanisms involved in the inhibitory effect of quercetin on the Wnt/β-catenin signaling pathway in NT2/D1 cells we investigated the effect of quercetin treatment on the activity of β-catenin, central player in the canonical Wnt pathway.

First, we analyzed the effect of quercetin on the β-catenin dependent transcriptional activation of Wnt target genes in NT2/D1 cells. We used TOPFlash reporter gene construct as well established and widely used system for the evaluation of β-catenin dependent transcriptional activation[23]. TOPFlash reporter gene construct contains four copies of an optimized wild type binding sites for Tcf/Lef transcription factors, downstream effectors of Wnt/β-catenin signaling[23]. FOPFlash is its negative control counterpart and contains four mutated Tcf/Lef binding sites[23]. NT2/D1 cells were transiently transfected with TOPFlash or FOPFlash and subsequentially treated with quercetin for 24 h. As shown in Figure 4, Panel A, quercetin significantly decreased β-catenin/Tcf transriptional activity by approximately 2,8 fold in NT2/D1 cells. As expected, FOPFlash activity remained unchanged after quercetin treatment (Figure 4, Panel A). Inhibitory effect of quercetin on β-catenin transriptional activity was further confirmed in experiments where TOPFlash was cotransfected with β-catenin expression vector (Figure 4, Panel B). Cotransfection with β-catenin activated TOPFlash reporter by approximately 3 fold in untreated NT2/D1 cells (Figure 4, Panel B), while treatment with quercetin completely abolished this effect and retained TOPFlash activity similar to the activity detected in vector control (Figure 4, Panel B). The control reporter construct FOPFlash was not activated in any of the conditions (Figure 4, Panel B).

Next, we wanted to investigate the potential change in the intracellular distribution of β-catenin in NT2/D1 cells upon quercetin treatment. The status of β-catenin in NT2/D1 cells and its redistribution upon quercetin treatment were examined using immunocytochemistry. Confocal microscopy (Figure 5, Panel A) showed that β-catenin was expressed predominantly at the cell membrane where it is involved in cell-cell adhesion. Upon quercetin treatment β-catenin levels decreased in the nucleus (Figure 5, Panel A) suggesting that quercetin exerts its effect through the block in β-catenin nuclear translocation in NT2/D1 cells. This was further confirmed by the Western blot analysis of the β-catenin expression in cytosolic and nuclear fraction of the NT2/D1 cells treated with quercetin. The nuclei of the treated cells contained decreased amounts of β-catenin while cytosolic level of β-catenin remains approximately the same, relative to control (Figure 5, Panel B). Data obtained by immunocytochemistry and Western blot provide the explanation for the reduced signaling activity of β-catenin in TOPFlash assay

upon quercetin treatment. It is very likely that quercetin inhibits β-catenin nuclear translocation and decreases β-catenin/Tcf dependent transcription in NT2/D1 cells.

Discussion

The present study demonstrated anticancer activity of quercetin in human *in vitro* model of teratocarcinoma, NT2/D1 cell line. We have also shown, for the first time, that quercetin downregulates the expression of three master regulators of pluripotency SOX2, Oct4 and Nanog in NT2/D1 cells. Our results suggest that quercetin exerts its effect in NT2/D1 cells through inhibition of Wnt/β-catenin signaling pathway.

By several lines of experimental evidence we have demonstrated antitumorous and antimetastatic activity of quercetin in NT2/D1 cell line. Quercetin inhibited proliferation, adhesion and migration of these cells and induced cell death. It has been shown previously that quercetin induces selective antiproliferative effects and cell death, predominantly in cancer cell lines but not in normal cells[25, 26, 27, 28]. Literature data suggest that capability of quercetin to prevent tumor growth is a multifunctional effect. Described mechanisms include activation of ERK and p38 pathways in osteoblastic cells[29], inhibition of H-Ras in human breast epithelial cells MCF10A[30] and down regulation of MMP-9 (matrix metalloproteinases-9) expression in melanoma models[31].

Various studies have demonstrated the role of constitutively active Wnt/β-catenin signaling and nuclear β-catenin accumulation in tumor progression and metastasis[32]. This loss of β-catenin regulation is accompanied by activation of genes involved in cell proliferation and migration[33]. Antimetastic effect of quercetin in NT2/D1 cells is probably caused by several co-existing molecular mechanisms. Inhibition of cell migration induced by β-catenin inactivation has been previously associated with downregulation of β-catenin downstream targets involved in tumor invasion and metastasis, such as Membrane type 1 matrix metalloproteinase (MT1-MMP)[34, 35] matrix metalloproteinase (MMP)-2[32], MMP-7[34], MMP-9[32], laminin-5 γ2 chain[35], etc. Also, quercetin affects some of the key targets in multiple cellular signaling pathways related to the cell migration and exerts different, more complex effects. For example, quercetin suppresses invasion and migration of MCF10A human epithelial cells by inhibiting

phosphatidylinositol 3-kinase (PI3K)/Akt signaling[30]. At the same time, AKT1 kinase, key mediator of the PI3K/AKT pathway, regulates β-catenin directly by increasing the activating phosphorylation of β-catenin[36], or indirectly as a result of the inhibition of GSK3β[37]. Another possible mechanism by which quercetin might suppress migration of NT2/D1 cells is via downregulation of pluripotency factors. It has been shown previously that SOX2[38], Oct4[39] and Nanog[40] play important role in tumor cell migration. For example, ectopic expression of Sox2 in malignant gliomas was sufficient to induce invasion and migration of these cells, while downregulation of Sox2 reduced migratory and invasive potential[38]. This reduction was the result of the decreased expression of migration and invasion markers EphA2, Rac, RHO, Rock1, Rock2, VEGF, TGFβ, MMP9, MMP2, Notch1, Notch2 and Notch3[38]. Although the basic mechanisms governing all these effects are not fully understood, the existing data support potential use of quercetin as an anticancer agent or an addition to cancer therapies.

Our findings that quercetin downregulates SOX2, Oct4 and Nanog in NT2/D1 cells may have important implications in understanding of human teratocarcinoma pathogenesis and treatment. It has been reported previously that elevated expression of pluripotency factors SOX2 and Oct4 in EC cells modified various biological processes such as cell cycle, differentiation, apoptosis and cell adhesion [41]. Association of SOX2 downregulation with EC cells apoptosis and cell cycle arrest led to the investigations of the therapeutic potential of SOX2 inhibition for embryonal carcinoma. The study reported SOX2 silencing induced apoptosis *in vitro* and growth suppression of small xenograft tumors derived from EC cells[42]. Normal fetal germ cells share the pattern of expression of pluripotency factors with human embryonal stem cells hESC[43]. Although expression of SOX2, Oct4 and Nanog is high, in contrast to hESC germ cells exhibit strong inhibition of pluripotency and self-renewal[43]. However, loss of this inhibition may trigger tumorogenesis and transformation of these cells to germ line cancer stem cells. This implies that quercetin has the potential to become a chemopreventive and/or chemotherapeutic agent for testicular carcinoma. Effects of quercetin on cancer stem cells (CSC) have emerged as a new field in cancer research and therapy[44]. In lung cancer stem cells (LCSC) and breast cancer stem cells (BCSC) quercetin suppress the expression of heat shock protein 27 (Hsp27)[44, 45]. Through this mechanism quercetin inhibits expression

of stemness genes SOX2, Oct4 and Nanog in LCSC[45] and suppress basic characteristics of BCSC: aldehyde dehydrogenase activity, mammosphere formation and epithelial-mesenchymal transition (EMT) [46].

Our results suggest that quercetin downregulates expression of pluripotency transcription factors in NT2/D1 cells through inhibition of Wnt/β-catenin signaling pathway. Quercetin blocked β-catenin nuclear translocation and consequently suppressed β-catenin dependent transcriptional activation in human testicular carcinoma cells. Previous studies have shown that activation of Wnt/β-catenin signaling is involved in maintenance of self-renewal and pluripotency of human and mouse ES cells[47]. Wnt signaling activation by specific GSK3β inhibitor, 6-bromoindirubin-3'-oxime (BIO), sustain pluripotency in mouse and human ES cells[48, 49]. *c-myc* gene, Wnt/β catenin target gene, has a critical role in maintenance of pluripotency of mouse ES cells[50, 51]. In addition, previous results emphasized functional importance of c-myc in the maintenance of pluripotency in human and mouse EC cells[52, 53, 54, 55]. In NT2/D1 cells *c-myc* expression is high in the undifferentiated state but significantly decreased during differentiation[53, 54]. Activation of Wnt/β-catenin signaling using lithium as an GSK3β inhibitor upregulates c-myc expression in NT2/D1 cells, while β-catenin inhibition significantly decreased c-myc level[19]. In addition, in P19 cells, mouse model of teratocarcinoma, activation of Wnt/β-catenin signaling promoted proliferation by inducing expression of *c-myc* and maintained pluripotency by supporting high expression of Sox2, Oct4, and Nanog[55].

These data suggest that activation of Wnt/β-catenin signaling plays an important role in the pathogenesis of testicular carcinoma. DeAlmeida et al.[56], have shown that treatment of NT2/D1 cells with specific extracellular antagonists of Wnt signaling (F8CRDhFc) inhibited activity of this signaling pathway *in vitro*[56]. In addition, in *in vivo* model system with xenograft tumors derived from NT2/D1 cells treatment with therapeutic doses of F8CRDhFc results in a significant suppression of these tumors[56]. Our findings further support the significance of Wnt signaling in pathogenesis, progression and potential treatment of human teratocarcinoma. Based on these findings we may speculate that quercetin as Wnt signaling antagonist might be effective therapeutic agent in cancers with aberrant activation of Wnt pathway. Future *in vitro* and

in vivo studies are needed to understand wide range of molecular mechanisms involved in complex anticancer activity of quercetin. They will also provide new insights into development of new targeted therapeutics that will be used in cancer therapy.

Conclusions

We have shown that quercetin inhibited proliferation and promoted cell death in human teratocarcinoma *in vitro* model. Quercetin decreased adhesion and reduced migratory potential of NT2/D1 cells. In addition, the expression of pluripotency factors SOX2, Oct4 and Nanog was downregulated in NT2/D1 cells after quercetin treatment.

Our results further suggest that quercetin exerts its anticancer effect through inhibition of Wnt signaling. Quercetin antagonized Wnt/β-catenin signaling pathway in NT2/D1 cells by inhibition of β-catenin nuclear translocation and consequent downregulation of β-catenin dependent transcription.

Presented data suggest that quercetin might be effective as a therapeutic agent against teratocarcinoma and other cancers with abnormal Wnt signalling activation.

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Notes

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References

1 A. K. Jain, K. Thanki and S. Jain, *Nanomedicine*, 2014,
2 A. Murakami, H. Ashida and J. Terao, *Cancer Lett*, 2008, **269**, 315-325.
3 P. Pratheeshkumar, A. Budhraja, Y. O. Son, X. Wang, Z. Zhang, S. Ding, L. Wang, A. Hitron, J. C. Lee, M. Xu, G. Chen, J. Luo and X. Shi, *PLoS One*, 2012, **7**, e47516.
4 W. K. Kim, M. H. Bang, E. S. Kim, N. E. Kang, K. C. Jung, H. J. Cho and J. H. Park, *J Nutr Biochem*, 2005, **16**, 155-162.
5 C. H. Park, J. Y. Chang, E. R. Hahm, S. Park, H. K. Kim and C. H. Yang, *Biochem. Biophys. Res. Commun.*, 2005, **328**, 227-234.
6 P. Polakis, *Cold Spring Harb Perspect Biol*, 2012, **4**,
7 H. Clevers and R. Nusse, *Cell*, 2012, **149**, 1192-1205.
8 A. J. Chien, W. H. Conrad and R. T. Moon, *J Invest Dermatol*, 2009, **129**, 1614-1627.
9 D. Nonaka, *Am J Clin Pathol*, 2009, **131**, 731-736.
10 L. H. Looijenga, H. Stoop, H. P. de Leeuw, C. A. de Gouveia Brazao, A. J. Gillis, K. E. van Roozendaal, E. J. van Zoelen, R. F. Weber, K. P. Wolffenbuttel, H. van Dekken, F. Honecker, C. Bokemeyer, E. J. Perlman, D. T. Schneider, J. Kononen, G. Sauter and J. W. Oosterhuis, *Cancer Res*, 2003, **63**, 2244-2250.
11 P. W. Andrews, *APMIS*, 1998, **106**, 158-167; discussion 167-168.
12 E. Dmitrovsky, V. V. Murty, D. Moy, W. H. Miller, Jr., D. Nanus, A. P. Albino, F. Samaniego, G. Bosl and R. S. Chaganti, *Oncogene*, 1990, **5**, 543-548.
13 H. Burger, K. Nooter, A. W. Boersma, C. J. Kortland and G. Stoter, *Br J Cancer*, 1998, **77**, 1562-1567.
14 S. Masui, Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, H. Okochi, A. Okuda, R. Matoba, A. A. Sharov, M. S. Ko and H. Niwa, *Nat Cell Biol*, 2007, **9**, 625-635.
15 K. Takahashi and S. Yamanaka, *Cell*, 2006, **126**, 663-676.
16 M. Herreros-Villanueva, J. S. Zhang, A. Koenig, E. V. Abel, T. C. Smyrk, W. R. Bamlet, A. A. de Narvajas, T. S. Gomez, D. M. Simeone, L. Bujanda and D. D. Billadeau, *Oncogenesis*, 2013, **2**, e61.
17 A. A. Avilion, S. K. Nicolis, L. H. Pevny, L. Perez, N. Vivian and R. Lovell-Badge, *Genes Dev*, 2003, **17**, 126-140.
18 J. de Jong, H. Stoop, A. J. Gillis, R. J. van Gurp, G. J. van de Geijn, M. Boer, R. Hersmus, P. T. Saunders, R. A. Anderson, J. W. Oosterhuis and L. H. Looijenga, *J Pathol*, 2008, **215**, 21-30.
19 M. Mojsin, V. Topalovic, J. Marjanovic and M. Stevanovic, *Arch. Biol. Sci.,*, 2013, **65**, 201-209.
20 P. W. Andrews, I. Damjanov, D. Simon, G. S. Banting, C. Carlin, N. C. Dracopoli and J. Fogh, *Lab. Invest.*, 1984, **50**, 147-162.
21 J. D. Dignam, R. M. Lebovitz and R. G. Roeder, *Nucleic Acids Res.*, 1983, **11**, 1475-1489.
22 P. Bernard, H. Sim, K. Knower, E. Vilain and V. Harley, *Int. J. Biochem. Cell Biol.*, 2008, **40**, 2889-2900.

23 M. van de Wetering, R. Cavallo, D. Dooijes, M. van Beest, J. van Es, J. Loureiro, A. Ypma, D. Hursh, T. Jones, A. Bejsovec, M. Peifer, M. Mortin and H. Clevers, *Cell*, 1997, **88**, 789-799.
24 T. Scholzen and J. Gerdes, *J. Cell. Physiol.*, 2000, **182**, 311-322.
25 J. M. Davis, E. A. Murphy and M. D. Carmichael, *Curr Sports Med Rep*, 2009, **8**, 206-213.
26 K. V. Hirpara, P. Aggarwal, A. J. Mukherjee, N. Joshi and A. C. Burman, *Anticancer Agents Med Chem*, 2009, **9**, 138-161.
27 S. Jagtap, K. Meganathan, V. Wagh, J. Winkler, J. Hescheler and A. Sachinidis, *Curr Med Chem*, 2009, **16**, 1451-1462.
28 T. Mainardi, S. Kapoor and L. Bielory, *J Allergy Clin Immunol*, 2009, **123**, 283-294; quiz 295-286.
29 T. W. Nam, C. I. Yoo, H. T. Kim, C. H. Kwon, J. Y. Park and Y. K. Kim, *J Bone Miner Metab*, 2008, **26**, 551-560.
30 N. R. Song, M. Y. Chung, N. J. Kang, S. G. Seo, T. S. Jang, H. J. Lee and K. W. Lee, *Food Chem.*, 2014, **142**, 66-71.
31 S. J. Pradhan, R. Mishra, P. Sharma and G. C. Kundu, *Exp Ther Med*, 2010, **1**, 915-920.
32 M. Vaid, R. Prasad, Q. Sun and S. K. Katiyar, *PLoS One*, 2011, **6**, e23000.
33 A. Klaus and W. Birchmeier, *Nat Rev Cancer*, 2008, **8**, 387-398.
34 T. Brabletz, A. Jung, S. Dag, S. Reu and T. Kirchner, *Verh Dtsch Ges Pathol*, 2000, **84**, 175-181.
35 F. Hlubek, S. Spaderna, A. Jung, T. Kirchner and T. Brabletz, *Int J Cancer*, 2004, **108**, 321-326.
36 D. Fang, D. Hawke, Y. Zheng, Y. Xia, J. Meisenhelder, H. Nika, G. B. Mills, R. Kobayashi, T. Hunter and Z. Lu, *J. Biol. Chem.*, 2007, **282**, 11221-11229.
37 D. A. Cross, D. R. Alessi, P. Cohen, M. Andjelkovich and B. A. Hemmings, *Nature*, 1995, **378**, 785-789.
38 M. M. Alonso, R. Diez-Valle, L. Manterola, A. Rubio, D. Liu, N. Cortes-Santiago, L. Urquiza, P. Jauregi, A. Lopez de Munain, N. Sampron, A. Aramburu, S. Tejada-Solis, C. Vicente, M. D. Odero, E. Bandres, J. Garcia-Foncillas, M. A. Idoate, F. F. Lang, J. Fueyo and C. Gomez-Manzano, *PLoS One*, 2011, **6**, e26740.
39 X. Dai, J. Ge, X. Wang, X. Qian, C. Zhang and X. Li, *Oncol Rep*, 2013, **29**, 155-160.
40 W. Ji and Z. Jiang, *Oncol Lett*, 2013, **6**, 367-374.
41 B. Greber, H. Lehrach and J. Adjaye, *BMC Dev Biol*, 2007, **7**, 46.
42 H. Ushida, T. Chano, K. Minami, H. Kita, T. Kawakami, H. Okabe, Y. Okada and K. Okamoto, *J Urol*, 2012, **187**, 1876-1881.
43 A. T. Clark, *Stem Cell Rev*, 2007, **3**, 49-59.
44 N. P. Nguyen, F. S. Almeida, A. Chi, L. M. Nguyen, D. Cohen, U. Karlsson and V. Vinh-Hung, *Cancer Treat Rev*, 2010, **36**, 485-491.
45 H. S. Hsu, J. H. Lin, W. C. Huang, T. W. Hsu, K. Su, S. H. Chiou, Y. T. Tsai and S. C. Hung, *Cancer*, 2011, **117**, 1516-1528.
46 L. Wei, T. T. Liu, H. H. Wang, H. M. Hong, A. L. Yu, H. P. Feng and W. W. Chang, *Breast Cancer Res*, 2011, **13**, R101.
47 T. Miki, S. Y. Yasuda and M. Kahn, *Stem Cell Rev*, 2011, **7**, 836-846.
48 J. Hao, T. G. Li, X. Qi, D. F. Zhao and G. Q. Zhao, *Dev Biol*, 2006, **290**, 81-91.

49 N. Sato, L. Meijer, L. Skaltsounis, P. Greengard and A. H. Brivanlou, *Nat Med*, 2004, **10**, 55-63.
50 P. Cartwright, C. McLean, A. Sheppard, D. Rivett, K. Jones and S. Dalton, *Development*, 2005, **132**, 885-896.
51 N. V. Varlakhanova, R. F. Cotterman, W. N. deVries, J. Morgan, L. R. Donahue, S. Murray, B. B. Knowles and P. S. Knoepfler, *Differentiation*, 2010, **80**, 9-19.
52 B. Bilir, O. Kucuk and C. S. Moreno, *J Transl Med*, 2013, **11**, 280.
53 S. J. Freemantle, A. V. Vaseva, K. E. Ewings, T. Bee, K. A. Krizan, M. R. Kelley, E. M. Hattab, V. A. Memoli, C. C. Black, M. J. Spinella and E. Dmitrovsky, *Int. J. Oncol.*, 2007, **30**, 333-340.
54 W. H. Miller, Jr., D. Moy, A. Li, J. F. Grippo and E. Dmitrovsky, *Oncogene*, 1990, **5**, 511-517.
55 S. Zhang, Y. Li, Y. Wu, K. Shi, L. Bing and J. Hao, *Anat Rec (Hoboken)*, **295**, 2104-2113.
56 V. I. DeAlmeida, L. Miao, J. A. Ernst, H. Koeppen, P. Polakis and B. Rubinfeld, *Cancer Res*, 2007, **67**, 5371-5379.

Figure captions

Fig. 1 The effect of quercetin on proliferation rate and apoptotic status of NT2/D1 cells **A** Ki-67 staining of NT2/D1 cells treated with vehicle control-DMSO-NT2/D1 (b) and quercetin (e) for 24 h. Nuclei were counterstained with DAPI (a, d). Scale bar: 50μm. The relative quantities of Ki-67 positive cells were calculated and represented as the percentage of control vs. treatment in the form of a bar graph **B** Propidium iodide (PI) and DAPI staining of NT2/D1 cells treated for 24 h with either DMSO as vehicle control (a), or quercetin (b and c). Images were examined under a fluorescence microscope (20 X objective lens). Boxed regions in a, b, and c are enlarged in the same figures. White arrows indicate viable cells with normal nuclei, yellow arrowhead indicates viable cell with chromatin condensation, white arrowheads indicate cells with morphological changes characteristic for late apoptosis, and yellow arrows indicate apoptotic bodies **C** Western blot analysis of PARP expression and cleavage after quercetin treatment of NT2/D1 cells. α-tubulin was used as loading control.

Fig. 2 Quercetin inhibits adhesion and migratory potential of NT2/D1 cells **A** Cell adhesion was measured using MTT test. Results are expressed as the mean percentage of the vehicle control (DMSO) assigned as 100% and are presented as the mean±S.E.M,

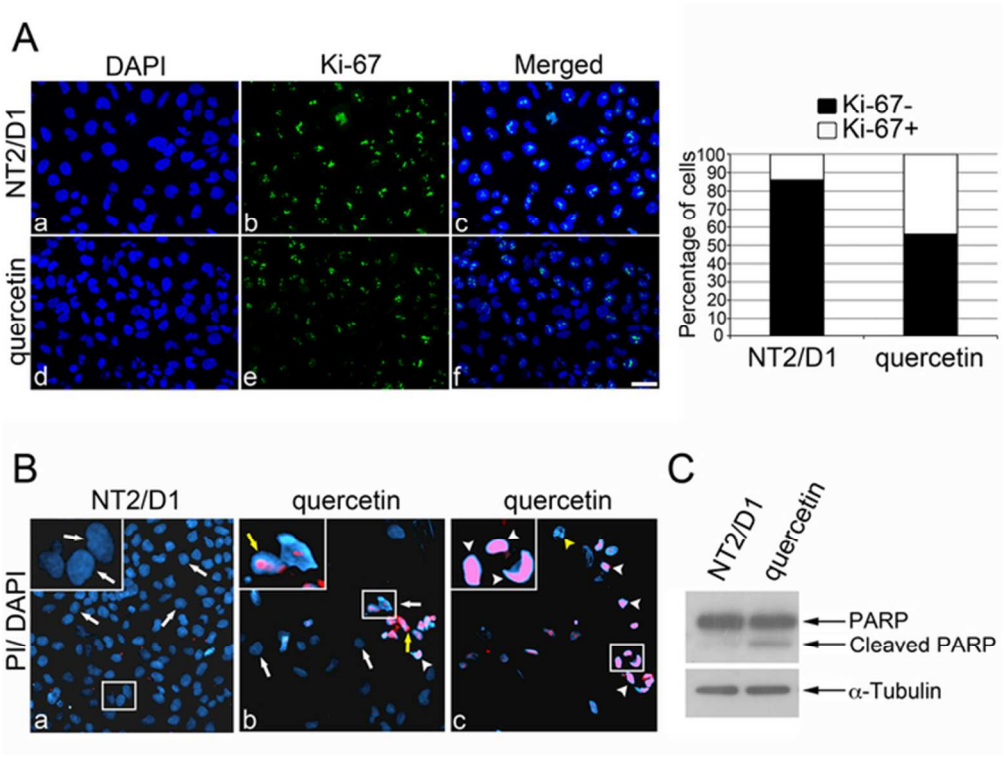
*P<0.01. Experiments were performed in triplicate and repeated three times **B** The wound migration assay of NT2/D1 cells treated with vehicle control (NT2/D1) and quercetin. Representative images of the wounds were captured using confocal microscopy at the indicated time points. Data were quantified and presented as the percentage of wound migration vs. treatment in the form of a bar graph. The error bars indicate the standard error of the mean. Experiments were performed in triplicate (*p<0.01). Scale bar: 100μm.

Fig. 3 Effects of quercetin on the expression of pluripotency markers *SOX2*/SOX2, *Oct4* and *Nanog* in NT2/D1 cells **A** Real-time PCR analysis of pluripotency markers *SOX2, Oct4* and *Nanog* expression in NT2/D1 cells treated with quercetin. Data were normalized by the amount of *GAPDH* mRNA expressed relative to the corresponding value for vehicle controle, DMSO-treated cells, and are means $\pm$ S.E.M. ,*$P < 0.01$ from triplicate data **B** Immunocytochemistry analysis of SOX2 protein expression in NT2/D1 cells treated with quercetin for 24 h. Significant downregulation of SOX2 expression was visible in all cells treated with quercetin (Left panels). Cell nuclei were stained with DAPI (Right panels). Scale bar: 50μm **C** SOX2 protein expression was analyzed by Western blot and relative protein expression levels were analyzed. One representative Western blot is presented, while quantification of the relative SOX2 protein level is based on the results obtained by 3 independent quercetin treatments. The normalized proteins activities were evaluated as the percentage of the activity in vehicle treated NT2/D1 cells which was set as 100% activity and are presented as the mean±S.E.M. of at least three independent experiments. *P<0.01

Fig. 4 Effects of quercetin on transcriptional activity of β-catenin/Tcf signaling in NT2/D1 cells **A** NT2/D1 cells were transfected with reporter gene constructs harboring Tcf binding sites (TOP) and mutant Tcf binding sites (FOP) and subsequently treated with vehicle control (DMSO) or 70 μM quercetin (Q) **B** TOPFlash and FOPFlash reporters were cotransfected with β-catenin expression vector in the presence or absence of 70 μM quercetin. The normalized Luc activities were evaluated as the percentage of the activity of vehicle control treated (DMSO) treated NT2/D1 cells transfected with

TOPFlash construct which was set as 100% activity and are presented as the mean ± S.E.M. of at least three independent experiments. $^{*}P<0.01$

Fig. 5 Cellular redistribution of β-catenin in NT2/D1 cells upon treatment with quercetin **A** Immunofluorescence analysis of β-catenin in vehicle control treated and NT2/D1 cells treated with quercetin for 24 hours. Note decrease of β-catenin in the nuclei after quercetin treatment (left panel). Cell nuclei were counterstained with DAPI (right panel). Scale bare: 20μm **B** Cells were incubated with 70 μM quercetin for 24 h fractionated into their cytosolic and nuclear components and subjected to Western blot analysis with antibody to β-catenin. α-tubulin was used to assess the purity of the nuclear extract preparation control. ce-cytosolic extracts; ne-nuclear extracts.



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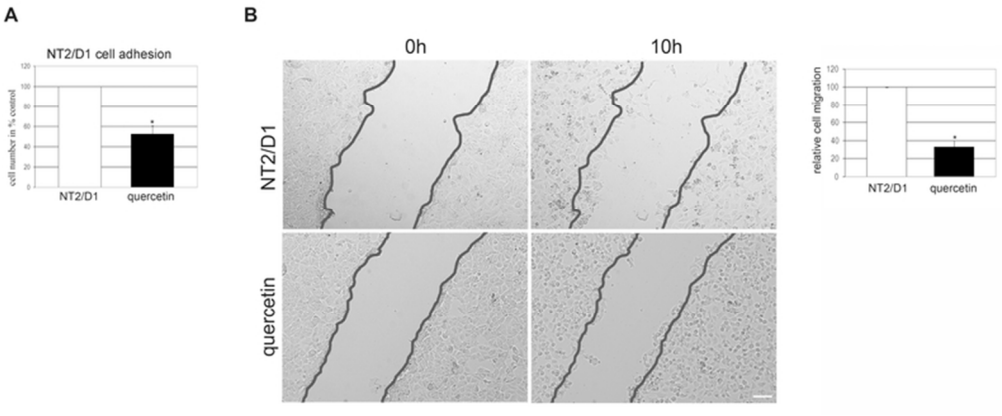

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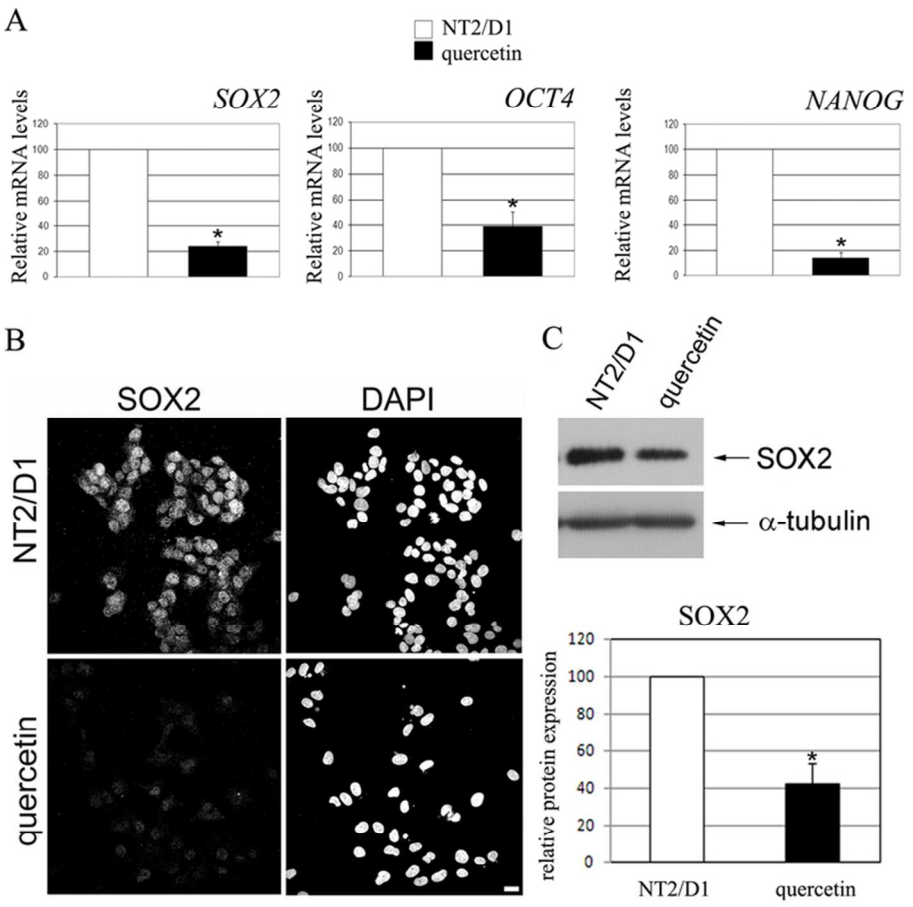

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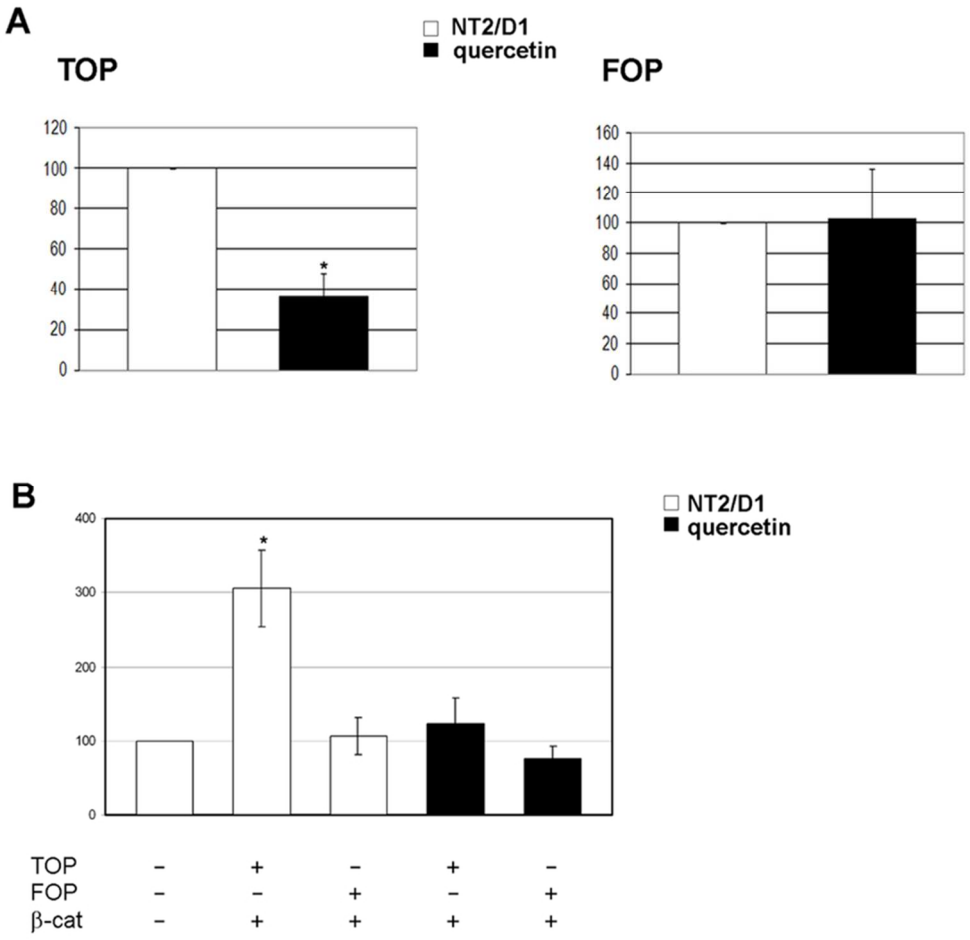

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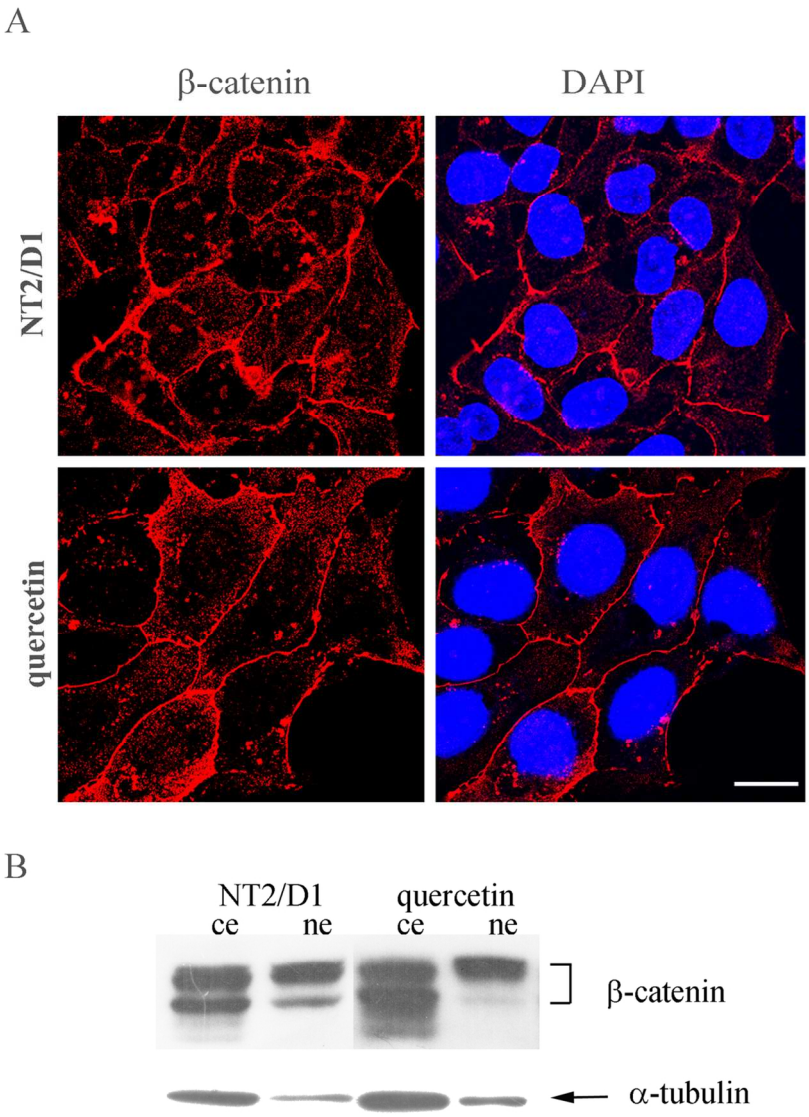

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