Metformin inhibits growth and decreases resistance to anoikis in medullary thyroid cancer cells

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Abstract

Medullary thyroid cancer (MTC) is associated with activation of mammalian target of rapamycin (mTOR) signaling pathways. Recent studies showed that the antidiabetic agent metformin decreases proliferation of cancer cells through 5′-AMP-activated protein kinase (AMPK)-dependent inhibition of mTOR. In the current study, we assessed the effect of metformin on MTC cells. For this purpose, we determined growth, viability, migration, and resistance to anoikis assays using two MTC-derived cell lines (TT and MZ-CRC-1). Expressions of molecular targets of metformin were examined in MTC cell lines and in 14 human MTC tissue samples. We found that metformin inhibited growth and decreased expression of cyclin D1 in MTC cells. Treatment with metformin was associated with inhibition of mTOR/p70S6K/pS6 signaling and down-regulation of pERK in both TT and MZ-CRC-1 cells. Metformin had no significant effects on pAKT in the cell lines examined. Metformin-inducible AMPK activation was noted only in TT cells. Treatment with AMPK inhibitor (compound C) or AMPK silencing did not prevent growth inhibitory effects of metformin in TT cells. Metformin had no effect on MTC cell migration but reduced the ability of cells to form multicellular spheroids in nonadherent conditions. Immunostaining of human MTC showed over-expression of cyclin D1 in all tumors compared with corresponding normal tissue. Activation of mTOR/p70S6K was detected in 8/14 (57.1%) examined tumors. Together, these findings indicate that growth inhibitory effects in MTC cells are associated with downregulation of both mTOR/6SK and pERK signaling pathways. Expression of metformin's molecular targets in human MTC cells suggests its potential utility for the treatment of MTC in patients.

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Introduction

Medullary thyroid cancer (MTC) derives from parafollicular C-cells of the thyroid and comprises ~4% of thyroid cancers in United States (Hundahl et al. 1998). MTC occurs in sporadic and hereditary forms. The heritable variants include three different clinical phenotypes: multiple endocrine neoplasia type 2A (MEN2A), MEN2B, and familial MTC-only syndrome (Pelizzo et al. 2007). Prognosis in patients with MTC mainly depends on the stage of tumor progression at the time of diagnosis, with a mean 10-year survival rate of 100, 93, 71, and 21% for stages I, II, III, and IV respectively (Modigliani et al. 1998).

Dominant activating mutations in the *RET* protooncogene have been identified as the underlying cause of the development of MTC (de Groot et al. 2006). The activation of antiapoptotic PI3K/AKT pathway and mitogenic MEK/ERK cascade are crucial events in cell transformation triggered by oncogenic *RET* (Segouffin-Cariou & Billaud 2000, Drosten et al. 2004). Recent studies demonstrated that the presence of the *RET* mutation is also associated with activation of the mammalian target of rapamycin (mTOR) signaling in MTC cells (Grozinsky-Glasberg et al. 2010, Faggiano et al. 2011).

The mTOR protein complex integrates signals from growth factors with protein translation. mTOR regulates cell growth through p70-S6 kinase (p70S6K), which leads to phosphorylation of ribosomal protein S6 (Wullschleger et al. 2006, Guertin & Sabatini 2007). The pathway from growth factor receptor stimulation to mTOR activation proceeds through PI3K/AKT and MAPK/ERK signaling pathways. AKT phosphorylates and inhibits tuberin sclerosis protein complexes (TSC1 and 2) leading to mTOR activation through interactions with the mTOR protein complexes mTORC1 and mTORC2 (Astrinidis & Henske 2005). In addition to being directly phosphorylated by AKT, TSC2 can be phosphorylated and inactivated by ERK as well as its downstream kinase RSK (Shaw & Cantley 2006).

Inhibition of signaling pathways activated by oncogenic *RET* emerged as an alternative strategy for the treatment of patients with MTCs (Lanzi et al. 2009). The kinase inhibitor that blocks activity of RET kinase (Vandetanib) is being used in clinical practice for the treatment of progressive MTC. Multikinase inhibitors targeting VEGFR (Sorafenib), as well as mTOR inhibitor (Everolimus), showed antitumor effects in preclinical studies and are in clinical trials (Lanzi et al. 2009, Lam et al. 2010, Faggiano et al. 2011). However, only partial responses and transient disease stabilization were reported from the clinical trials, and most of the MTC patients eventually developed progressive disease (Cabanillas et al. 2010). Therefore, the understanding of mechanisms controlling MTC progression vs metastatic phenotypes is important for development of new therapeutic approaches.

There is limited information on how cell signaling activation may control invasion of MTC cells, and there are no studies that address molecular mechanisms underlying survival of MTC cells in blood and lymphatic vessels. We previously demonstrated that invasive thyroid cancer cells are characterized by a gene expression signature that is consistent with epithelial to mesenchymal transition (Vasko et al. 2007). Studies on various cancer cell lines showed that activation of MEK–ERK and PI3/AKT/mTOR signaling pathways contributes to cancer

cell resistance to anoikis (apoptosis induced by detachment from extracellular matrix; Zugasti et al. 2001, Goldstein et al. 2009, Jensen et al. 2011). It has also been reported that concurrent inhibition of MEK–ERK and mTOR/p70S6K pathways selectively targets anoikis-resistant cancer cells with acceptable effects on normal cells in their proper tissue context (Fukazawa et al. 2002). Therefore, pharmacological agents with ability to inhibit both MEK/ERK and PI3/AKT/mTOR signaling pathways could play a role in prevention of metastases.

Analysis of cell signaling network interactions reveals that both MEK/ERK and mTOR/p70S6K pathways can be inhibited by 5′-AMP-activated protein kinase (AMPK). AMPK directly phosphor-ylates the TSC2 tumor suppressor on conserved serine sites distinct from those targeted by other kinases, leading to the inhibition of mTORC1 (Shaw 2009, Luo et al. 2010). Recent studies also demonstrated interrelationships between AMPK and mitogenic pathways via kinase suppressor of ras 2 (KSR2; Costanzo-Garvey et al. 2009). It has been suggested that under metabolic stress, binding of AMPK to KSR2 prevents RAF/MEK to be targeted to the plasma membrane for their activation (Luo et al. 2010).

The antidiabetic drug metformin has been shown to be a potent AMPK activator. On the intracellular level, metformin inhibits mitochondrial complex I, which leads to an altered AMP/ATP ratio and activation of AMPK (El-Mir et al. 2000). Studies on various cancer cell lines and animal models demonstrated that metformin inhibits growth via activation of AMPK pathway (Shaw 2009, Ben Sahra et al. 2010, Martin-Castillo et al. 2010). A recent study demonstrated growth inhibitory effects of metformin on anaplastic thyroid cancer cell lines (Chen et al. 2012).

In the current study, we assessed the *in vitro* effect of metformin on proliferation and activation of AKT/mTOR/S6K, MAPK/ERK, and AMPK signaling pathways in MTC-derived cells. We have also examined the effects of metformin on MTC cell properties that are required for development of metastases (migration and resistance to anoikis). We also determined the expression of metformin molecular targets in human MTC tissue samples.

Materials and methods

Human thyroid tissue and cell culture

The study protocol was approved by the Institutional Review Boards at the Washington Hospital Center and the Uniformed Services University of the Health Sciences. The paraffin-embedded thyroid tissue samples from 14 patients with MTC were selected from a thyroid tumor bank maintained at USUHS. There were 11 samples from patients with sporadic MTC and three samples from patients with hereditary MTC (two MEN2A and one familial MTC).

Human medullary thyroid cancer cell lines harboring C634W *RET* mutation (TT cells) and M918T *RET* mutation (MZ-CRC-1 cells) were obtained from Dr Motoyasu Saji (The Ohio State University). Cells were maintained in RPMI1640 medium supplemented with heat-inactivated 20% fetal bovine serum and 1×nonessential amino acids (1%) at 37 °C and humidified 5% CO_2. For an *in vitro* model of anoikis, TT and MZ-CRC-1 cells were cultured on poly-HEMA-treated low-adherent plates. Cells were incubated with either

control medium or medium containing metformin (Sigma Chemical Co.). The pharmacological inhibitor of pAMPK compound C was purchased from EMD Biosciences (San Diego, CA, USA).

siRNA transfections

TT cells were transfected with AMPK siRNA specific to α1 and α2 isoforms of catalytic subunit or with Stealth RNAi siRNA-negative control (Invitrogen). RNAi duplex–lipofectamine complexes were prepared using RNAiMAX transfection reagent (Invitrogen Corp.). The efficiency of silencing was assessed by Block-it fluorescent reagent and by detection of αAMPK protein level. Assays were performed 48 h after the initiation of the transfections.

Protein extraction and western blot analysis

Thyroid cancer cells were incubated with ice-cold cell lysis buffer, scraped, centrifuged, and the supernatant was stored at $-80\ ^\circ$C. Total protein lysate (25 μg) was suspended in reduced SDS sample buffer and protein lysates were subjected to SDS–PAGE (7%). The separated proteins were transferred to nitro-cellulose membranes (0.2 μm pore size; Invitrogen) by electrophoretic blotting (Invitrogen). Nonspecific binding was prevented by blocking with 0.1% Tween 20 in PBS (PBS-T) containing 5% nonfat dry milk overnight at 4 °C.

Membranes were incubated overnight with the primary antibody against phospho-p70S6K (Thr389), total p70S6K, phospho-pS6 (Ser235/236), total pS6, phospho-AKT (Ser473), total AKT, phospho-ERK (Thr202/Tyr204), total ERK, phospho-AMPKα (Thr172), total AMPKα, PARP, and cleaved caspase 3 (Cell Signaling, Boston, MA, USA); and cyclin D1, E-cadherin, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then incubated with the secondary antibody in PBS-T containing 5% nonfat dry milk for 1 h at room temperature. After washing with PBS-T four times (15 min/wash), protein bands on the blots were visualized using Li-COR Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Cell viability and proliferation assays

Cell viability was determined by Alamar blue assay (Invitrogen) according to the manufacturer's instructions. Cells were plated in 96-well plates and grown until 50% confluence was reached. Cells were treated with metformin at concentrations varying from 0.1 to 5 mM for 24, 48, and 72 h. Ten microliters (10% of sample volume) of Alamar blue solution were added to each well, and cells were subsequently returned to the incubator for 4 h. The absorbance of Alamar blue reagent was monitored at 570 and 600 nM by spectrophotometer.

For cell proliferation assay, TT and MZ-CRC-1 cells were plated at a density of 50 000 cells/well in 6-well plates. Cells were allowed to adhere overnight and then treated with metformin at concentrations varying from 0.1 to 5 mM. After incubation for 24, 48, or 72 h, cell proliferation rate was determined by cell counting using Vi-CELL Cell Viability Analyzer from Beckman Coulter (Fullerton, CA, USA).

In low-adherent conditions, cell viability was determined by evaluation of mitochondrial membrane potential with a fluorogenic lipophilic cation (JC-1; Cayman Chemical Company, Ann Arbor, MI, USA). All experiments were repeated at least three times, and the average values ±s.D. of representative experiments are reported.

Immunostaining of human thyroid tissue samples

Immunostaining was performed on paraffin-embedded tissue sections. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Sections were incubated overnight with cyclin D1 or phospho-p70S6K antibodies. Immunostaining was performed using Vector kits (Vector Labs, Burlingame, CA, USA) according to the manufacturer's instruction. For quantification of cyclin D1 expression, at least 200 cells in three different areas of tumor were examined and percentage of positive nuclei was calculated. For phospho-p70S6K staining, the results of staining were interpreted as follows: negative – no staining or focal/low intensity of staining in < 20% of cells and positive staining – strong staining in more than 20% of cells. The intensity of immunostaining was scored independently by two investigators (J Klubo-Gwiezdzinska and V Vasko).

Results

Metformin inhibits growth of medullary thyroid cancer cells

To determine the effects of metformin on MTC-derived cell lines viability, we performed an Alamar blue assay. Treatment with increasing concentration of metformin was associated with inhibition of TT cell growth in dose- and time-dependent manner (Fig. 1A). Significant inhibition of TT cells growth was detected after cell exposure for 72 h with micromolar concentration of metformin. In MZ-CRC-1 cells, significant inhibition of growth was observed after exposure to metformin at concentration 1 and 5 mM for 48 or 72 h. Direct cell counts using Vi-CELL Cell Viability Analyzer confirmed data obtained by Alamar blue assay in TT and MZ-CRC-1 cell lines.

The reduction of cell number after treatment with metformin may be the result of either cell cycle arrest or cell death. To address this question, we performed western blot analysis with antibody against proliferation marker (cyclin D1) and apoptotic markers (cleaved caspase 3 and PARP). Treatment with metformin was associated with inhibition of cyclin D1 expression in TT and MZ-CRC-1 cell lines (Fig. 1B) without significant changes in cleavage of caspase 3 and PARP. The intensity of immunostaining with antibodies against cyclin D1 was decreased in metformin-treated cells (Fig. 1C) corroborating western blot data. These data indicate that metformin inhibits MTC cell growth through inhibition of proliferation but not induction of apoptosis.

Metformin inhibits mTOR signaling in MTC-derived cells

As growth inhibitory effects of metformin in various cancer cell lines were attributed to downregulation of mTOR signaling, we next examined activation of well-established downstream targets of mTOR-p70S6K and its substrate ribosomal protein S6 using specific antibodies. Western blot analysis showed that treatment with metformin was associated with dose-dependent inhibition of phosphop-p70-S6K and phospho-pS6 in MTC-derived cells

(Fig. 2A). Immunostaining also showed that intensity of immunostaining with anti-phospho-pS6 was decreased in MTC cells after treatment with metformin (Fig. 2B).

We also examined the effects of metformin on phosphorylation status of AKT, ERK, and AMPK in MTC-derived cells by western blot analysis. The level of pAKT was not significantly affected by treatment with increasing concentration of metformin in any of the examined cell lines (Fig. 2C). Metformin inhibited pERK in both TT and MZ-CR-1 cells. Induction of pAMPK was observed in TT cells but not in MZ-CRC-1 cells after treatment with metformin (Fig. 2C).

Consistent with the results of western blot analysis, the intensity of immunostaining with anti-pAMPK was increased in TT cells treated with metformin (Fig. 2D) but not in MZ-CRC-1 cells.

The role of AMPK in metformin-inducible mTOR inhibition

To clarify the role of pAMPK in TT cell response to metformin, we performed experiments using pharmacological AMPK inhibitor (compound C). Treatment with compound C (2 μM) decreased metformin-inducible activation of pAMPK (Fig. 3A). However, compound C did not prevent metfromin-inducible inhibition of phospho-pS6 and downregulation of cyclin D1 in TT cells.

We also performed silencing experiments and examined the effects of metformin on αAMPK-deficient TT cells. Combined inhibition of both α1 and a α2 isoforms of catalytic subunit prevented metformininducible AMPK activation (Fig. 3B). However, loss of *AMPK* expression did not prevent metformin-inducible downregulation of phospho-pS6 and only partially rescued metformin-inducible inhibition of cyclin D1. These results showed that loss of AMPK activity is not sufficient to completely abrogate inhibitory effects of metformin on mTOR signaling in TT cells.

The effects of metformin on MTC cell migration and resistance to anoikis

We next examined the effects of metformin on migration of MTC-derived cells and their resistance to anoikis. TT and MZ-CRC-1 cells intrinsically express E-cadherin and do not efficiently migrate through 8 μM pore membrane in the Boyden chamber migration assay. Treatment with metformin had no effects on E-cadherin expression and did not affect migratory ability of TT or MZ-CRC-1 cells (data not shown).

For analysis of anoikis, TT and MZ-CRC-1 cells were maintained in either adherent cell culture plates or low-adherent cell culture conditions. In low-adherent conditions, both MTC-derived cell lines were characterized by establishment of cell-to-cell contacts and formation of multicellular spheroids. MTC-derived cancer cells that formed spheroids were viable as demonstrated by JC-1 staining (Fig. 4A).

To determine whether metformin can affect spheroid formation, we pretreated TT and MZ-CRC-1 cells for 24 h with increasing concentrations of metformin in adherent plates and then transferred these cells into nonadherent plates. As shown in Fig. 4A, the number and size of spheroids were decreased in MTC cells pretreated with metformin. The viability of

these cells was significantly reduced as demonstrated by JC-1 staining. We also performed analysis of pro-survival signaling in nonadherent cells. Western blot analysis showed that TT and MZ-CRC-1 cells maintained activation of pAKT, pERK, and p70S6K/pS6 in low-adherent conditions. Pretreatment with metformin resulted in downregulation of pAKT, pERK, and p70S6K/pS6 in MTC cells growing in nonadherent conditions (Fig. 4B) and induction of apoptosis (Fig. 4C). Together, these data demonstrate that metformin impairs MTC cell survival after detachment from extracellular matrix and sensitizes MTC cells to anoikis.

Activation of mTOR signaling in human MTCs

Our *in vitro* findings demonstrated that metformin inhibits cell growth, decreases cyclin D1 expression, and inhibits mTOR/p70S6K signaling in both MTC-derived cell lines. To determine whether these *in vitro* findings could have relevance in human MTC, we assessed expression of cyclin D1 and activation of mTOR/p70S6K signaling in human MTC tissue samples. The level of immunostaining with anti-cyclin D1 was increased in all examined MTCs compared with the corresponding normal thyroid tissue. Cyclin D1 expression was detected in nuclei of MTC cells (Fig. 5A). The number of positive nuclei in tumors varied from case to case and ranged from 10 to 60%. The highest level of cyclin D1 expression was found in MTC samples from patients with hereditary forms of MTC (two patients with MEN2A and one patient with familial MTC).

Immunostaining with phospho-p70S6K was increased in 8/14 (57.1%) examined MTCs compared with the corresponding normal thyroid tissue (Fig. 5A). All hereditary MTCs and 5/11 sporadic MTCs showed strong immunoreactivity with anti-phospho-p70S6K antibody. In two cases of widely invasive MTCs, we detected cancer cells that were located inside the vessels. Positive immunostaining with p-p70S6K was observed in intravascular MTC cells. Together, these data showed that human MTC cells are characterized by increased expression of cyclin D1 and activation of mTOR/p70S6K signaling pathway.

Discussion

Targeting cancer cell metabolism is a promising strategy to treat cancer. There is increasing evidence of the potential efficacy of a commonly used antidiabetic drug, metformin, as an anticancer agent. The primary systemic effect of metformin is thelowering of bloodglucose levels through reduction of the hepatic glucose production and increased insulin sensitivity enabling appropriate usage of glucose by muscles and adipocytes (Ben Sahra et al. 2010). As insulin is a growth-promoting hormone with mitogenic effects, it has been suggested that beneficial effects of metformin in cancer patients are related to its systemic insulin-lowering effects. However, numerous studies demonstrated that in addition to its systemic action, metformin has direct growth inhibitory effects on cancer cells through inhibition of mTOR/S6K signaling (Guertin & Sabatini 2007, Ben Sahra et al. 2008, Luo et al. 2010). In this study, we used MTC-derived cells to examine the effects of metformin on medullary thyroid cancer cell growth, migration, and resistance to anoikis.

Metformin inhibited MTC cell growth in a dose- and time-dependent manner. In fact, MTC cells seem to be specifically sensitive to metformin. In contrast to previous studies

documenting anticancer effects of millimolar concentrations of metformin in breast, ovarian, and pancreatic cancer cell lines, we observed significant inhibition of MTC proliferation with micro-molar concentrations applied for 72 h. Inhibition of growth was associated with downregulation of cyclin D1 but not induction of apoptosis. Our findings are similar to previously reported observations in breast, prostate, and ovarian cancer cell lines showing that metformin induces blockade of cell cycle progression in G0/G1 phase (Ben Sahra et al. 2008, Zhuang & Miskimins 2008). In MTC cells, treatment with metformin resulted in inhibition of mTOR signaling pathway as demonstrated by down-regulation of phospho-p70S6K and phospho-pS6. Activation of p70S6K appears to be necessary for translation of specific set of mRNA, encoding ribosomal protein and translation elongation factors (Vesely et al. 1994, Sonenberg & Gingras 1998). It has also been demonstrated that expression of other genes important for cell cycle progression, such as Myc and cyclin D, may be controlled by the p70S6K pathway. These findings could explain the reason why inhibition of this pathway induces blockade of proliferation in MTC-derived cancer cells.

We demonstrated that metformin-inducible inhibition of mTOR/S6K signaling was associated with increased AMPK phosphorylation only in TT cells but not in MZ-CRC-1 cell line. Previous studies on breast and ovarian cancer cell lines demonstrated that the inhibitory effects of metformin on cancer cell growth were mediated via activation of AMPK (Dowling et al. 2007, Gotlieb et al. 2008). In contrast, studies using prostate cancer cells showed that metformin inhibits cell growth independently of its effect on AMPK (Ben Sahra et al. 2008). In the current study, pharmacological inhibition of AMPK activity or AMPK silencing did not prevent metformin-inducible inhibition of growth and downregulation of mTOR/S6K signaling in MTC cells. These data suggest that in addition to AMPK, other mechanisms could be involved in metformin-inducible downregulation of mTOR/S6K signaling and inhibition of cell growth in MTC cells. We also demonstrated that treatment with metformin was associated with inhibition of MEK/ERK signaling in TT and MZ-CRC-1 cells. Taking into consideration the role of both MEK/ERK and mTOR/S6K cascades in development of MTC, the dual inhibition of these signaling pathways by metformin could represent an attractive strategy for the treatment of MTC.

As local and distant metastases are common in MTC patients, we also examined the effects of metformin on cancer cell properties that are required for development of metastases (migration and resistance to anoikis). We found that MTC-derived cells were intrinsically resistant to anoikis and maintained activation of AKT/mTOR/p-70S6K and ERK signaling after detachment from extracellular matrix. Treatment with metformin sensitized MTC cell to anoikis and dramatically impaired their ability to survive in nonadherent conditions. Resistance to anoikis is a critical element of the metastatic cascade and our findings suggest the possible utility of metformin as an agent that may prevent metastases.

These findings are specifically promising because we found increased expression of metformin molecular targets in a series of human MTC samples. We found that activation of mTOR signaling is common in human MTCs. Positive staining with anti-phospho-p70S6K antibody in human MTC cells that invaded vessels corroborated *in vitro* results and suggested the role of mTOR signaling in MTC cell resistance to anoikis. Our results are similar to recently published findings (Rapa et al. 2011) demonstrating activation of mTOR/

p70S6K signaling in patients with MTCs. Taking into consideration that antineoplastic effects of metformin are mediated through inhibition of phospho-p70S6K, it is tempting to speculate that detection of phospho-p70S6K in samples from MTC patients could represent a marker of tumor cell response to the treatment with metformin.

In the current study, growth inhibitory effects of metformin were observed at concentrations that were significantly higher than those achievable in humans. The therapeutic concentrations of metformin in human serum range from 5 to 20 μM (Dell'Aglio et al. 2009). It is noted that in animal models, antitumor effects of physiological concentration of metformin (3–13 μM) were observed only after chronic exposure (for up to 13 weeks). The inhibition of tumor growth was attributed not only to direct effects of metformin on cancer cells but also to its indirect effects through diminished levels of circulating growth factors. It is possible that a combination of direct effects of metformin on MTC cells with its systemic insulin-lowering effects may result in inhibition of MTC growth with concentration that is achievable in human.

Retrospective data from population-based studies demonstrated that treatment with metformin reduced cancer risk in humans and improved outcome of different cancers. On the basis of these studies, metformin is now being used in various clinical trials including in a neoadjuvant setting and in combination with other drugs. Our *in vitro* data showed that metformin inhibits growth and induces anoikis in MTC-derived cells. Analysis of human MTC samples demonstrated expression of metformin molecular targets in MTC cells. In summary, these data suggest that the antidiabetic drug metformin can inhibit growth and prevent development of metastases in MTC.

Acknowledgements

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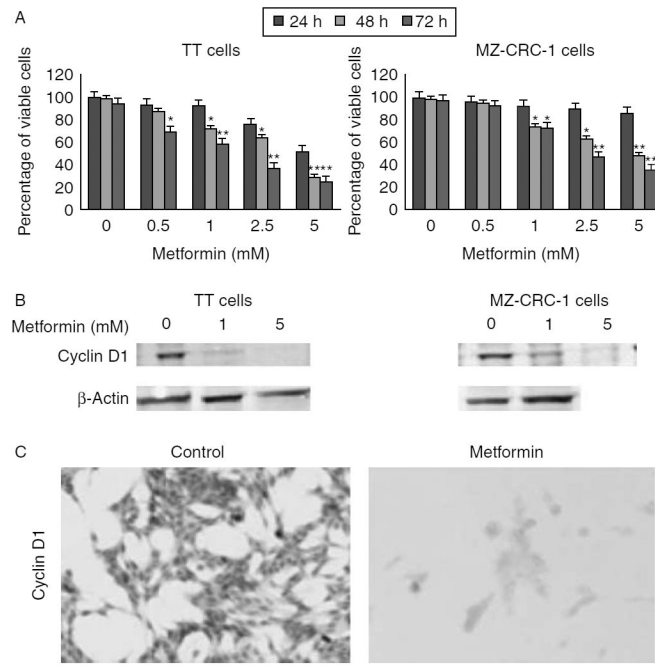
Figure 1.

The effects of metformin on MTC cells growth. (A) Dose and time dependent effect of metformin on TT and MZ-CRC-1cells growth. The decrement in number of viable cells was dependent upon both the concentration of metformin and the duration of exposure (*P value <0.05; **P value <0.001). (B) Western Blot analysis demonstrating metformin-inducible down-regulation of cyclin D1 expression in dose dependent manner. (C) TT cells were grown on LabTech chamber slides and treated with metformin (5 mM) for 24 h. Treatment with metformin was associated with inhibition of cyclin D1 expression.
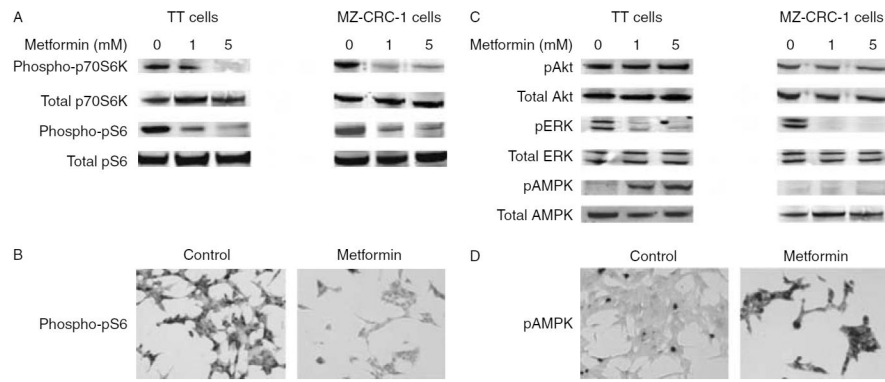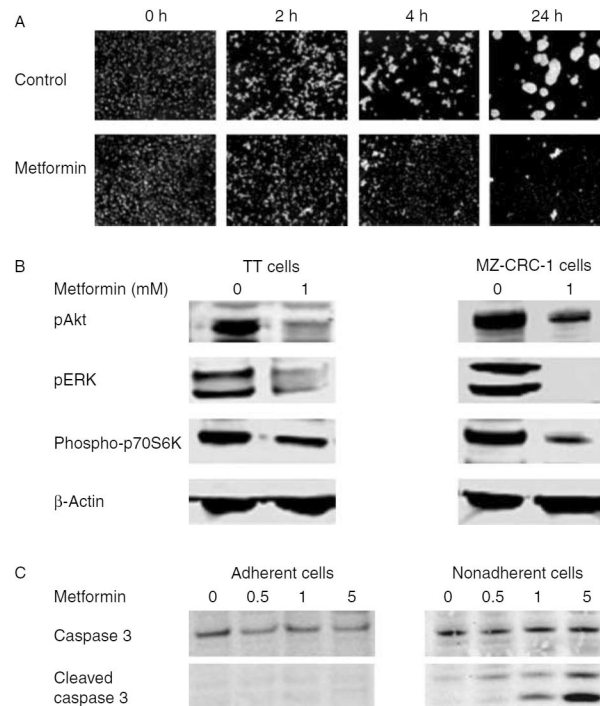
Figure 2.
The effects of metformin on cell signaling activation in MTC-derived cells. (A) Western blot analysis showing dose dependent inhibition of p-p70-S6K and p-pS6 in TT and MZ-CRC-1cells treated with metformin. (B) Immunostaining showed decreased phosphorylation of pS6 in TT cells treated with metformin (5 mM for 24 h). (C) Western blot analysis showing that metformin had no effect on pAkt, but inhibited pERK in TT and MZ-CR-1 cells. Metformin increased phosphorylation of AMPK in TT cells, but not in MZ-CRC-1 cells. (D) Immunostaining with anti-pAMPK showed increased immunoreactivity in TT cells that were treated with metformin (5 mM for 24 h).
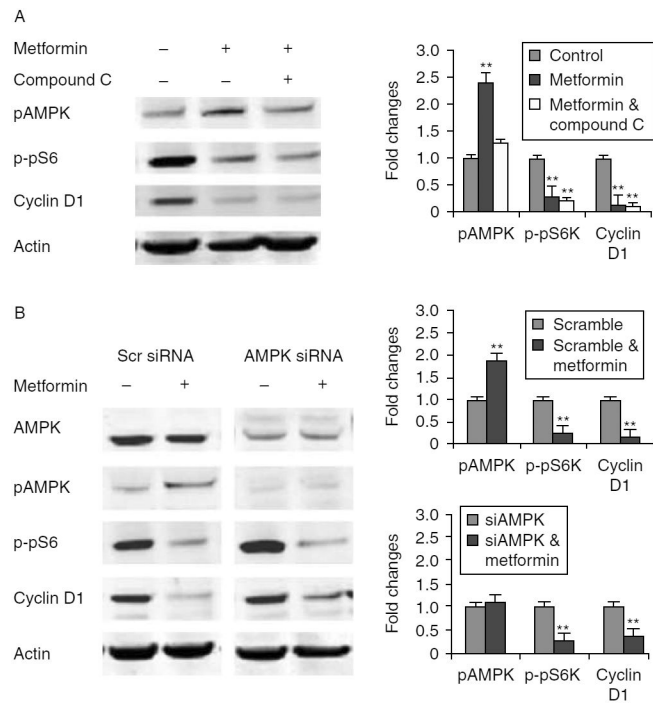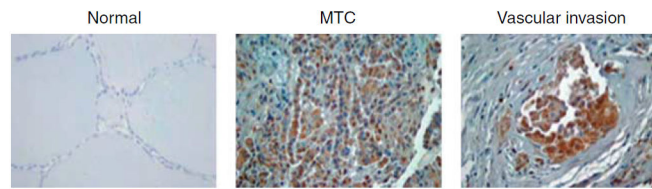
Figure 3.
The effect of AMPK inhibitor and AMPK silencing on TT cell response to treatment with metformin. (A) TT cells were treated with metformin alone or in combination with the AMPK inhibitor compound C. Western blot analysis showing that compound C attenuates metformin inducible AMPK phosphorylation with no significant effect on metformin inducible down-regulation of p-pS6 and cyclin D1 in TT cells. (B) Silencing of α1 and α2 isoforms of the catalytic subunit of AMPK attenuated metformin inducible AMPK activation, but did not prevent down-regulation of p-pS6 in TT cells. Loss of AMPK only partially rescued metformin-inducible inhibition of cyclin D1. **$P<0.001$.

Figure 4.
The effects of metformin on spheroid formation in low adherent condition and detachment induced apoptosis (anoikis). (A) After trypsinization TT cells were places on non adherent culture plates and morphological changes were monitored by microscopy. At 4 h post-plating, MTC cells formed multi-cellular conglomerates and at 24 h formed spheroids-like structures. Pretreatment with metformin 1 mM/24 h resulted in reduced size and number of spheroids and decreased viability of the cells as documented by JC-1 staining. (B) Western blot showing that pretreatment with metformin (1 mM/24 h) resulted in significant downregulation of pAKT, pERK, p-p70S6K in TT and MZ-CRC-1 cells growing in low adherent (LA) conditions compared to the LA cells not pre-treated with metformin. (C) Treatment with increasing concentrations of metformin for 24 h did not induce apoptosis in adherent TT cells. Pre-treatment with metformin sensitized TT cells to anoikis.

Figure 5.
Immunohistochemical staining with anti-p-p70S6K antibody in human thyroid tissue samples. The level of p-p70S6K immunostaining is increased in MTC cells compared to normal thyroid cells. MTC cells that invaded into the vessel show high level of immunoreactivity with anti-p-p70S6K.