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DISSERTATION

Effect of metformin on growth of differentiated thyroid cells and thyroid carcinoma cells and their derived cancer stem cells: implication for metformin as adjuvant treatment for undifferentiated thyroid cancer

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To my parents

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Abbreviations

ABCG2	ATP-binding cassette transporter G2
AMPK	5’ adenosine monophosphate-activated protein kinase
ATC	anaplastic thyroid carcinoma
ºC	Degree Celsius
cDNA	complementary deoxyribonucleic acid
CSC	cancer stem cell
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
×g	G-force (unit of measurement of rotation of a centrifuge)
HBSS	Hank’s balanced salt solution
IGF-1	insulin-like growth factor 1
MDR1	multidrug resistance transporter 1
MEM	non-essential amino acids
$MgCl_2$	magnesium chloride
ml	Milliliter
mRNA	messenger ribonucleic acid
MTT	methyl thiazolyl tetrazolium
µM	micromole per liter solution (unit of concentration)
µg	microgram
µl	Microliter
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
PH	potentia hydrogenii (negative decimal logarithm of hydrogen-ion concentration)
PI	Propidium iodide
pNA	p-nitroaniline
rpm	Revolutions per minute

RT	reverse transcription
SP	side population
TBE	Tris-borate EDTA
TBS	Tris buffered saline
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
UV	Ultraviolet

1 Introduction

1.1 Epidemiology of thyroid cancer

Thyroid cancer is a relatively rare cancer, representing ~1% of all malignancies and accounting for ~0.5% of all deaths caused by malignant tumors (1). However, it is the most frequent endocrine malignancy and accounts for the majority of endocrine cancer deaths. There are four main histological types of thyroid carcinoma: three well-differentiated carcinomas including papillary, follicular, medullary, and the undifferentiated anaplastic carcinoma. The well-differentiated thyroid carcinomas account for >90% of all thyroid cancers (2) and are generally characterized by slow growth and a low mortality rate (3-5). In contrast, anaplastic thyroid carcinomas, about 2% to 5% of all thyroid cancers, are responsible for most thyroid cancer deaths (6-9). This type is characterized by rapid growth and dissemination, with a mean survival rate of only 8 months after diagnosis, according to the statistics of American Cancer Society (Cancer facts and figures 2011, available at http://www.cancer.org/docroot/stt/stt_0.asp). Anaplastic thyroid carcinomas fail to concentrate radioiodine and to respond to conventional chemotherapy.

Epidemiological studies have reported an increasing incidence of thyroid cancer worldwide. The American Cancer Society estimates about 48,020 (11,470 male and 36,550 female) new cases, with 1,740 (760 male and 980 female) deaths in the United States in 2011 (10). In Europe, approximately 25,000 people are diagnosed as having thyroid cancer each year. In Germany, the incidence of thyroid cancer was about 1670 male and 3680 female per 100,000 in 2010. Thyroid cancer is believed to become one of the top ten leading cancer types in females.

1.2 Treatment of thyroid cancer and cancer stem cells

The treatment strategy for anaplastic thyroid carcinoma (ATC) is multimodal. It remains controversial whether primary chemotherapy or radiotherapy results in longer survival than primary surgical intervention. Regardless, there is no effective therapeutic regimen for ATC until now.

Although chemotherapeutic agents can suppress cancer growth, a considerable number of cancer cells do not respond to conventional chemotherapy. Patients often present with resistance to these drugs and recurrence of the tumor. To explain this phenomenon, the cancer stem cells (CSCs) hypothesis has been put forward (11-13). This notion argues that a sub-population of tumor cells is biologically distinct (14). These cells, designated CSCs or tumor-initiating cells, have the ability of sustained self-renewal and tumor propagation. Unlike the main population

within the tumor, CSCs are relatively resistant to radiation and chemotherapy. After treatment they can regenerate all cell types in the tumor through their stem cell-like characteristic. The CSC hypothesis demands a new treatment strategy that targets this small faction of tumor cells.

Toxicity of chemotherapeutic agents is another major problem of patients who accept chemotherapy. The patients may suffer from serious side effects including anemia, nausea, vomiting, appetite changes, fatigue, hair loss (15). Since these side-effects are, at least in part, dose-dependent, the identification of agents that can be combined with lower doses of the existing chemotherapeutic drugs is of high clinical value.

1.3 Diabetes mellitus and cancer risk

Diabetes mellitus (DM) is a worldwide prevalent disease with increasing incidence annually. Nowadays, about 250 million people live with diabetes globally, with this figure expected to reach 380 million within 20 years (16). People with diabetes suffer from variously acute and chronic complications that negatively influence the quality of life and their survival. Moreover, recent studies presume that patients with type 2 diabetes mellitus also have higher risk of cancer and cancer-related mortality.

There is growing epidemiological evidence that type 2 diabetes mellitus and obesity are associated with a higher risk of several types of cancer (16). Recently, it has been confirmed that the risk of hematological and several solid malignancies (including liver, pancreas, colorectal, kidney, bladder, endometrial and breast cancer) is increased in diabetic patients (17-25). Meta-analyses indicate that the strongest association between diabetes and increased cancer risk is with pancreatic and liver cancers (16), organs that play an important role in the pathogenesis of diabetes. The relative risk (RR) of liver and pancreatic cancer in diabetic patients is ~1.94 and ~2.5, respectively (17, 18). Data on cancer mortality in diabetic patients are less abundant and less homogeneous than data on cancer incidence. However, a positive association between the mortality of breast, colorectal, endometrial cancer and diabetes was found (22, 24, 26-29).

Besides diabetes, a positive association between obesity and carcinogenesis is also widely accepted. Accumulating epidemiologic evidence shows that overweight or obesity is linked with an increased risk of common cancers, such as breast, endometrial, prostate and colon cancer, which are the most cited malignancies (30-32).

Given the notion that cancer is more frequent in diabetic patients, is there also an association between thyroid cancer and diabetes?

Indeed, type 2 diabetic patients are at higher risk of thyroid cancers. Recently, clinical evidence showed that in type 2 diabetics the thyroid volume and the incidence of thyroid nodules are

increased (33). Furthermore, insulin resistance and hyperinsulinemia were also identified as a risk factor for developing differentiated thyroid carcinomas (34). In animal experiments, Sakano K. et al. (35) examined the carcinogenic potency of N-nitrosobis(2-oxopropyl)amine (BOP) in the Otsuka Long-Evans Tokushima Fatty (OLETF) rats, model animals for non-insulin-dependent diabetes mellitus, to determine whether diabetic and/or hyperlipidemic conditions influence carcinogenesis by BOP. They demonstrated that the diabetic/hyperlipidemic state enhances BOP-induced carcinogenesis of the thyroid gland and colon in OLETF rats.

Most prospective studies also suggest the existence of a positive relationship between obesity and thyroid cancer for men and women. In a pooled analysis of five prospective U.S. studies, the hazard ratios (HRs) for overweight (25.0-29.9 kg/m^2) and obesity (≥30 kg/m^2) compared with normal-weight (18.5-24.9 kg/m^2) were 1.20 and 1.53, respectively (36). A team in Italy studied a large population of obese individuals and found a 31% prevalence of single cold nodule (37) with an 8% occurrence of carcinoma (38) which was considered higher than the one reported in their normal-weight subjects (5-6%).

Overall, most studies suggest that both type 2 diabetes and obesity are independent risk factor for thyroid caner in men and women.

1.4 Pathophysiological mechanisms that link type 2 diabetes to cancer

The role of insulin/insulin-like growth factor 1 (IGF1) in increasing cancer risk of diabetic patients

The main mechanism that underlines the association between type 2 diabetes/obesity and cancer is hyperglycemia and hyperinsulinemia as a consequence of insulin resistance in these patients. Insulin exerts its growth-promoting effect via activation of insulin receptor- and insulin-like growth factor (IGF) receptor-dependent signaling (39).

Insulin receptor (IR) and IGF-1R share a signaling pathway, which is represented by two main branches: the mitogenic pathway (RAS/RAF/MEK/ERK) and the metabolic pathway (PI3K/AKT). The metabolic pathway can be further divided into two subpathways: one is the mTOR pathway, which plays a role mainly in metabolic but also in part in mitogenic regulation; the other is the Foxo pathway, which is mainly involved in cell survival in response to nutrient availability.

Under the conditions of normal insulin levels (Figure 1.1), insulin exerts a metabolic effect on its target tissue via the activation of the PI3 kinase pathway. In contrast, in hyperinsulinemic patients (Figure 1.2), insulin receptor signaling may be attenuated for the metabolic branch, but

not for the mitogenic branch (40, 41). This implies that hyperinsulinemia and insulin resistance mainly blunts the metabolic but not mitogenic effect of insulin. This unbalanced insulin receptor signaling may have different effects in different tissues, depending on predominant enzymatic machinery of the cell: it may cause impaired glucose homeostasis in typical insulin target tissue like live, muscle, and adipose tissue, whereas it will result in increased cell proliferation in other tissues (including ovary and cancer cells), which may finally favor neoplasms formation (42, 43). The AMP-activated protein kinase (AMPK), mammalian target of rapamycin (mTOR), and insulin signaling pathway are three interrelated components of a complex mechanism controlling cell response to nutrient availability. Their dysregulation may favor malignant cell proliferation in response to hyperinsulinemia.

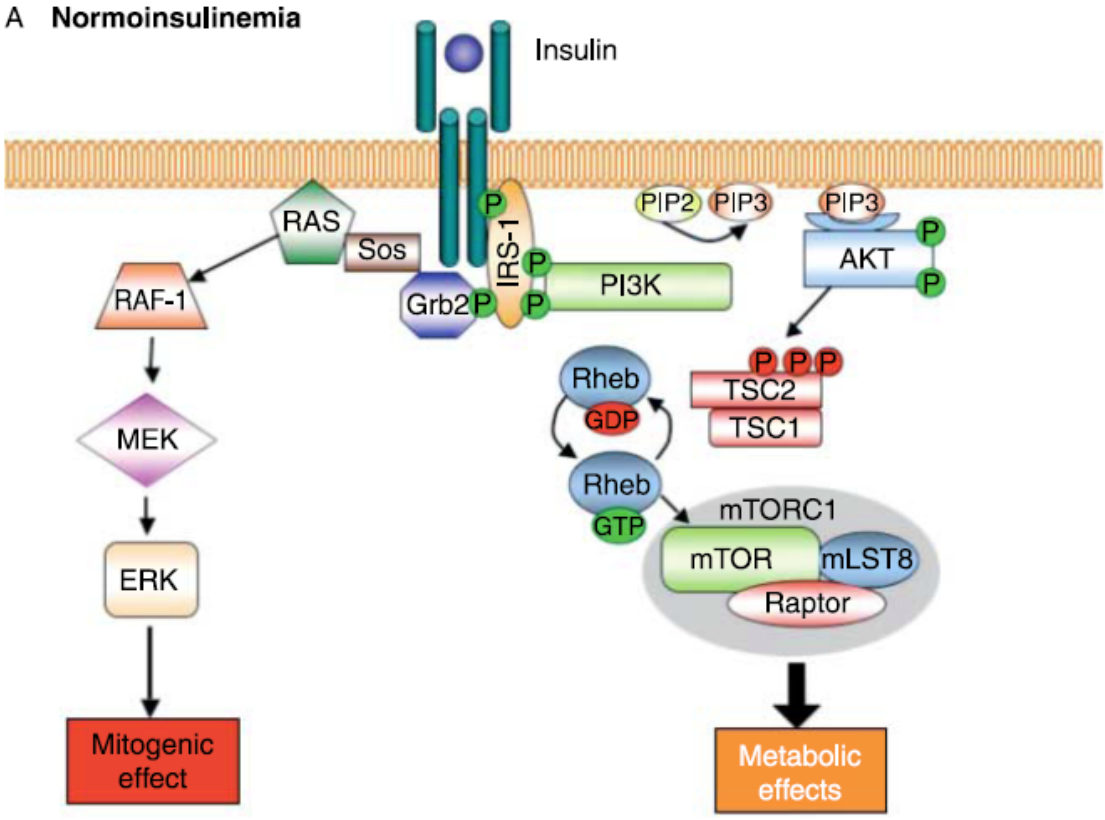

Fig1.1 The signaling pathway of insulin in normoinsulinemic person. (Paolo Vigneri, et al. Endocrine-Related Cancer, 2009, 16: 1103–1123)

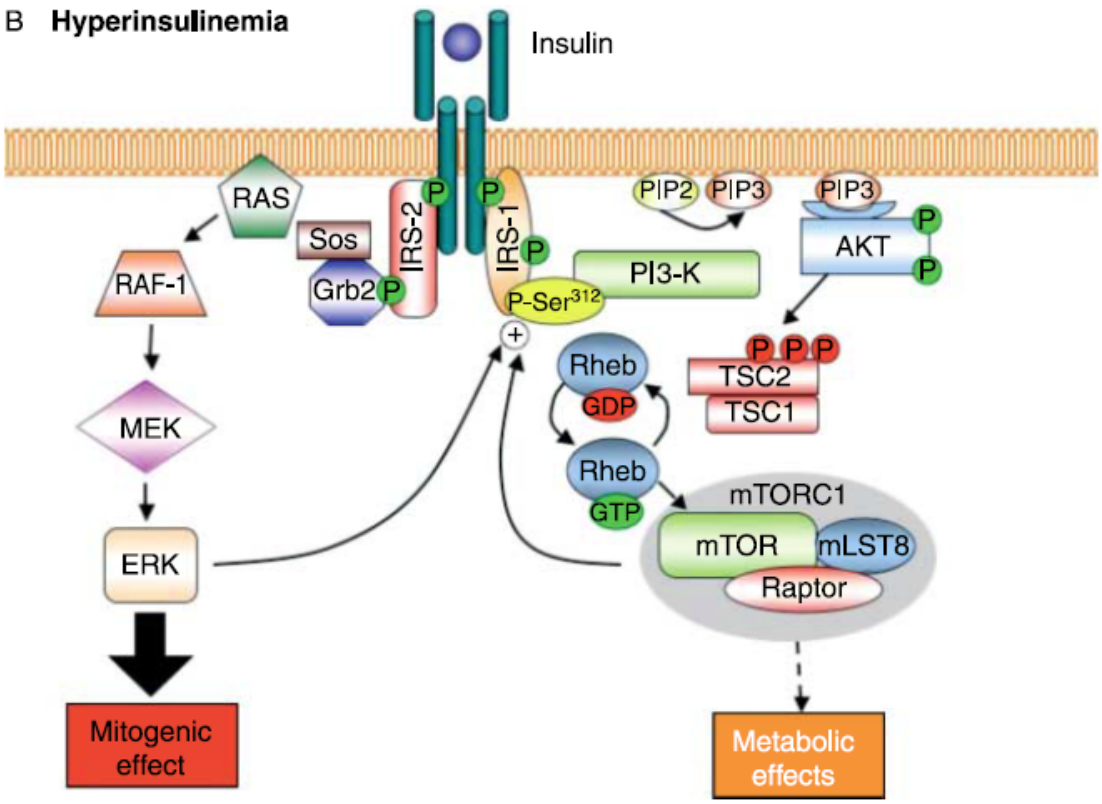

Fig 1.2 The signaling pathway of insulin in hyperinsulinemic patient. (Paolo Vigneri, et al. Endocrine-Related Cancer, 2009, 16: 1103–1123)

In the thyroid gland, insulin/IGF-1 has an effect on the expression of the TSH receptor gene and the thyroglobulin gene at transcriptional level. These two hormones are necessary for the action of TSH on thyroid cells: they work together with TSH to induce thyroid cell proliferation and maintain differentiation (44). The traditional view is that the major role of insulin/IGF-1 in the thyroid is not the mitogenic effect but permissive effect for TSH mediated thyroid cell stimulation (45, 46). However, there is increasing evidence showing over-activation of the insulin/IGF-1 system in thyroid cancer and their mitogenic signaling in these tumors. Ciampolillo et al. reported over-expression of IGF-1 and IGF-1 receptor in thyroid cancer with positive correlation with tumor diameter and intrathyroidal extension (47). Rezzonico et al. have recently shown that patients with insulin resistance have larger thyroid volumes and a higher prevalence of thyroid nodules (33). In addition, they reported that thyroid carcinoma produces increased levels of IGF-1, and patients with thyroid cancer presented with higher incidence of insulin resistance compared to the cancer-free controls (50% vs 10%, $P<0.001$) (34).

Other factors that may influence the risk of cancer in diabetes

Besides the role of insulin/IGF-1 signaling pathway, there are some other factors and proposed mechanisms linking diabetes to an increased risk of cancer and cancer-related mortality. Evidence from preclinical experiments suggests that cytokines produced by adipose tissue (leptin, adiponectin), hyperglycemia, free fatty acids, chronic inflammation and oxidative stress are all related to carcinogenesis.

1.5 Metformin reduces cancer risk and cancer-related mortality

Given that hyperinsulinemia plays a role in increasing cancer risk and progression in diabetic and obesity patients, it may be reasonable to presume that the reduction of insulin levels may decrease the risk of cancer (including thyroid carcinomas) in type 2 diabetics and obesity patients.

The three major families of oral anti-diabetic drugs include insulin secretagogues (sulfonylureas, glinides), insulin sensitizers (biguanides and thiazolidinediones) and DPPIV inhibitors. They have different mechanisms of action. Sulphonylureas stimulate endogenous insulin secretion and cause hyperinsulinemia. Therefore, they have been associated with an increased risk of cancer (48). Data on insulin sensitizer thiazolidinediones are inconclusive. A beneficial, neutral, or even deleterious effect has been reported in different types of cancer (49-51). In several in vitro experimental models, studies attributed the anti-cancer effect of glitazones to the activation of PPARγ receptors in addition to lower effect on hyperinsulinemia (52). However, the use of these compounds is too recent and too limited to consider the present meager epidemiologic observations reliable. Thus, metformin, which belongs to the biguanides, is preferred due to its long history as an excellent anti-diabetic agent.

Metformin, an oral anti-diabetic drug, has been in general use for more than 50 years and is currently suggested as first-line therapy in type 2 diabetics with obesity, insulin resistance and consecutive hyperinsulinemia. It improves insulin resistance and glycemic control through reduced hepatic gluconeogenesis and increased glucose uptake in skeletal muscles (53, 54). On the cellular level metformin inhibits hepatic glucose production through activation of LKB1/AMPK pathway (55). In addition to glucose control, metformin lowers cardiovascular mortality by about 25% compared with other oral diabetic drugs or placebo and was demonstrated to reduce the incidence of diabetes in persons at high risk, with beneficial effects persisting for at least 10 years (56-58). Metformin can be safely combined with other kinds of anti-diabetic drugs. It has a good safety profile with transient nausea and diarrhea being the most common side effects (59). The most serious but rare adverse reaction is lactic acidosis.

Importantly, the cost of metformin is extremely low, thus making it easily accessible in clinical practice.

Beyond the effects on insulin-responsive tissues, the effect of metformin on other cell types favours its use in cancer prevention and treatment. Preclinical studies have shown that metformin inhibited the growth of cancer cells in vitro and in vivo (60-64). Initial experiments showed that metformin was capable of reducing proliferation in breast, prostate, colon, pancreatic, and endometrial cancer cell lines (60, 61, 63, 65, 66). Subsequent in vivo experiments using intraperitoneal or oral metformin in a xenograft mouse model resulted in tumor growth inhibition and prolonged remission (67-70).

Epidemiologic studies linked the treatment of metformin with a lower risk of cancer in patients. In a pilot observational study, Evans et al. reported that among patients with type 2 diabetes, those treated with metformin had lower incidence of cancer compared with untreated controls (71). In a population-based cohort study using administrative databases from Saskatchewan Health, Bowker et al. reported that type 2 diabetes patients who use metformin have a lower cancer-related mortality compared with those using sulfonylureas or insulin (48). In 2010, a meta-analysis by DeCensi et al. concluded that metformin is associated with a 30% reduction in cancer incidence in individuals with type 2 diabetes compared with other diabetic treatments (72). These extensive preclinical and epidemiological data provide the rationale to consider metformin as a potential drug for adjuvant anti-cancer therapy (73). Despite the wide use of metformin in diabetic patients and the link to cancer risks, there is still no clear understanding of the effect of metformin on tumor growth. However, the insulin/insulin-like growth factor-1 (IGF-1) signaling pathway and the 5’ adenosine monophosphate-activated protein kinase (AMPK) pathway are two important pathways involved in cancer growth and survival. It was reported that metformin exerts an anti-mitogenic effect by activation of the AMPK and by inhibition of the mitogen-activated protein (MAP) kinase pathway, which is a major part of insulin/IGF-1 signaling that regulates cell growth (64).

1.6 Aim of the present study

There is growing epidemiological and experimental evidence that type 2 diabetes mellitus and obesity are associated with a higher risk of several types of cancer. Studies also demonstrate the link between insulin resistance/hyperinsulinemia and a high risk of differentiated thyroid cancer. Furthermore, it is proven that the antidiabetic drug metformin has an anti-mitogenic effect on several cancer growth both in vitro and in vivo by activation of AMPK and by inhibition MAP

kinase pathway. From these, it is rational to consider metformin as a potential drug for adjuvant anti-cancer therapy in type 2 diabetes.

Up to now, there are no data related to the role of metformin on growth of differentiated thyroid cells and thyroid cancer.

Therefore, the aim of the present work was (1) to identify whether metformin exerts an anti-mitogenic effect on differentiated human thyroid cells and on thyroid carcinoma cells, and to elucidated the underlying mechanism at the cellular level (cell cycle progression and apoptosis), (2) to prove whether metformin targets cancer stem cells in a doxorubicin-resistant anaplastic thyroid carcinoma cell line which are enriched with cancer stem cells and to analyse whether it affect clonal cell growth or thyroid cancer sphere formation, (3) to investigate the effect of metformin on insulin-mediated cell growth, and to find out if metformin inhibits growth by activation of the AMPK/mTOR pathway, and if it antagonizes the growth stimulatory effect of insulin by inhibition of MAP kinase pathway, (4) to detect whether metformin is capable of amplifying the anti-mitogenic effect of chemotherapeutic agents such as doxorubicin and cisplatin on undifferentiated thyroid carcinoma cells.

2 Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

All cell lines were stored in liquid nitrogen at -196°C. For defrosting, cells were placed in 37°C water bath and shaken gently for 1 min and then immediately suspended in pre-warmed culture medium.

Human anaplastic thyroid carcinoma cell lines (HTh74, HTh74Rdox, C643 and SW1736), human follicular carcinoma cell line (FTC133), and FRTL-5 cell line were used in the experiment.

The HTh74 human anaplastic thyroid carcinoma cell line was isolated from thyroid carcinoma tissue of a 73-year-old woman (74). HTh74 cells were shown to express some thyroid-specific genes like functional TSH receptor protein (74), and mRNA for thyroglobulin, albeit both at very low levels (75).

The C643 cell line was established from a fine-needle biopsy of an anaplastic thyroid carcinoma of a 76-year-old man (75). The patient died within 5 months after diagnosis. Demonstration of thyroglobulin mRNA ascertained a thyroid epithelial origin of the cell line (76).

The SW1736 cell line was originally developed by Leibowitz and McCombs III at the Scott and White Memorial Hospital (Temple, TX) in 1977. Their epithelial origin was confirmed by demonstration of cytokeratin expression (77).

All these three cell lines were kindly provided by Prof. Nils-Erik Heldin (Uppsala University, Uppsala, Sweden).

A doxorubicin-resistant HTh74 sub-line (HTh74Rdox) was developed by continuous exposure of HTh74 cell line to doxorubicin (Sigma, St. Louis, USA) starting at 10 ng/ml and increasing in a stepwise manner to 500 ng/ml. The resistant cell line was maintained in the medium containing doxorubicin for more than 6 months (78). The IC50 value for doxorubicin was 153.53±16.43 μg/ml, which corresponds to a 85-fold increase of resistance compared to parental HTh74 cells.

The permanent cell line FTC-133 was established from differentiated follicular human thyroid cancer cells. FTC-133 cells proved to be particularly useful in assessing growth regulation of human thyroid tissue. These cells could be propagated in serum-free medium, and showed thyroglobulin immunoreactivity and EGF receptors (79). The FTC133 cell line was kindly provided by Dr Goretzki, Neuss, Germany.

The FRTL-5 cell line, which is a stable thyroid cell line derived from the thyroid of Fisher rats

under defined culture conditions, has been widely adopted as a model for the study of differentiated thyroid cell function. It was obtained from American Type Culture Collection (Bethesda, MD).

2.1.2 Preparation of primary thyroid cell cultures

Thyroid tissues were obtained from 12 patients with nodular goiters after thyroidectomy, provided by the Endocrine Surgery Department of the St. Hedwig Clinic in Berlin. Malignancy was ruled out in all cases by means of fine needle aspiration biopsy and cytological examination prior to the operation, rapid excision biopsy during the operation, and histological examination of tissue after surgery. Informed consent was obtained from all patients prior to the operation. The study was approved by the ethics committee of the Charité, Humboldt University of Berlin. The patient group consisted of 12 patients, 7 female (58%) and 5 male (42%). The mean age of the patients was 56.25 years, with a range of 30-77 years.

Preparation of primary thyroid cultures from human nodule tissue was performed as described previously (80, 81). Firstly, in the operating room, separated tissue specimens were put in sterile transport tubes containing 20 ml transport medium (HBSS solution with 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B), placed on ice and transported to the laboratory. Then all work with cell cultures was performed in a laminar flow cabinet. Employing sterile techniques, macroscopically visible capsule material and other connective tissues were removed using scalpel and tweezers, the remaining tissue was grossly chopped with a scalpel to facilitate subsequent dispartment in follicles. Enzyme digestion was carried out in fresh HBSS containing 5 mg/ml Collagenase A (Roche, Mannheim, Germany) and 30 mg/ml dispase II (Roche, Mannheim, Germany) at 37 °C for 1.5 h in a shaking bath with slow swirling motion at about 80 rpm. After 90 min, the digest was filtered through a sterile mesh sieve, leaving behind the undigested tissue fragments. The flow-through was centrifuged for 5 min at 310 × g. The supernatant was returned to the remaining tissue to continue digestion, while the thyroid cell pellet was re-suspended in pre-warmed (37 °C) primary cell culture medium, transferred to 100 mm cell culture dishes (Sarstedt, Nümbrecht, Germany) and placed in a humidified incubator at 37 °C, with a 5 % CO_2 atmosphere for 20 min. This was the pre-plating step which helps to reduce fibroblast contamination since fibroblasts adhere to the dish surface more readily than thyrocytes. After 20 min the medium with unattached cells was transferred to another dish and placed in the incubator for culture. The digestion procedure for undigested tissue fragments was repeated several times until only white connective tissue remained.

2.1.3 Culture conditions

HTh74, HTh74Rdox, and FTC133 cells were cultured in Ham's F-12 medium (Gibco, Karlsruhe, Germany) with L-glutamine, supplemented with 10 % fetal calf serum (FCS, v/v) (Gibco, Karlsruhe, Germany), 1 % non-essential amino acids (MEM, v/v) (Gibco, Karlsruhe, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Roche, Mannheim, Germany), and 2.5 µg/ml amphotericin B (Sigma, USA). HTh74Rdox cells were grown in the same medium but supplemented with 0.5 µg/ml doxorubicin (Sigma, St. Louis, MO, USA).

C643 and SW1736 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 10 % FCS (v/v), 1 % MEM (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B.

FRTL-5 cells were grown in Ham's F-12 medium supplemented with 5 % FCS, 1 % MEM (v/v), 5 mU/mL TSH (from bovine pituitary, Sigma, St. Louis, MO, USA), H5-mix (10 ng/ml glycyl-histidyl-lysine, 10 µg/ml insulin, 10 ng/ml somatostatin, 5 µg/ml transferrin, 3.2 ng/ml hydrocortisone, all from Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Human primary thyroid cells were cultured in the same medium as FRTL-5 but supplemented with 10 % FCS.

For most experiments, cells were grown as monolayer in 100 mm or 60 mm plastic dishes and kept in a humidified incubator at 37 °C under 5 % CO_2, with a medium change every 3-4 days. Upon reaching the confluency of 70-80 %, cells were passaged after trypsinization using trypsin (0.05%)/EDTA-4Na (0.53 mM) solution.

2.1.4 Cell counting

After trypsinization and neutralization protocol for cell cultures, a cell suspension was obtained in a centrifuge tube. A 1:2 diluted cell suspension in trypan blue (Sigma, Steinheim, Germany) was loaded on both counting chambers of the hemacytometer with the coverslip placed over them. The cells were counted under a microscope at 100 × magnification. Cell numbers (total and viable: Trypan blue-unlabeled) overlaying four × 1 mm^2 areas of the counting chamber were determined. And then the total (or viable) cell number of the original cell suspension can be calculated as follows:

Total (or viable) cells recovered = cells/mm^2 divided by dilution (1/2), and multiplied by 10^4 and total volume of cell suspension

2.2 Cell viability assay

Cell viability was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) assay. Cells ($4\text{-}10\times10^3$) were seeded in 96-well plates. After preculture, cells were treated with medium containing different doses of metformin (Sigma, St. Louis, MO, USA) and/or different agents as described in Results and Figure legends. MTT assay was performed by incubating the cells with 0.5 mg/ml MTT for 4 h at 37 ˚C in 5% CO_2. The formazan product was dissolved in DMSO and the absorbance were read at 490 nm. All experiments were repeated at least three times in at least quadruplicate per group each time. Cell viability is presented as relative change compared to the control value (untreated cells) arbitrarily set to 1 or as % of control value.

2.3 Cell cycle distribution and cell apoptosis by flow cytometry

Analysis of cell cycle arrest was performed in low density cultures (1×10^5 cells per 60 mm dish, 40-50% confluency), whereas apoptosis was investigated in high density cultures (4×10^5 cells per 60 mm dish, confluent > 90%) (82).

The cell cycle was analyzed by measuring the amount of propidium iodide (PI)-labeled DNA in ethanol-fixed cells. Cells were plated in 60 mm dishes in their corresponding media for 24 h. Subsequently, the cells were starved overnight and then treated with 10% serum and metformin at various concentrations for 48 h. After that, cells were collected, washed with PBS, fixed in cold 70% ethanol solution and then stored at −20 °C until flow cytometric analysis was performed. On the day of analysis, cells were washed and centrifuged using cold PBS, suspended in 1 ml PI staining solution (PBS with 200 µg/ml RNase A, 50 µg/ml PI and 0.1% Triton X-100) followed by incubation for 30 min at room temperature. Flow cytometric analysis was performed on a FACS LSRII flow cytometer (BD, Heidelberg, Germany) using a peak fluorescence gate to exclude cell aggregates during cell cycle analysis.

Apoptotic and necrotic cell death was analyzed by double staining with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI, in which annexin V bound to the apoptotic cells with exposed phosphatidylserine, while PI labeled the necrotic cells with membrane damage. Staining was performed according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). Briefly, cells treated with or without metformin for 24 h were harvested and resuspended in 1 × Binding Buffer at a concentration of $\sim1\times10^6$ cells/ml. 5 µl of 50 µg/ml annexin V FITC conjugate and 10 µl of 100 µlg/ml PI solution were added to each 500 µl cell suspension. The tubes were incubated at room temperature for exactly 10 min and protected from light. The green and red fluorescence of annexin V/PI-stained live cells was analyzed with a FACS LSRII flow cytometer.

The numbers of viable (annexin V–/PI–), apoptotic (annexin V+/PI–) and necrotic (annexin V+/PI+) cells as well as the proportion of cells in different cell cycle phases were determined with FACSDiva software (BD). All experiments were performed in triplicate and repeated twice to assess for consistency of response.

2.4 Colorimetric assay of caspase-3 activity

The caspase-3 colorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. p-Nitroaniline has a high absorbance at 405 nm (εmM = 10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

2×10^6 cells were plated in 100 mm culture dishes. After attaching to the flasks overnight, cells were treated with metformin, and the control was treated with the culture medium. Caspase-3 activity was determined using a colorimetric assay kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

2.5 In vitro clonal analysis

HTh74 and HTh74Rdox cells were plated at clonal density (200 cells per well) in triplicates in 6-well plates and treated with indicated concentrations of metformin. The cells were allowed to form colonies for up to 2 weeks and medium was replaced every third day. Colonies were stained with Giemsa and counted as indicated. The percentage of cells that initiated a clone was presented as cloning efficiency. Triplicate samples were run for 3 independent experiments.

2.6 Tumor-sphere culture and sphere formation efficiency

Tumor spheres were generated by placing HTh74 cells (1×10^4 cells/ml) into serum-free DMEM/F-12 (1:1, v/v) medium containing B27 (1:50 dilution, Invitrogen), bFGF (20 ng/ml, Invitrogen) and EGF (20 ng/ml, Invitrogen). Every 2-3 d, B27, bFGF and EGF were added. After 7 d, some cells had formed intact floating spheres. Sphere-forming efficiency (SFE) was calculated as the number of sphere-like structures (large diameter > 50 μm) formed in 7 days divided by the original number of cells seeded and expressed as percentage means (±SD).

2.7 Reverse transcription, polymerase chain reaction (RT-PCR) and quantitative real-time reverse transcription-PCR

2.7.1 Total RNA isolation

Total RNA was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. In brief, after aspiration of the culture medium, 350 µl buffer RLT, supplemented with β-mercaptoethanol (β-ME, add 10 µl β-ME per 1 ml buffer RLT), was added to the monolayer cultures. Cell lysates were then collected using a rubber policeman, pipetted in QIA Shredder microcentrifuge tubes (Qiagen, Hilden, Germany) and centrifuged for 2 min at maximum speed for homogenization. Equal volumes of 70 % ethanol were added to the homogenized lysate and mixed by pipetting. Samples were then applied to RNeasy mini columns placed in 2 ml collection tubes and centrifuged for 15 sec at 8000 ×g. Flow-through was discarded and 700 µl of RW1 buffer was added to the RNeasy columns and centrifuged for 15 sec at 8000 ×g to wash the columns. Flow-through and collection tubes were discarded and the RNeasy columns were transferred into new 2 ml collection tubes. 500 µl of buffer RPE was pipetted onto the RNeasy columns which were then centrifuged again for 15 sec at 8000 ×g to wash. Flow-through was discarded and another 500 µl buffer RPE was added to the RNeasy columns. Tubes were then centrifuged again for 2 min at 8000 ×g to dry the RNeasy silica-gel membrane. For elution, the RNeasy columns were transferred to new 1.5 ml collection tubes and 30 µl of RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. Tubes were closed gently and centrifuged for 1 min at 8000 ×g. The resulting RNA was either stored at - 20ºC or used directly for spectrophotometry and RT-reaction (see below).

For the measurement of RNA concentration, 1 µl of RNA was added and absorption was calculated at OD_{260} (NanoDrop, Spectrophotometer).

2.7.2 Reverse transcription reaction

For reverse transcription, 4 µl of iScript reaction mix and 1 µl iScript reverse transcriptase were added to the volume of mRNA solution containing 1 µg of mRNA (iScriptTM cDNA Synthesis Kit, BIORAD, Hercules, CA). RNase-free water was added to a final volume of 20 µl and the mix was heated to 25°C for 5 min, 42°C for 30 min, 85°C for 5 min. The cDNA samples were stored at -20°C for later use.

2.7.3 Primer preparation

All primers were obtained in powder form from Invitrogen Inc. Upon delivery, primers were diluted in RNAse-free water (concentration 10 pmol/µl), aliquoted and stored at -20°C.

2.7.4 Polymerase chain reaction

For PCR amplification, the Hot Start method was used. In brief, 4 µl of complementary DNA was added to a 45.5 µl master mix containing 5 µl 10 × reaction buffer, 1.5 mM $MgCl_2$, 1 µl dNTPs (10 mM) and 0.5 µM primers. Negative controls without template cDNA were included in all cases to exclude carry-over contamination with genomic DNA. 50 µl of mineral oil was added to each reaction tube, the lids were closed and the mixture was preheated to 95 ºC for 10 min before adding Taq polymerase (Invitrogen, Karlsruhe, Germany) to reduce non-specific annealing and primer elongation events. The samples were then cooled to 80 ºC and 0.5 µl of Taq DNA polymerase (5 U/µl) was added to each reaction tube. Using a thermocycler, cycling conditions were carried out at 95 °C for 30 sec (initial denaturation), 52-63 °C for 30 sec (primer specific, annealing) and 72 °C for 1 min (extension), followed by a final extension at 72 °C for 10 min and termination at 4 °C. The number of cycles used was determined by the log-linear phase of the amplification reaction. In all PCR analyses, β-actin was served as an internal control. Primer pair sequences, product lengths and annealing temperatures were as follows:

Table 2.1 Primer sequences, annealing temperatures, cycles and product sizes for PCR

Target gene	Primer sequences[#]	Annealing Temp.(ºC)	Cycles	Expected size(bp)
β-actin	S: 5'-CCCAGGCACCAGGGCGTGAT-3' AS: 5'-TCAAACATGATCTGGGTCAT-3'	59	25	280
18sRNA*	S: 5'-CTCAACACGGGAAACCTCAC-3' AS: 5'-CGCTCCACCAACTAAGAACG-3'	58	-	110
CyclinD1*	S: 5'-ACAAACAGATCATCCGCAAACAC-3' AS: 5'-TGTTGGGGCTCCTCAGGTTC-3'	60	32	144

S: sense primer; AS: antisense primer

* The primers were used for real-time PCR

2.7.5 Agarose gel electrophoresis

PCR products were separated on 1.5 % or 2 % (w/v) agarose gels (2 % for expected fragment sizes of 250 bp or lower) containing 0.5 µg/ml ethidium bromide. Gels were run at a voltage of 90 V in 1 × TBE running buffer for 60 min. Bands were visualized on a UV-transilluminator at 312 nm.

2.7.6 Quantitative real-time reverse transcription-PCR

Real-time quantitative RT-PCR was performed with iCycler iQ Real time PCR detector system (BIORAD) using iQ™ SYBR® Green Supermix (BIORAD, Hercules, CA). The PCR reaction

was performed in a 96 well plate. Cycling conditions were as follows: initial enzyme activation at 95 ºC for 3 min, followed by 50 cycles at 95 ºC for 15 s; 58 ºC for 30 s; 72 ºC for 30 s. Relative expression levels of each gene in real time were analyzed using the $2^{-\Delta\Delta Ct}$ method and normalized to the expression of the housekeeping gene 18s rRNA. Each sample was replicated twice from 3 independent sets of RNA preparations. Data were presented as mean of fold change ± SEM vs the control.

2.8 Western blot analysis

Cells following different treatment were lysed with RIPA buffer. The proteins were treated with 4× sample buffer containing dithiothreitol and boiled for 10 min. An equal amount of protein (30 µg) was subjected to 12.5 % SDS polyacrylamide gel and separated proteins were transferred to NC membranes. The membranes were blocked in 5% skim milk for 1 h at room temperature. The immunoblots were incubated overnight at 4 ˚C with anti-AMPKα, anti-phosphorylated AMPKα (Thr^{172}) (both from Cell Signaling Technology, MA, USA), anti-ERK, and anti-phosphorylated ERK1/2 (Thr^{202} and Tyr^{204}) (both from Santa Cruz, CA, USA) antibodies in 5% BSA/TBST at a dilution of 1:1000, 1:1000, 1:10000, and 1:2000, respectively. Next day, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz, CA, USA) for 1 h at room temperature. The immunoreactive bands were detected with chemiluminescence substrate kit (ProteinSimple, CA, USA) under the Fluor Chem FC2 system.

2.9 Cell transfection with siRNA targeting the α1 and α2 catalytic subunits of AMPK

HTh74 and HTh74Rdox cells were transfected with siRNA targeting the α1 and α2 catalytic subunits of AMPK or a negative control (all siRNA from Qiagen) using Lipofectamine RNAiMAX (Invitrogen) as described by the manufacturer's instructions. Cell cultures were incubated for 24 h with various concentrations of siRNA before metformin treatment. Proteins were extracted 48h after addition of metformin. The expression of AMPK, mTOR, pmTOR (Ser^{2448}) (both mTOR and pmTOR antibodies are from Cell Signaling Technology, MA, USA) were detected by western blot as above.

2.10 Side population detection by flow cytometry after Hoechst 33342 staining

To isolate the thyroid cancer side population fraction, FACS was performed using the Hoechst staining method as outlined by Goodell et al. (83). Before Hoechst staining, the water bath was kept at precisely 37 °C, and DMEM+ medium (see below) was pre-warmed. Cancer cells were harvested, suspended at a density of 10^6 cells /ml in DMEM+ medium, and pre-incubated at

37°C for 10 min. The cells were then labeled in the same medium with 5 µg/ml Hoechst 33342 dye (Sigma, USA) at 37°C for 120 min with periodic agitation, either alone or in combination with 50 µM verapamil (Sigma, USA), an inhibitor of ABCG2 transporter. During the incubation, tubes were gently inverted every 30 min to prevent cell settling and clumping. Finally, the cells were centrifuged and resuspended in cold HBSS+ medium and counterstained with 1 µg/ml propidium iodide (PI) to exclude dead cells.

A 350-nm UV laser was used to excite Hoechst 33342 and PI. Analysis was performed on FACS LSRII flow cytometer (Becton-Dickinson Biosciences, Heidelberg, Germany) by using a dual-wavelength analysis (blue, 424-444 nm, red, 675 nm) in the facilities of the German Rheumatology Research Center, Berlin. PI-positive dead cells were excluded from the analysis. The side population was identified and selected by gating on the characteristic fluorescence emission profile.

Reagent solutions:

Hoechst 33342: Obtained from Sigma (called Bis-Benzimide) as a powder. Re-suspended at 1 mg/ml (stock concentration) in water, filter sterilized, and frozen in small aliquots.

HBSS+: Hanks Balanced Salt Solution (Gibco, Germany) with 2 % FCS and 10 mM HEPES buffer.

DMEM+: DMEM with 2 % FCS and 10 mM HEPES buffer.

Propidium iodide: Obtained from Sigma. Frozen stock is 10 mg/ml in water. Working stock (covered with aluminium foil and kept in the fridge) is at 200 µg/ml in PBS. Final concentration of PI in samples is 2 µg/ml.

2.11 Experimental equipment

2.11.1 Apparatus

FACS LSRII flow cytometer	Becton-Dickinson Biosciences
Laminar flow cabinet	Heraeus, Laminair HB 2448
Cell culture incubator	Heraeus
Phase contrast microscope	Nikon, TMS
Fluorescence microscope	Carl Zeiss
Digital camera	Sony DSC-W7
Water bath	Kottermann Labortechnik
UV Illuminator	Bachofer Laboratoriumsgeräte

UV camera	Polaroid MP4 Land Camera with Polaroid 545 4X5 film holder
Scanner	CanoScan 5000
Thermocycler	Biometra, Trio-Thermoblock
Spectrophotometer	NanoDrop, ND-1000, Biotechnologie
Autoclave	H+P Varioklav
Shake incubator	Infors HT
Precision scale	Advenurer OHAUS
Voltage generators	Consort, Electrophoresis power supply, E455
Homogenisator	Eppendorf Thermomixer 5436
Centrifuges	Hettich Mikro 200R
	Hettich Rotina 46R
Heating oven	Memmert
Pipets and tips	Eppendorf
Plastic centrifuge tubes	Sarstedt
Plastic culture dishes and plates	Sarstedt
Falcon tubes	Becton Dickinson
Polystyrene slide flasks	Nunc
Elisa machine	Biotek
	BIO-RAD Model 3550 Microplate reader
Fluor Chem FC2	Alpha Innotech
Single Color Real-Time PCR Detection System	BIO-RAD MyiQ™

2.11.2 Software

FACSDiva	Flow cytometry analysis
iCycler	Real-time PCR analysis
Fluo Chem FC2	Western blot analysis
Adobe photoshop CS3	Figure preparation
GraphPad Prism 5.0	Figure preparation and statistical analysis
SPSS 13.0	Statistical analysis

3 Results

3.1 Effect of metformin on proliferation

3.1.1 Metformin inhibited proliferation of thyroid cancer cells

The effects of metformin on thyroid cancer cell growth were investigated in anaplastic thyroid carcinoma cell lines HTh74, SW1736 and C643 and in follicular thyroid cancer cell line FTC133. As shown in Fig. 3.1, metformin significantly inhibited growth of thyroid carcinoma cells in a dose-dependent manner. The mean EC50 values in the 48 hours cell viability assays were approximately 21.21±4.60 mM for HTh74 cells, 11.56±6.07 mM for FTC133 cells, 19.85±7.12 mM for C643 cells and 38.90±9.79 mM for SW1736 cells indicating that FTC133 cells displayed the highest sensitivity to metformin (Fig. 3.1). The growth inhibitory effect of metformin was time-dependent. For HTh74, metformin had a maximum effect at 72 h (Fig. 3.1 A).

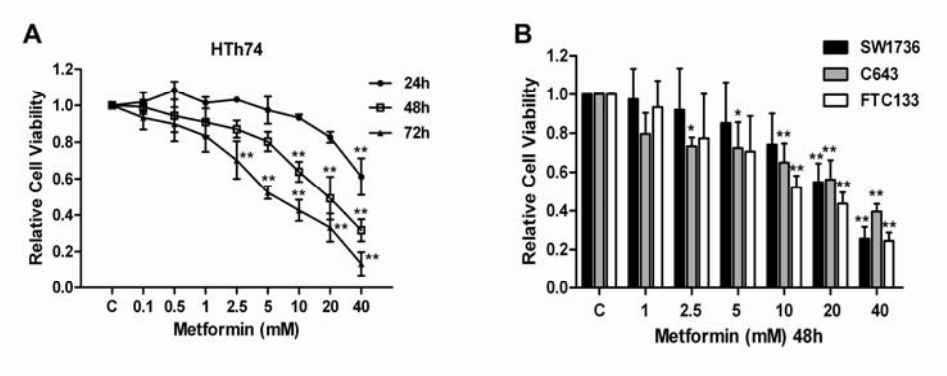

Figure 3.1 Metformin inhibited proliferation of thyroid cancer cells. Cells were plated in 96-well plates, after 24 hours, the culture medium was replaced with fresh medium or same medium containing increasing doses of metformin (0.1-40 mM) for 24-72 hours incubation. The percentages of surviving cells of each cell line in relation to controls, which were arbitrarily set to 1, were determined by MTT. Data show the representatives of at least three independent experiments done in six replicates. (C: control; vs control, * $P<0.05$, ** $P<0.01$)

Remarkably, metformin also inhibited growth of human thyrocytes, derived from goiters and of differentiated FRTL-5 rat thyroid cells (Fig. 3.2 A-B). However, a moderate but significant anti-proliferative effect of metformin on human thyrocytes and on FRTL-5 cells was only observed

after prolonged incubation for 48 h (FRTL-5 cells) - 72 h (primary thyrocytes), which corresponds to the slower growth rate of these differentiated thyrocytes (Fig. 3.2 C).

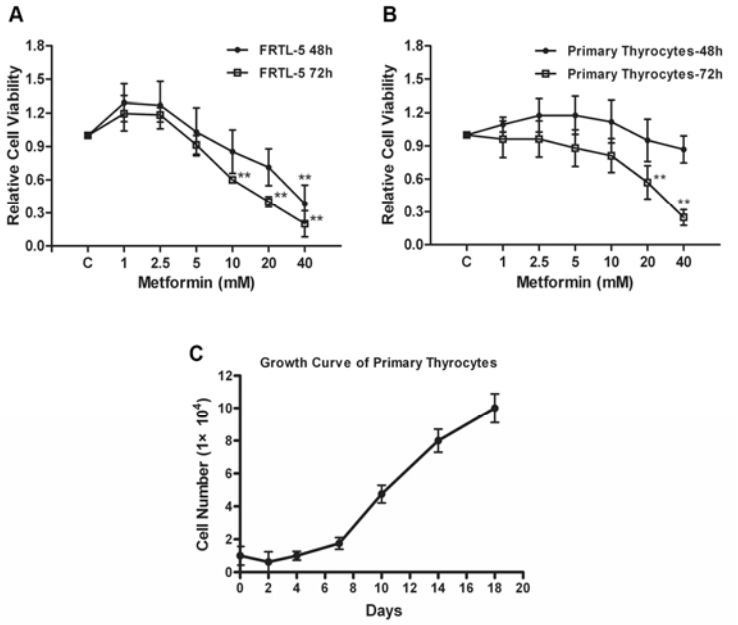

Figure 3.2 FRTL-5 cell line (A) and primary thyroid cells (B) were treated with metformin with indicated doses. MTT tests were performed after 48 h and 72h stimulation. Data shows the representative of at least three independent experiments done in quadruplicates. C: Growth curve of primary thyrocytes. Data were represented as mean ± SD of three independent experiments. (C: control; vs control, * $P<0.05$, ** $P<0.01$)

3.1.2 Metformin inhibited proliferation of CSCs enriched HTh74Rdox cells

The stable doxorubicin-resistant thyroid carcinoma cell line HTh74Rdox was established as described before (78). Flow cytometry of Hoechst staining demonstrated that about 70% of HTh74Rdox cells were detected as a side population cell fraction, which is enriched with cancer stem cells that express transporters of the ABC gene family.

After exposure to metformin with different concentrations for 24-72 h, HTh74Rdox cells displayed a significant decrease of cell viability (Fig 3.3). At each time point, the growth inhibitory effect on HTh74Rdox cells that are enriched with CSCs was more pronounced than in HTh74 cells (Fig. 3.1). The mean EC50 values in the 48 hours cell viability assay were only 8.92±3.12 mM for HTh74Rdox but 21.21±4.60 mM for HTh74. This indicates that HTh74Rox cells are more sensitive to metformin treatment.

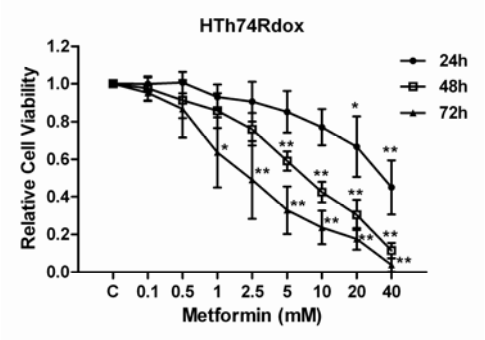

Figure 3.3 Metformin inhibited proliferation of CSCs enriched HTh74Rdox cells. HTh74Rdox cells were cultured with increasing doses of metformin (0.1-40 mM) for 24-72 hours incubation. The percentages of surviving cells in relation to control, was determined by MTT. Each data-point represents the mean of at least three independent experiments in six replicates. (C: control; vs control, * $P<0.05$, ** $P<0.01$)

Consistent with cell growth inhibition, microscopic examination revealed a significant decrease of cell density and change of cell morphology in metformin-treated HTh74 and HTh74Rdox cells, which showed a smaller, elongated and granulated shape (Fig. 3.4).

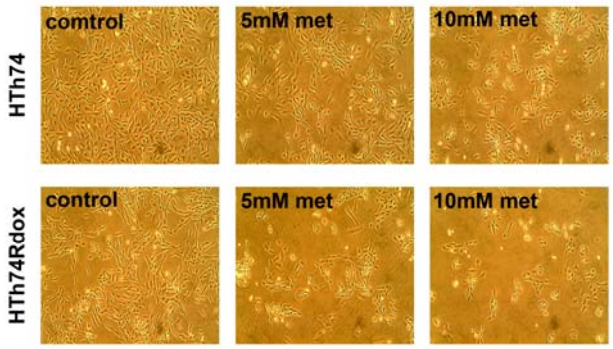

Figure 3.4 Morphology of HTh74 and HTh74Rdox cells after treated with metformin for 48h (10× magnifications).

3.2 Cell cycle arrest and induction of apoptosis

3.2.1 Metformin caused cell cycle arrest in G1 phase in thyroid cancer cells

To determine if metformin-induced decrease in cell viability is accompanied by cell cycle arrest, cell cycle progression was analyzed by flow cytometry after propidium iodide (PI) staining. Metformin treatment led to accumulation of cells in G1 phase with a consecutive decrease in the percentage of cells in S phase in HTh74 and HTh74Rdox cells (Fig. 3.5).

To determine whether metformin could induce changes in molecular signaling associated with the G1 checkpoint, expression of cyclin D1 mRNA was detected. As compared to controls, cyclin D1 mRNA was significantly reduced in metformin-treated cells (Fig 3.6). Since cyclin D1 plays a key role in promoting the G1-S cell cycle transition, a reduction in its expression may explain the growth inhibitory effect of metformin.

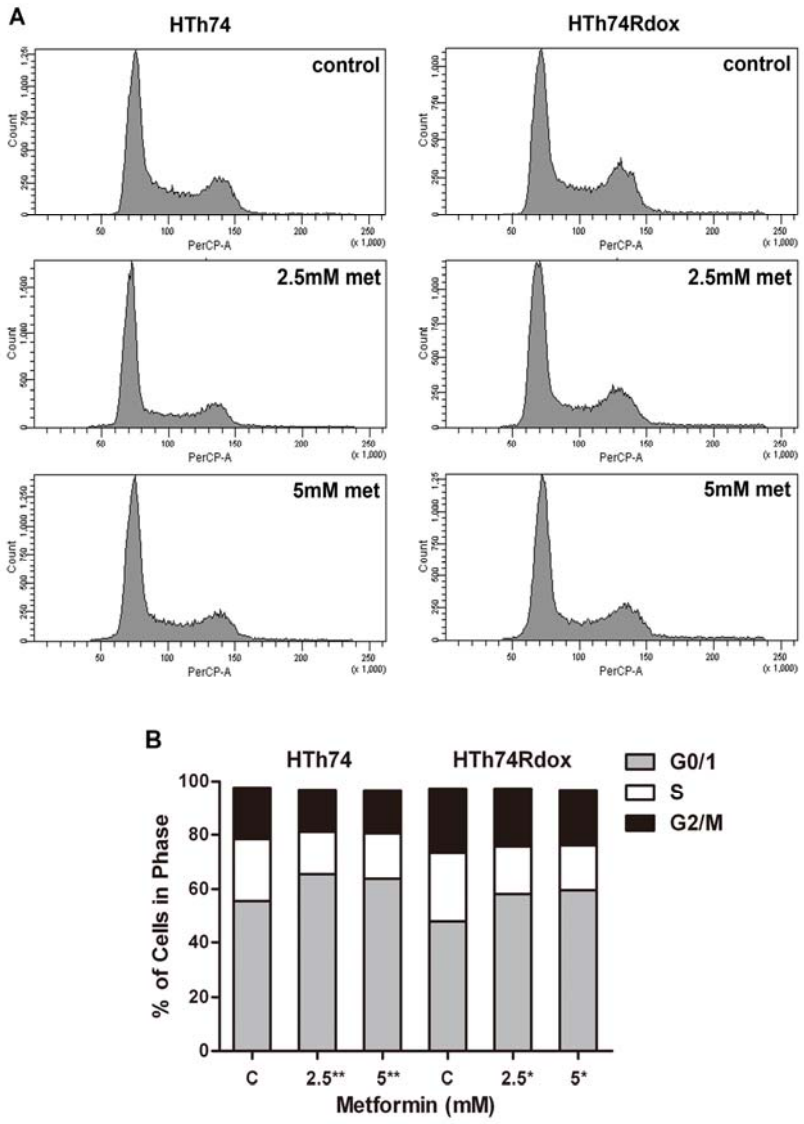

Figure 3.5 Metformin caused cell cycle arrest in G1 phase in thyroid cancer cells. A: HTh74 and HTh74Rdox cells were exposed to the indicated concentrations of metformin for 48 h. Cell cycle progression was analyzed by flow cytometry. B: Histogram shows cell cycle distribution of HTh74 and HTh74Rdox cell. (C: control; vs control, * $P<0.05$, ** $P<0.01$)

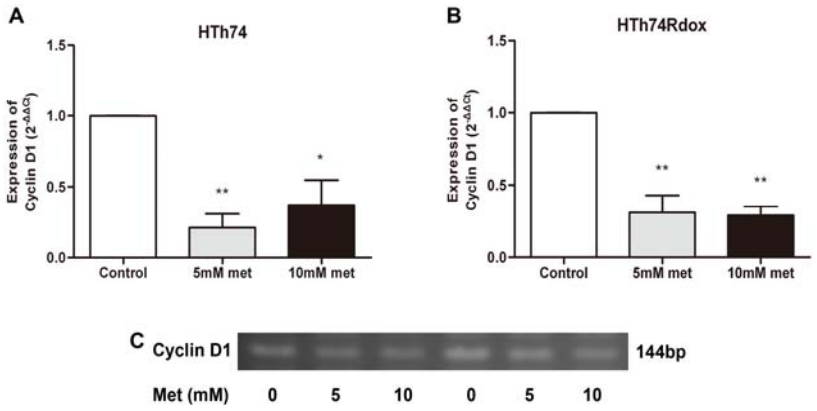

Figure 3.6 Metformin decreased cyclin D1 mRNA expression in thyroid cancer cells. A-B, Real-time PCR analysis of cyclin D1 mRNA expression in HTh74 (A) and HTh74Rdox (B) cells treated with various concentrations of metformin. The relative expression of cyclinD1 was calculated by the $2^{-\Delta\Delta Ct}$ methods. Data were presented as mean of fold change vs. control and were derived from at least three independent experiments. C: RT-PCR assays of cyclinD1 expression. (vs control, * $P<0.05$, ** $P<0.01$)

3.2.2 Metformin induced apoptosis in thyroid cancer cells

Next, cell apoptosis was detected by flow cytometry after double staining with annexin V and PI. Metformin enhanced apoptosis of both HTh74 and HTh74Rdox cells. Addition of 10 mM metformin increased the percentage of apoptotic and necrotic cells from 0.7% to 1.3% and 3.5% in HTh74 cells and HTh74Rdox cells, respectively (Fig. 3.7 A).

In addition, metformin-induced apoptosis was assayed by detection of caspase-3 activity in HTh74 and HTh74Rdox cells. In response to metformin (5 mM or 10 mM for 24 h), caspase-3 activity significantly increased by 19.5% and 26.8% in HTh74 cells and 26.9% and 34.1% in HTh74Rdox cells, respectively (Fig. 3.7 B), indicating a more pronounced anti-apoptotic effect of metformin in HTh74Rdox than in HTh74 cells. This indicates a slightly higher pro-apoptotic effect of metformin in HTh74Rdox than HTh74 cells. In comparison to caspase-3 assay, flow cytometry of phosphatidylserine externalization indicated a more pronounced induction of apoptosis by metformin in HTh74Rdox than HTh74 (Fig. 3.7 A). This maybe explained by apoptotic pathways independent of caspases which account for induction of up to 50% of apoptosis (84).

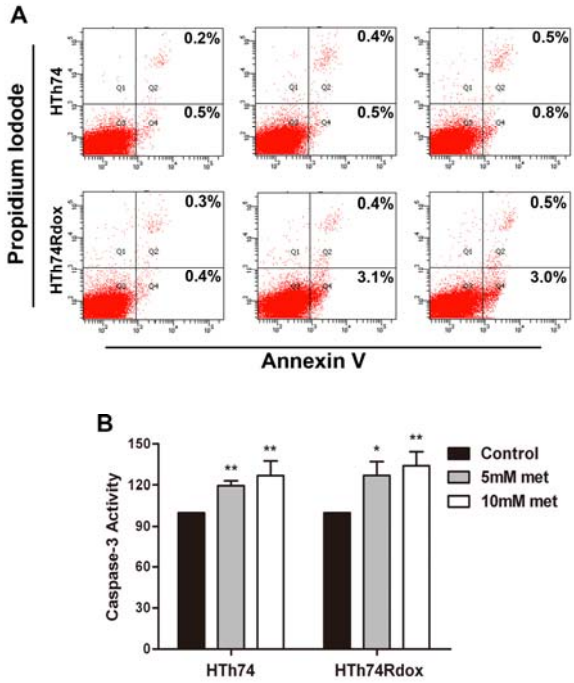

Figure 3.7 Metformin induced apoptosis in thyroid cancer cells. A: Apoptosis of HTh74 and HTh74Rdox cells in response to metformin (left to right: 0, 5 mM, 10 mM for 24 h) were determined by annexin V/propidium iodide staining. The percentage of early apoptosis (bottom right quadrangle) and late apoptosis/necrosis (top right quadrangle) are indicated. M10 vs control, $P<0.05$ (in both HTh74 and HTh74Rdox cells) B: Relative caspase-3 activity of HTh74 and HTh74Rdox cells after the treatment with metformin for 24 h. Activity of caspase-3 in untreated cells was set as 100%. (C: control; vs control, * $P<0.05$, ** $P<0.01$)

3.3 Inhibition of colony and sphere formation in response to metformin

3.3.1 Metformin inhibited colony formation of thyroid cancer cells

The self-renewal capacity of HTh74 and HTh74Rdox cells was analyzed by clonal formation assay. As shown in Fig. 3.8, HTh74Rdox cells were more clonogenic than HTh74 cells (48% vs 7%, $P<0.05$). After treatment with metformin, the number of HTh74Rdox colonies formed was reduced in a dose-dependent manner (Fig. 3.8). Metformin was already effective at concentrations as low as 0.5 mM. At the concentration of 5 mM, it suppressed almost 90% of HTh74Rdox colony formation as compared to untreated controls.

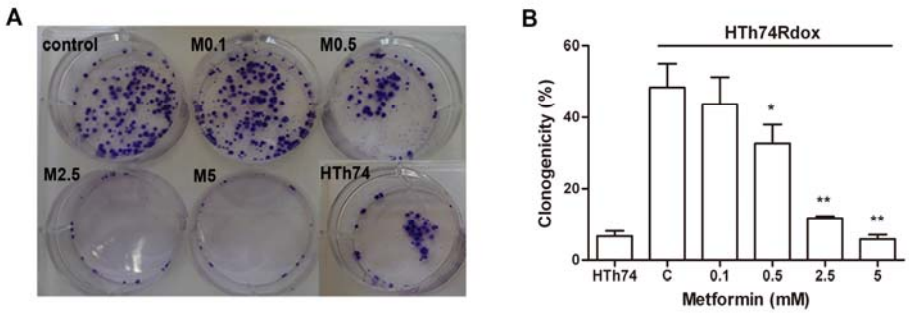

Figure 3.8 Metformin inbibited colony formation of thyroid cancer cells. A: Cells were grown in six-well plates (200 cells/well) in triplicates. After 24 hours, the culture medium was replaced with fresh medium containing 0.1 mM, 0.5 mM, 2.5 mM, 5 mM metformin or 2% FBS as control. After 14 d, the colonies were stained with Giemsa and counted. B: Histogram of colony formation ability. Data shows the representative of three independent experiments. (C: control; vs control, * P<0.05, ** P<0.01)

3.3.2 Metformin decreased sphere formation efficiency of thyroid cancer cells

Since HTh74Rdox cells are enriched with cancer stem cells (78), the effect of metformin on doxorubicin-resistant cancer stem cells was analyzed by sphere formation assay. Both HTh74 and HTh74Rdox cells cultured in sphere medium for 7 days formed tumor spheres, however, HTh74Rdox derived spheres formed larger and irregular structures. When the medium was supplemented with increasing doses of metformin, sphere formation efficiency was significantly lower in both cell lines, with about 38% and 67% reduction at concentration of 5 mM, and 66% and 76% reduction at concentration of 10 mM in HTh74 and HTh74Rdox, respectively (Fig. 3.9).

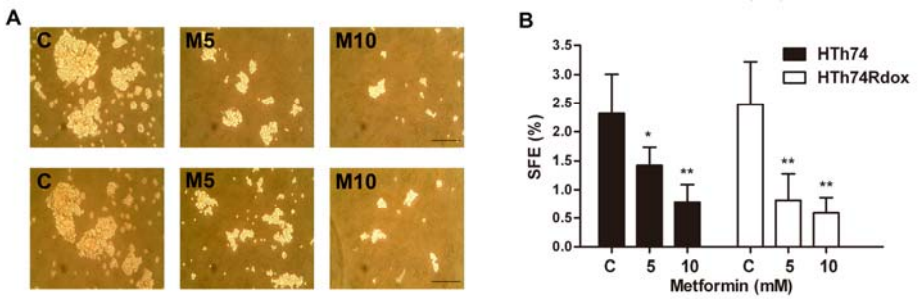

Figure 3.9 Metformin decreased sphere formation efficiency of thyroid cancer cells. A: Figure shows representative spheres formed by HTh74 (up) and HTh74Rdox (down) cells growing in sphere medium for 7 days in the absence or presence of 5 mM or 10 mM metformin, as specified (10× magnifications). B: Sphere-forming efficiency (SFE) of HTh74 and HTh74Rdox cells were calculated as the number of spheres formed in 7 days divided by the original number of cells

seeded and expressed as percentage means ± SD. Data shows the representative of three independent experiments. (C: control; vs control, * P<0.05, ** P<0.01; Scale bars: 100μm)

3.4 Synergistic effect of metformin with other agents

3.4.1 Additive anti-proliferative effect with doxorubicin or cisplatin

As depicted in figure 3.10 the combination of metformin with either doxorubicin or cisplatin was more effective than each agent alone. Whereas metformin inhibited cell viability of doxorubicin-resistant HTh74Rdox cells by 60% (Fig. 3.3) but doxorubicin did not (Fig. 3.10), addition of metformin plus doxorubicin further decreased cell viability which indicates that doxorubicin-resistance can be overcome by metformin (Fig. 3.10). However, in cells pretreated with metformin higher concentrations of doxorubicin (HTh74 cells) or cisplatin (both HTh74 and HTh74Rdox cells) did not further decrease cell viability.

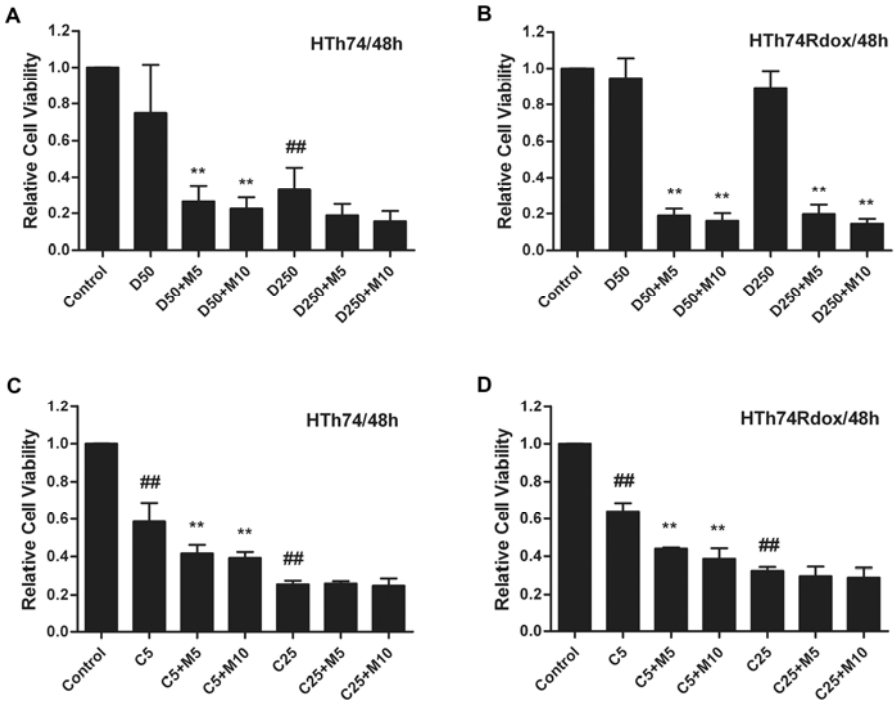

Figure 3.10 Synergistic effect of metformin and chemotherapeutic agents on proliferation of thyroid cancer cells. HTh74 and HTh74Rdox cells treated with doxorubicin (50 ng/ml, 250 ng/ml) or cisplatin (5 μM, 25 μM) with or without metformin (5 mM, 10 mM) for 48 hours. The results are expressed as percentage of viable cells compared with the control. The data represents at least three separate experiments. (##, vs control, P<0.01; **, vs D50, D250, or C5, P<0.01)

3.4.2 Effect of metformin on insulin-stimulated cell proliferation

To investigate whether metformin has an effect on insulin stimulated cell growth in thyroid cancer cells, HTh74 and HTh74Rdox cells were incubated with insulin in the absence or presence of metformin. Insulin significantly stimulated cell growth of HTh74 and HTh74Rdox cells. However, when metformin was added, the insulin-induced increase of cell proliferation was almost abolished (Fig. 3.11).

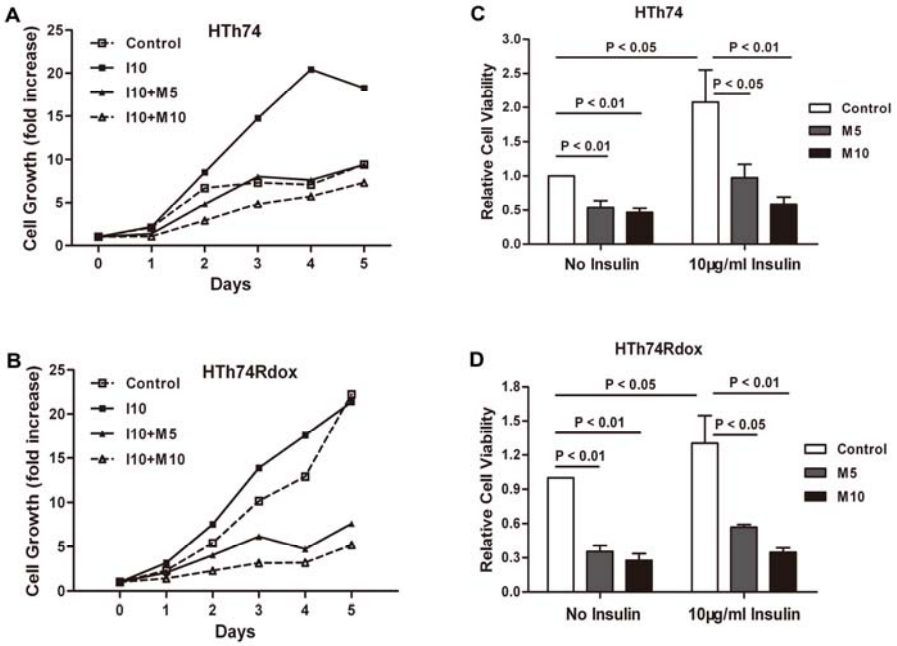

Figure 3.11 Metformin inhibited insulin-stimulated proliferation of thyroid cancer cells. HTh74 (A) and HTh74Rdox (B) cells were cultured with 10 μg/mL insulin (I10) or without (control), or a combination of insulin and 5 mM or 10 mM metformin (I10+M5, I10+M10). C-D: Cell viability of HTh74 and HTh74Rdox cells after treated with metformin with or without insulin for 72 hours. Similar results were obtained in three independent experiments.

3.5 Effect of metformin on the side population of HTh74Rdox cells

Our previous study established a stable doxorubicin-resistant cell line and proved that the resistant cell line consisted of a 80% side population (SP) fraction enriched with Oct4-positive cancer stem cells (78). To investigate the effect of metformin on the proportions of SP and non-SP cells of the HTh74Rdox cell line, the SP content of HTh74Rdox cells was analyzed using flow cytometry with Hoechst 33342 dye staining. In response to 10 mM metformin for 48 h, there was a slight but not significant decrease of the proportion of SP cells (Fig. 3.12). This suggests that metformin has the equivalent potency to kill thyroid cancer cells and thyroid cancer stem cells, i.e. it decreased the absolute number of cells but not its proportion.

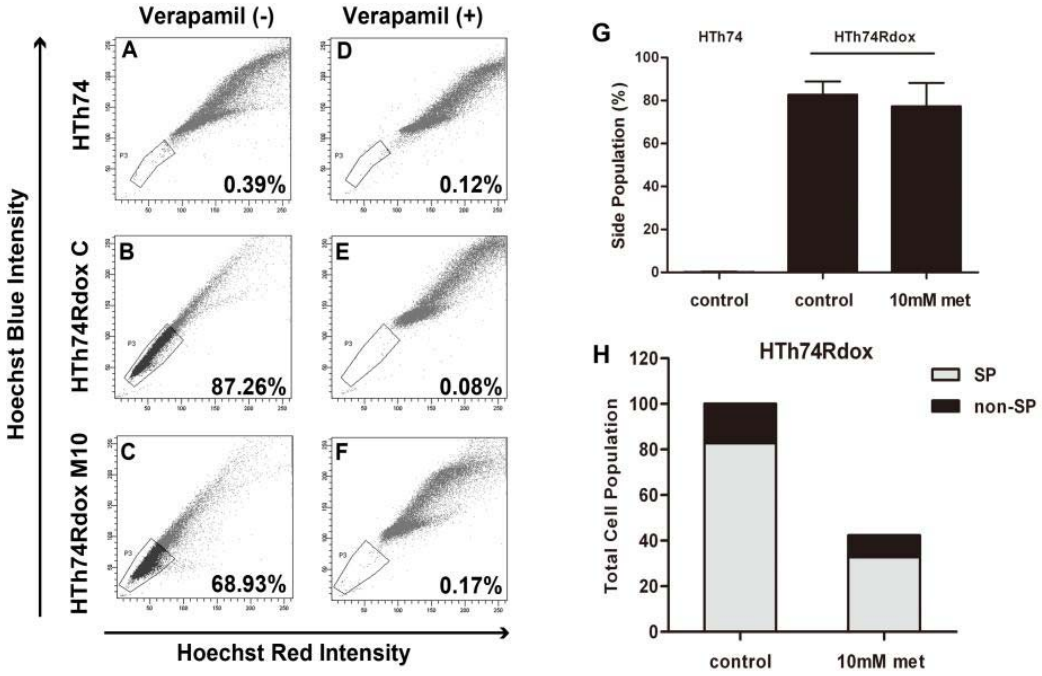

Figure 3.12 Effect of metformin on the proportion of side population (SP) cells and non-side population cells in HTh74Rdox cells. HTh74 and HTh74Rdox cells treated with metformin for 48 h were labeled either with Hoechst 33342 dye alone (A-C) or in combination with verapamil (D-F) for 120 min and then analyzed by FACS. The SP cells (A-C), which disappeared in the presence of verapamil (D-F), were outlined and shown as a percentage of the gated cell population. G: Bar graph represents the mean percentages of SP ± SD (n = 6). H: Metformin decreased the total cell numbers but did not change the ratio of side population and non-side population cells in HTh74Rdox cells.

3.6 Metformin targeted insulin/IGF signaling and AMPK pathway

To evaluate the specific effect of metformin on insulin-dependent MAP kinase pathway, phosphorylation of extracellular signal-regulated kinase (ERK) was analyzed in HTh74 and HTh74Rdox cells. Western immunoblot analysis revealed that metformin dramatically decreased the phosphorylation of ERK in a dose-dependent manner (Fig. 3.13 B). When metformin was added together with insulin, it abolished the increase in phosphorylation of ERK induced by insulin (Fig. 3.13 C). In addition, metformin induced phosphorylation of AMPK at Thr-172 (Fig. 3.13 A) in HTh74 and HTh74Rdox cells. However, insulin did not phosphorylate AMPK in these cells (data not shown).

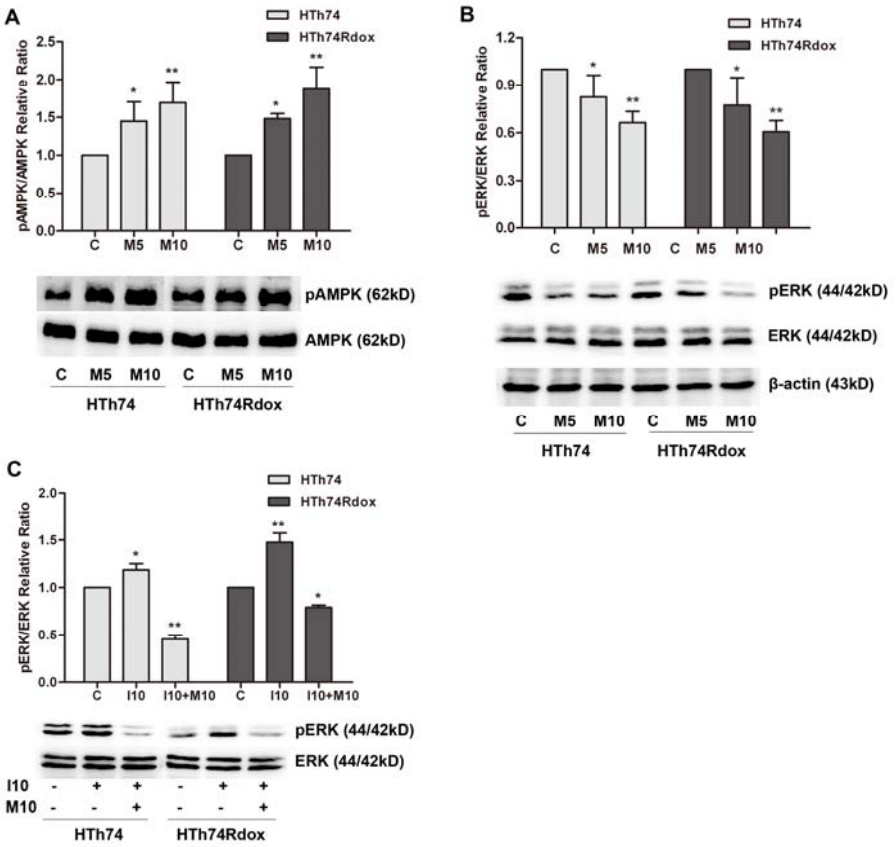

Figure 3.13 Metformin decreased phosphorylation of ERK and increased phosphorylation of AMPK. A-B: HTh74 and HTh74Rdox cells were treated with metformin (0, 5 mM and 10 mM) for 24 h, AMPK phosphorylation at Thr^{172} and ERK phosphorylation at Thr^{202} and Tyr^{204} were analyzed by western blot as indicated in Materials and Methods. Similar results were obtained in three independent experiments. C: HTh74 and HTh74Rdox cells were treated with insulin (10 μg/ml) or/and metformin (10 mM) for 24 h. Then cell lysates were analyzed for ERK phosphorylation at Thr^{202} and Tyr^{204}.

3.7 The antiproliferative effect of metformin is also mediated by AMPK/mTOR pathway

To analyze if the growth-inhibitory effect of metformin is also mediated by activation of the AMPK signaling way, the pathway was blocked using siRNA directed against the α1 and α2 catalytic subunits of AMPK. SiRNA interference decreased the expression of the AMPK proteins (Fig. 3.14 A). Activation of AMPK by metformin resulted in a diminished phosphorylation of mTOR, a downstream target of AMPK pathway. However, prior transfection of cell lines with specific siRNA reduced the effect of metformin on phosphorylation of mTOR, whereas pre-treatment with control siRNA did not affect it (Fig. 3.14 B-C).

Finally, we investigated if blocking of AMPK/mTOR pathway affects cell growth after metformin treatment. As revealed by MTT assay, transfection of HTh74 and HTh74Rdox cells with α1 and α2 siRNA slightly increased metformin-inhibited growth (Fig. 3.14 D-E). This indicates that the antiproliferative effect of metformin partly depends on activation of the AMPK/mTOR pathway.

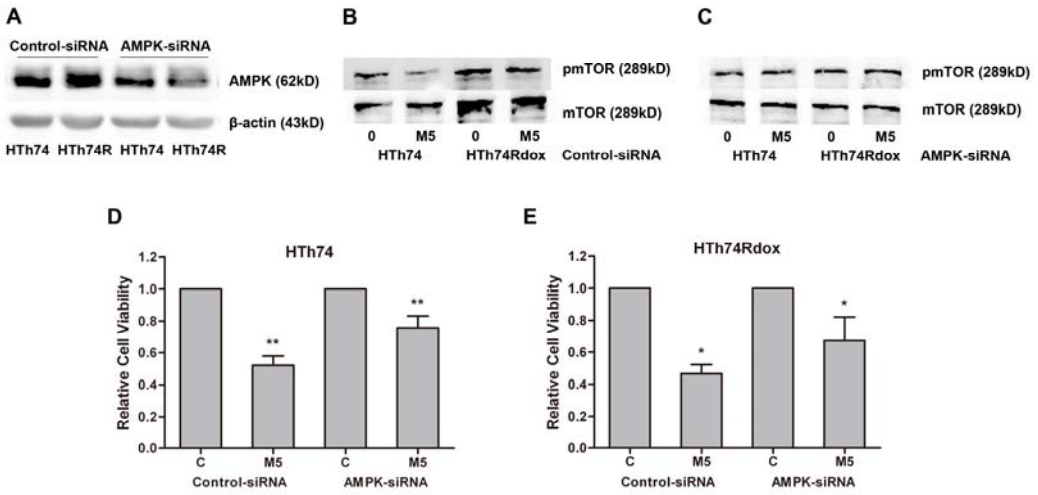

Figure 3.14. Metformin inhibited phosphorylation of mTOR, while AMPKα1 and α2 siRNA partly recover pmTOR expression and in part rescue HTh74 and HTh74Rdox cells from the inhibitory effect of metformin. Cells were transfected with various concentrations of AMPK α1 and α2-siRNA or with control siRNA using Lipofectamin RNAiMAX. Twenty-four hours after transfection, cells were treated with metformin for 48 hours. Protein expression of mTOR and pmTOR was detected by Western blots (B-C). Cell proliferation was measured by MTT assays (D-E), differences between the metformin treated cells transfected with AMPK α1 and α2-siRNA and control siRNA were significant. (*, $P<0.05$; **, $P<0.01$)

4 Discussion

It is well established that type 2 diabetes and obesity are associated with an elevated cancer risk and cancer-related mortality (16, 30, 31). Type 2 diabetes is characterized by insulin resistance and hyperinsulinemia. Most obesity patients also present with insulin resistance. Hyperinsulinemia is generally considered as the possible link between type 2 diabetes and cancer. Aside from its metabolic effects, insulin also has mitogenic effects that are mediated through the insulin receptor and IGF-1 receptor (as shown in Figure 1.1). Insulin is considered to be a potent growth factor that binds, albeit with a lower affinity, to the IGF receptor, a tyrosine kinase receptor for IGF-I and IGF-II, that plays a key role in malignant transformation, tumor progression and metastasis in different malignancies (85). The well documented relevance of insulin/IGF signaling for benign and malignant thyroid growth (86) and the recent finding of a higher incidence of thyroid nodules and differentiated carcinoma in type 2 diabetes with insulin resistance and consecutive hyperinsulinemia (33, 34) prompted us to analyze the effect of metformin, an anti-diabetic drug, on differentiated thyroid cells, undifferentiated thyroid carcinoma cells and their derived cancer stem cells.

An anti-mitogenic effect of metformin has been suggested by in vitro and epidemiological studies (48, 64, 71, 73). Furthermore, retrospective analysis of data from neo-adjuvant breast cancer therapy indicated that metformin may potentiate the effect of chemotherapy (73). In line with this finding, a recent population study and a retrospective cohort study demonstrated that metformin treatment is associated with lower cancer mortality in type 2 diabetes (87, 88). Metformin directly inhibits cell growth by activation of AMP kinase that occurs through activation of liver kinase B1 which results in the inhibition of mammalian target of rapamycin (mTOR) pathway, a major growth regulatory signaling way (62). MTOR signaling is frequently activated in many human cancers (89-92).

In the present work it was shown that metformin exerted a dose- and time-dependent growth-inhibitory effect on slowly proliferating differentiated human thyroid cells, FRTL5 rat thyroid cells and in a more pronounced manner on various undifferentiated thyroid carcinoma cell lines (Figure 3.1-3.2). Corresponding to the lower growth velocity of primary thyroid cells, a significant anti-mitogenic effect of metformin was not observed before 72 h of treatment in these cells, whereas in carcinoma cells metformin was already effective after 24-48 h in culture. Also the sensitivity to metformin treatment was higher in carcinoma cells with the lowest EC50 of

only 8.92±3.12 mM in doxorubicin-resistant HTh74Rdox cells. Among the wild-type carcinoma cell lines, the lowest EC50 of metformin was detected in FTC133 cells, followed by C643 and HTh74 cells (Figure 3.1-3.2).

It was initially demonstrated that metformin was capable of reducing proliferation in breast, colon, and prostate cancer cell lines through cell cycle arrest as demonstrated by a decrease of cyclinD1 protein level (61). However, there are no data available to date on the metformin effect on thyroid cancer cells. In the present work, it was shown that metformin treatment resulted in a significant reduction of cell viability in undifferentiated (HTh74, SW1736, C643) and differentiated (FTC133) thyroid carcinoma cell lines. Furthermore, a moderate effect on cell viability in primary human thyroid cells and FRTL-5 rat thyroid cell lines was observed, which corresponds to a report on normal prostate epithelial cells by Ben Sahra et al. (93). In contrast, Isakovic et al. (82) reported that rat primary astrocytes were completely resistant to the anti-proliferative action of metformin, whereas glioma cell lines U251 and C6 were sensitive to metformin.

The stable doxorubicin resistant thyroid anaplastic cancer cell line (HTh74Rdox) was established by our group after long-term culture with increasing amounts of doxorubicin, stepwise for more than 6 months. The EC50 of HTh74Rdox cells was 85-fold higher than that of parental HTh74 cells. HTh74Rdox cells consisted of about 70% side population which is considered to be enriched with cancer stem cells and be responsible for doxorubicin resistance (78).
In this study, metformin not only inhibited the growth of various thyroid carcinoma cell lines, but also reduced the proliferation of doxorubicin resistant HTh74Rdox cells. The effect was both dose- and time-dependent as in other thyroid carcinoma cell lines. However, the inhibition was much stronger than in the parental HTh74 cell line (Figure 3.1 and 3.3). This intriguing result that metformin also targets cancer stem cells may provide a new strategy of cancer treatment and prevention of relapse.

To study the biological mechanisms by which metformin inhibits cell growth, the effect on cell cycle progression and cell apoptosis of thyroid carcinoma cell line was evaluated. Metformin reduced cell viability of thyroid carcinoma cells by dual cellular action. On the one hand, it blocked the cell cycle progression via G_1 phase arrest in non-confluent cultures of proliferating cells, accompanied with a decrease of cyclinD1 mRNA expression (Figure 3.5-3.6). On the other

hand, it induced apoptosis through caspase-3 activation (Figure 3.7). The proapoptotic effect is believed to be a mitochondria-dependent process (82).

The cell cycle arrest effect of metformin is not surprising, given that it may activate AMP kinase, further inhibiting mTOR, which controls cell growth and proliferation as discussed in details later. In vitro studies have shown that metformin arrests cells in G1 phase in a number of different cancer cells, including endometrial, ovarian, breast, prostate cancer cells and glioma cells. But it is debatable whether metformin may also induce apoptosis. In the endometrial cancer cells, metformin was able to induce apoptosis but only at high concentrations of treatment (2mM and 5mM) (66). In contrast, metformin failed to induce apoptosis in prostate and breast cancer cells at a similar dose of treatment but did block cell cycle progression at G1 phase (60, 93). However, metformin was found to induce apoptosis in colon cancer cells but only in the cells that lacked the tumor suppressor p53 (63). Furthermore, in glioma cells, metformin was able to induce mitochondria-dependent apoptosis (82). In the present work, a significant apoptosis after metformin treatment (5mM, 10mM) was also observed. Discrepancy between different findings may be due to the concentrations of metformin used and/or may indicate distinct mechanisms regulating cell processes in different cells in response to metformin.

In the present study, metformin activated AMPK by phosphorylation at Thr^{172} in HTh74 and HTh74Rdox cells (Figure 3.13A) and inhibited phosphorylation of mTOR (Figure 3.14B), a downstream target of AMPK pathway, which is increased in many human cancers (89). Metformin also inhibited insulin/IGF signaling. As shown in Figure 3.13C, insulin increased phosphorylation of ERK, a key enzyme of MAP kinase pathway, whereas metformin decreased phosphorylation and abolished the insulin effect.
Transfection of thyroid cancer cells with siRNA directed against the α1 and α2 catalytic subunits of AMPK inhibited AMPK activation and thus diminished the metformin-induced inhibition of mTOR (Figure 3.14). From these data it is evident that metformin exerts its growth-inhibitory effect both via insulin/IGF signaling and AMPK/mTOR pathway.

The molecular mechanism of growth inhibitory effect of metformin on cancer cells can be explained by two ways: one is insulin-dependent and the other insulin-independent. Both mechanisms of metformin have been linked to activation of the AMP-activated protein kinase (AMPK) (94). AMPK is a heterotrimer serine/threonine protein kinase which is composed of a catalytic subunit α, and regulatory subunits β and γ. AMPK regulates energy metabolism and is

activated by an increase in the intracellular ratio of AMP/ATP. Activation of AMPK requires an allosteric change induced by AMP, as well as phosphorylation on Thr^{172} within the catalytic domain of the α subunit (95, 96). The upstream LKB1 is the kinase responsible for phosphorylation of AMPK, and its activity is required for AMPK activation in response to energy stress in cell culture (97-99). LKB1 is a serine/threonine kinase previously described as a tumor suppressor gene whose inactivation leads to Peutz-Jeghers syndrome, a rare autosomal syndrome characterized by colorectal polyps and a significantly increased lifetime risk of various epithelial cancers, including the testes, colon, and breast (100, 101).

AMPK, which is also activated by weight loss, hypoxia, and physical activity; once activated, it phosphorylates a number of effector proteins leading to the activation of ATP-generating pathways (glycolysis, fatty acid β oxidation), and the inhibition of ATP-consuming pathways (glucoeogenesis, protein and fatty acid synthesis, cholesterol synthesis) (95). A direct consequence of AMPK activation is the inhibition of the mTOR pathway via phosphorylation and activation of tuberous sclerosis complex 2 (TSC2) (102), a subunit of the TSC1/TSC2 (hamartin/tuberin) complex that negatively regulates mTOR signaling (103). mTOR upregulates many energy consuming cellular processes and has a central role in regulating cell growth by controlling mRNA translation and ribosome biogenesis (104). Therefore, the inhibition of mTOR via AMPK activation represents a novel approach for the treatment of cancer (105). Metformin has been proposed to be a promising candidate for inhibition of cell proliferation through AMPK/mTOR axis. Figure 5.1 summarises the mechanism of the effect of metformin on cancer cells.

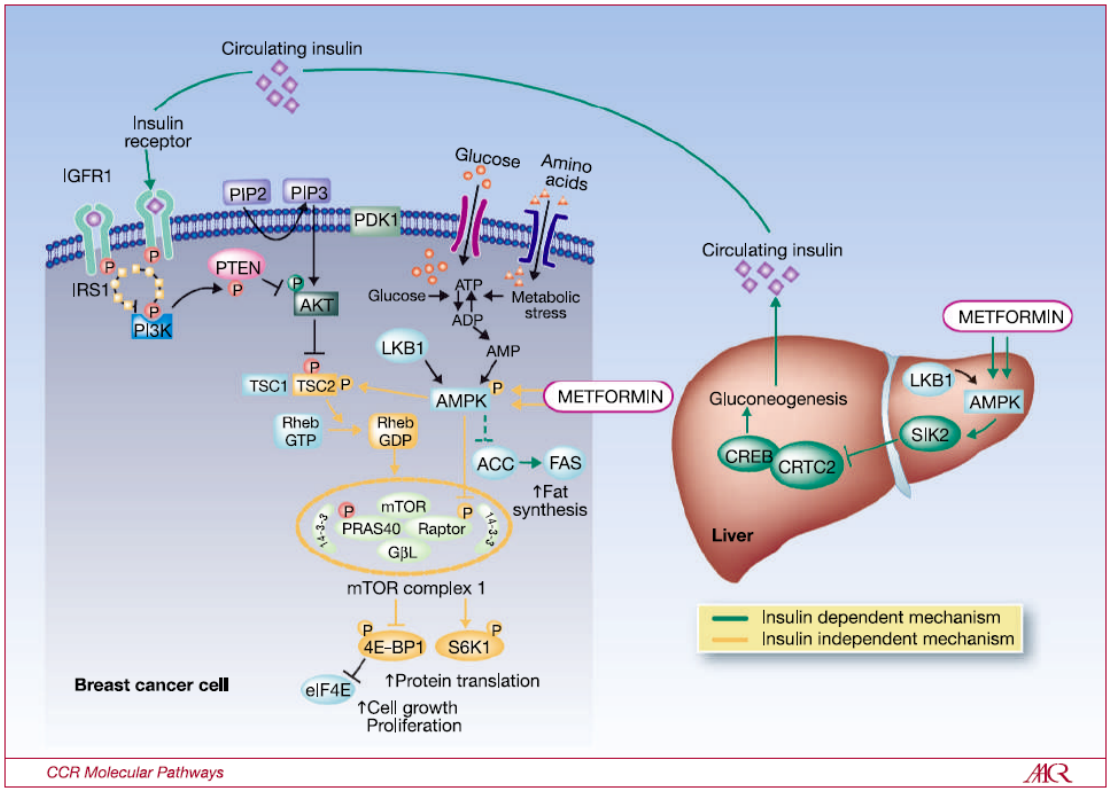

Figure 5.1 Mechanisms of metformin action. Metformin exerts its cell growth-inhibitory effects through two distinct mechanisms: a direct mechanism that inhibits the mTOR pathway and an indirect mechanism that depends on insulin levels. Metformin activates AMPK, the cellular energy sensor. Activation of AMPK leads to suppression of many of the processes highly dependent on ATP. It inhibits transcription of gluconeogenesis genes in the liver and increases glucose uptake in skeletal muscle. Thus it reduces the levels of circulating glucose, increases insulin sensitivity, and reduces the hyperinsulinemia associated with insulin resistance. In cancer cells, the mTOR signaling pathway promotes cell growth and proliferation by interplay of two opposing upstream pathways involving the Akt pathway, which signals availability of nutrients, and the AMPK pathway, which signals lack of energy. The AMPK pathway exerts inhibitory effects on mTOR via phosphorylation of TSC2 and raptor similarly to the Akt pathway, which exerts stimulatory effects via phosphorylation of TSC2 and PRAS40. (Ana M. Gonzalez-Angulo and Funda Meric-Bernstam, Clin Cancer Res; 2010, 16(6); 1695–700.)

For any disease, understanding of the pathogenesis is a prerequisite to adopting effective and rational strategies of treatment. However, the exact mechanisms of tumourigenesis are still not completely understood. Some evidence suggests that a small population of tumor cells have stem-like properties. This has lead to the evolution of the cancer stem cells (CSCs) hypothesis which states that the tumour initiation, progression, metastasis and recurrence are related to a

small population of cancer stem cells. With the technological advancement in isolating and identifying single cells, the cancer stem cell hypothesis has been emphasized. With new techniques, the existence of CSCs in hematopoietic malignancies and in solid tumors such as brain, breast, lung, and thyroid cancer was confirmed (106-109). These cancer stem cells, also termed tumour-initiating cells (110), have the capability of self-renewal and giving rise to phenotypically diverse progeny similar as normal stem cells.

Clonal growth and sphere formation are a hallmark of cancer stem cells including thyroid cancer stem cells (78, 111). Clonogenic formation assay that measures the potency to form single cell-derived clones in culture is a useful method to estimate self-renewal ability. It was used in many normal and malignant cell lines (112-116), since it provides a reliable method for the identification and isolation of cells with stem cell properties from tumors or cancer cell lines and is capable of characterizing CSCs responses to various factors and therapeutic agents.
Sphere formation assay culture cancer cells as non-adherent, three-dimensional spheres in the serum-free medium containing EGF and bFGF. This technique has been proposed as a reliable method for enriching sub-populations of putative cancer stem cells (117-119). In this culture, the small fraction of cancer cells is able to form multi-cellular “microtumors” in non-adherent and non-differentiating conditions. Thyrosphere culture provides a highly appropriate model for studying the sensitivity of the cancer stem cells within a heterogeneous thyroid cancer population to therapeutic agents. In breast cancer cells, it has been shown that mammosphere number reflects stem cell self-renewal, whereas mammosphere size reflects progenitor cell proliferation (118, 120, 121).

As shown in the present work, HTh74Rox cells were more clonogenic than HTh74 cells: only about 7% of total HTh74 cells grew as colonies, whereas about 50% of the HTh74Rdox cells sustained a clonal growth and formed characteristic compact circular colonies with a cobblestone appearance (78). In the present study, it was demonstrated that metformin significantly reduced clonal growth of HTh74Rdox thyroid carcinoma cells (Figure 3.8). In addition, metformin inhibited sphere formation and reduced sphere size in HTh74 and HTh74Rdox thyroid cancer cells which reflects suppression of self-renewal and proliferation of cancer stem cells. The inhibitory effect was only slightly higher in doxorubicin-resistant cells than in HTh74 wild-type cells (Figure 3.9), which corresponds to a recent report on the effect of metformin on sphere formation and size in resistant and wild-type breast cancer cells (121).

Many currently available chemotherapeutic agents can shrink tumour size but often only transiently, since existing therapies may kill the bulk of cancer cells but may fail to eradicate cancer stem cells that are more resistant to chemotherapeutics (112). Reversing drug resistance is one of the major issues in the treatment of malignant tumors. Another major problem of tumor treatment is the high toxicity of some chemotherapeutic drugs. Thus, search for any agents that can be combined with lower doses of the existing chemotherapeutic drugs is of high clinical relevance.

Several studies have considered metformin as such a compelling agent. Most achievement in this area came from studies on breast cancer cells (60, 64, 122). In 2009, Hirsch and his colleagues reported that in genetically distinct breast cancer cell lines, metformin selectively inhibited the growth of chemotherapy resistant cancer stem cells. Furthermore, in mouse xenografts involving a human breast cancer cell line, co-injection of metformin and doxorubicin intraperitoneally (i.p.) was more effective than either drug alone in blocking tumor growth and preventing relapse (70). In 2011, the same group showed that metformin, also given orally, works together with a variety of standard chemotherapeutic agents, such as paclitaxel and carboplatin. In addition, metformin has comparable effects on tumor regression and preventing relapse when combined with a fourfold reduced dose of doxorubicin that is not effective as a monotherapy. Furthermore, these authors proved that the combination of metformin and doxorubicin also prevents relapse in xenografts generated with prostate and lung cancer cell lines (69). Very recently, others confirmed that metformin synergistically interacts with trastuzumab (Tzb) to suppress self renewal and proliferation of Tzb resistant (HER2-positive carcinoma cells showing acquired and de novo resistance to Tzb) tumor-initiating cells (121).

In the present work, an additive anti-mitogenic effect of metformin was observed when thyroid carcinoma cells were treated with either doxorubicin or cisplatin (Figure 3.10). This points to an important supportive effect of metformin and may offer an option for the adjuvant treatment of dedifferentiated thyroid cancer, also for emerging the new therapies (123, 124). Interestingly, when doxorubicin-resistant HTh74Rdox cells were treated with metformin, cells became again sensitive to doxorubicin with a significant decrease of cell viability (Figure 3.10B). However, the additive anti-mitogenic effect of metformin and doxorubicin or cisplatin had a maximum at 50ng/ml and 5μM, respectively (Figure 3.10). A further increase of chemotherapeutic doses did not affect cell viability. These data suggest that addition of metformin may save chemotherapeutic drugs and thus reduce their putative toxicity of high concentrations. This

protective effect of metformin has also been reported by Janjetovic et al. in cisplatin-mediated apoptotic death of cancer cells (125).

The doxorubicin-resistant thyroid carcinoma cell line HTh74Rdox, as previously established in our lab, overexpresses the stem cell marker Oct4 and genes of the ABC transporters (ABCG2, MDR1), which export doxorubicin out of the cells and thus confer doxorubicin resistance to these carcinoma cells (78). It has been proven that more than 80% of these cells express these resistance transporters and are identified as side population cells that are enriched with cancer stem cells. In the present study, when the doxorubicin-resistant cell line was treated with metformin, cell viability was further decreased (Figure 3.3). However, the relative proportion of side population cells enriched with cancer stem cells and the major population of thyroid carcinoma cells remained almost unchanged (Figure 3.12). This indicates that metformin inhibits growth of both cancer cells and cancer stem cells to a comparable extent. This is in contrast to recent findings of Hirsch and co-workers in breast cancer cells who reported that metformin selectively targets breast cancer stem cells (70).

The concentrations of metformin used in our study are in the range of 1 mM to 40 mM. Indeed, comparable metformin dosage (1-50 mM) was used to exhibit a strong and consistent anti-proliferation action on several other cancer cell lines, including breast, colon, ovary, pancreas, lung and prostate cells. However, the plasma concentrations of metformin in diabetic patients are estimated to be consistently less than 50 μmol/L (126, 127). Thus, in vitro metformin at a dosage of about 10 to 1000 fold of the recommended therapeutic levels was used.
There are several explanations for this discrepancy between in vitro concentrations and the clinical application of metformin. Firstly, in hepatic tissue, it was reported that metformin at concentration of 8 mmol/L represents physiological doses of metformin in hepatic tissue because liver receives the majority of the blood via the portal vein, which may contain concentrations of metformin substantially higher than those present in the circulation (128). As shown by Carvalho et al. (129) the positive charge of metformin could promote its accumulation within the mitochondrial matrix by 1000-fold (>20 mmol/L). Furthermore, it has been reported that metformin accumulates in tissues (such as small intestine, stomach, colon, kidney, liver) at concentrations several-fold higher than in blood (130). Secondly, the effects of high metformin doses on various cancer cells in vitro occur after a short-term intervention, while in patients comparably low metformin concentrations are operative for a long time. Thirdly, in vitro, the cells are cultured under condition of overabundant nutrients including high concentration of

glucose and 10% fetal bovine serum, which results in excessive growth stimulation, which may also explain that higher doses of metformin are necessary in cell culture.

At present, there are few data on the minimal dose needed for an anti-proliferative effect, and the safety of metformin in cancer patients without diabetes. In a recent pilot clinical trial with 26 non-diabetic patients with aberrant crypt foci, treatment with 250mg/ml metformin for 1 month suppressed the mean number of aberrant crypt foci per patient, compared with control subjects (131). As for breast cancer, there are several ongoing clinical trails to assess the antiproliferative effect of metformin on malignant, premalignant, and hyperplastic breast cells (132), as well as to establish the suitable dose for the patient without diabetes. These data may suggest that supra-therapeutic doses of metformin used *in vitro* are not mandatory to achieve a growth-inhibitory effect of metformin *in vivo*.

There is increasing evidence that thyroid cancer stem cells play an important role for the pathogenesis of thyroid carcinomas, their recurrence and metastasis (133). In this context, the recent finding of overexpression of insulin and IGF receptor in cancer stem cells derived from differentiated thyroid carcinomas is of particular interest (134). Since hyperinsulinemia in patients with type 2 diabetes mellitus and/or obesity is associated with a higher incidence of benign and malignant tumors (16) and metformin diminished insulin-stimulated cell proliferation of thyroid carcinoma cells and their cancer stem cells (Figure 3.11), it seem to be reasonable to postulate a significant role for this drug in the adjuvant chemotherapy of undifferentiated thyroid cancer. A potentiating effect of metformin may also apply to the recently developed multikinase inhibitors which may be more effective than doxorubicin and cisplatin (124). Since the anti-mitogenic effect of metformin on malignant growth is also observed in absence of (high) doses of insulin, metformin may also be a therapeutic option in patients with undifferentiated thyroid carcinoma but without diabetes mellitus and hyperinsulinemia.

In conclusion, the present work suggests that metformin exerted an anti-mitogenic effect on differentiated human thyroid cells and on thyroid carcinoma cells by inhibition of cell cycle progression and induction of apoptosis. Metformin inhibited growth by activation of the AMPK pathway, antagonized the growth stimulatory effect of insulin by inhibition of MAP kinase pathway and inhibited clonal cell growth and thyroid cancer sphere formation, a hallmark of cancer stem cells. Furthermore, metformin amplified the anti-mitogenic effect of chemotherapeutic agents such as doxorubicin and cisplatin on undifferentiated thyroid carcinoma

cells. Remarkably, this anti-proliferative effect was also observed in the doxorubicin-resistant thyroid carcinoma cell line. The growth-inhibitory effect of metformin was, however, not restricted to undifferentiated thyroid carcinoma cells but was also demonstrated in thyroid carcinoma stem cells. These data provide the rationale for combining metformin with chemotherapy as a new treatment option for undifferentiated thyroid cancers.

5 Summary

Insulin resistance and consecutive hyperinsulinemia and are the major reasons for a higher prevalence of several cancer entities in type 2 diabetes mellitus and obesity. Insulin exerts its growth-promoting effect via activation of insulin receptor- and insulin-like growth factor (IGF) receptor-dependent signaling. Metformin, an oral anti-diabetic drug, exerts a growth-inhibitory effect by reducing hyperinsulinemia and by a direct cellular action. This dual mode of metformin action is the rationale to consider metformin as a potential drug for adjuvant anti-cancer therapy.

The aim of the present work was (1) to identify whether metformin exerts an anti-mitogenic effect on differentiated human thyroid cells and on thyroid carcinoma cells, and to elucidate the underlying mechanism at the cellular level, (2) to prove that metformin targets cancer stem cells in a doxorubicin-resistant anaplastic thyroid carcinoma cell line which are enriched with cancer stem cells, (3) to investigate the effect of metformin on insulin-mediated cell growth, and to find out if metformin inhibits growth by activation of the AMPK pathway, and if it antagonizes the growth stimulatory effect of insulin by inhibition of MAP kinase pathway, (4) to detect whether metformin is capable of amplifying the anti-mitogenic effect of chemotherapeutic agents such as doxorubicin and cisplatin on undifferentiated thyroid carcinoma cells.

The study was performed in differentiated human thyroid cells, anaplastic thyroid carcinoma cells, a doxorubicin-resistant thyroid carcinoma cell line and thyroid cancer stem cells. The anti-mitogenic effect of metformin was studied in thyroid cells derived from goiters and in thyroid carcinoma cell lines by analysis of cell growth, cell cycle progression and apoptosis. Furthermore, the influence of pretreatment with insulin or with chemotherapeutic agents on metformin-induced growth inhibition was investigated in thyroid carcinoma cells, in a doxorubicin-resistant thyroid carcinoma cell line and in derived carcinoma stem cells.

This work demonstrated that metformin exerted an anti-mitogenic effect on differentiated human thyroid cells and on thyroid carcinoma cells by inhibition of cell cycle progression and induction of apoptosis. Metformin inhibited growth by activation of the AMPK pathway, antagonized the growth stimulatory effect of insulin by inhibition of MAP kinase pathway and inhibited clonal cell growth and thyroid cancer sphere formation, a hallmark of cancer stem cells. Furthermore, metformin amplified the anti-mitogenic effect of chemotherapeutic agents as doxorubicin and cisplatin on undifferentiated thyroid carcinoma cells. Remarkably, this anti-proliferative effect

was also observed in doxorubicin-resistant thyroid carcinoma cell line. The growth-inhibitory effect of metformin was, however, not restricted to undifferentiated thyroid carcinoma cells but was also demonstrated in thyroid carcinoma stem cells.

In conclusion, metformin markedly diminished growth stimulation by insulin and showed a cumulative anti-mitogenic effect to chemotherapeutics agents. Therefore, our results suggest this drug as adjuvant treatment for undifferentiated thyroid cancer in type 2 diabetic patients and obese patients with hyperinsulinemia.

ZUSAMMENFASSUNG IN DEUTSCHER SPRACHE

Die Hyperinsulinämie, die als Folge einer Insulinresistenz auftritt, ist ein wesentlicher pathogenetischer Faktor für die höhere Prävalenz verschiedener Tumorentitäten bei Patienten mit Typ 2 Diabetes und/oder einer Adipositas.
Insulin stimuliert das Zellwachstum durch Aktivierung der Insulinrezeptor- und der IGF-Rezeptor- abhängigen Signalwege. Metformin, ein orales Antidiabetikum, hemmt diesen Wachstums-stimulierenden Effekt durch Reduktion der Hyperinsulinämie und durch einen direkten zellulären Mechanismus. Diese duale Wirkung von Metformin stellt die Rationale dar, Metformin als ein potentielles Medikament für eine adjuvante Tumortherapie zu postulieren.

Das Ziel der vorliegenden Arbeit war es:

1. zu analysieren, ob Metformin einen anti-mitogenen Effekt auf differenzierte humane Schilddrüsenzellen und Schilddrüsenkarzinomzellen hat, und die dieser Wirkung zugrunde liegenden molekularen Mechanismen auf der zellulären Ebene aufzuklären.
2. zu untersuchen, ob Metformin Karzinom-Stammzellen in einer Doxorubicin-resistenten anaplastischen Schilddrüsenkarzinomlinie, die angereichert mit Karzinomstammzellen ist, in ihrem Wachstum beeinflusst.
3. die Wirkung von Metformin auf das Insulin-vermittelte Zellwachstum zu analysieren und speziell zu differenzieren, inwieweit die Wachstumshemmung über eine Aktivierung des AMPK-Signalweges und/oder eine Antagonisierung der wachstumsstimulierenden Wirkung von Insulin durch eine Hemmung des MAP-Kinase-Signalweges erfolgt.
4. zu untersuchen, ob Metformin den anti-mitogenen Effekt von Chemotherapeutika, wie Doxorubicin und Cisplatin, auf undifferenzierte Schilddrüsenkarzinomzellen verstärken kann.

Die Studie wurde durchgeführt an differenzierten menschlichen Schilddrüsenzellen, anaplastischen Schilddrüsenkarzinomzellen, einer Doxorubicin-resistenten Schilddrüsenkarzinomzelllinie und an Schilddrüsenkarzinom-Stammzellen. Der Anti-mitogene Effekt von Metformin auf differenzierte Schilddrüsenzellen, isoliert aus menschlichen Strumen, und auf Schilddrüsenkarzinomzelllinien wurde durch Zellwachstums-Analysen, Untersuchungen des Zellzyklus und der Apoptose analysiert. Ferner wurde der Einfluss einer Vorbehandlung der Zellen mit Insulin oder mit Chemotherapeutika auf die Metformin-induzierte

Wachstumshemmung in Schilddrüsenkarzinomzelllinien in einer Doxorubicin-resistenten Schilddrüsenkarzinomzelllinie und davon abgeleiteten Karzinomstammzellen untersucht.

In der Arbeit wurde gezeigt, dass Metformin anti-mitogen auf differenzierte menschliche Schilddrüsenzellen und Schilddrüsenkarzinomzelllinien wirkt, indem es die Zellzyklusprogression hemmt und eine Apoptose induziert. Metformin hemmte das Zellwachstum durch Aktivierung des AMPK-Signalweges, antagonisierte die Wachstumsstimulation von Insulin durch Hemmung des MAP-Kinase-Signalweges und hemmte ferner das klonale Wachstum und bei Schilddrüsenkarzinomzellen die so gen. Bildung von „spheres“, ein Charakteristikum von Karzinomstammzellen. Ferner verstärkte Metformin den anti-mitogenen Effekt von Chemotherapeutika wie Doxorubicin und Cisplatin auf undifferenzierte Schilddrüsenkarzinomzellen. Interessanterweise wurde dieser antiproliferative Effekt auch in einer Doxorubicin-resistenten Schilddrüsenkarzinomzelllinie beobachtet. Der Wachstums-hemmende Effekt war nicht beschränkt auf undifferenzierte Schilddrüsenkarzinomzellen sondern auch in Schilddrüsenkarzinomstammzellen nachweisbar.

Zusammenfassend wurde in dieser Arbeit nachgewiesen, dass Metformin die Wachstumsstimulation durch Insulin signifikant hemmt und in der Behandlung undifferenzierter Schilddrüsenkarzinomzellen bei gleichzeitiger Gabe von Chemotherapeutika einen additiven anti-mitogenen Effekt aufweist. Diese Ergebnisse sprechen dafür, Metformin zur adjuvanten Therapie undifferenzierter Schilddrüsenkarzinome bei Typ 2 Diabetikern und ggf. auch bei adipösen Patienten mit Hyperinsulinämie einzusetzen.

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Erklärung

„Ich, Guofang Chen, erkläre, dass ich die vorgelegte Dissertation mit dem Thema: „Effect of metformin on growth of differentiated thyroid cells and thyroid carcinoma cells and their derived cancer stem cells: implication for metformin as adjuvant treatment for undifferentiated thyroid cancer“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

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