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Insulin sensitizing therapy attenuates type 2 diabetes-mediated mammary tumor progression

Running Title: Anti-diabetic therapy inhibits mammary tumor progression

Yvonne Fierz, Ruslan Novosyadlyy, Archana Vijayakumar, Shoshana Yakar and Derek LeRoith

Division of Endocrinology, Diabetes and Bone Diseases, The Samuel Bronfman Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA

Corresponding author:
Derek LeRoith, MD, PhD
E-mail: derek.leroith@mssm.edu

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Objective: Type 2 diabetes increases breast cancer risk and mortality and hyperinsulinemia has been identified as a major factor linking these two diseases. Thus, we hypothesized that pharmacological reduction of elevated insulin levels would attenuate type 2 diabetes-mediated mammary tumor progression.

Research Design and Methods: We studied mammary tumor development in $MKR^{+/+}$ mice, a non-obese, hyperinsulinemic mouse model of type 2 diabetes. $MKR^{+/+}$ mice were either crossed with mice expressing the Polyoma Virus middle T (PyVmT) oncogene specifically in the mammary gland, or inoculated orthotopically with the mouse mammary tumor cell lines Met-1 and MCNeuA. $MKR^{+/+}$ or control mice harboring tumors were treated with CL-316243, a specific β_3-adrenergic receptor agonist, which sensitizes insulin action but has no direct effect on the mouse mammary epithelium or Met-1 and MCNeuA cells.

Results: CL-316243 treatment significantly reduced the elevated insulin levels in $MKR^{+/+}$ mice and, as a consequence, attenuated mammary tumor progression in the three tumor models tested. This effect was accompanied by reductions in phosphorylation of the insulin and IGF-I receptors in transformed mammary tissue.

Conclusion: Insulin sensitizing treatment is sufficient to abrogate type 2 diabetes-mediated mammary tumor progression. Therefore, early administration of insulin sensitizing therapy may reduce breast cancer risk and mortality in patients with type 2 diabetes.

Type 2 diabetes has become a major public health problem worldwide and is associated with severe acute and chronic complications. Recently it has been shown that the disease increases breast cancer risk and mortality (1-4). In our previous studies, we have identified hyperinsulinemia as the predominant factor responsible for diabetes-mediated mammary tumor progression (5). Elevated insulin levels are observed mainly at early stages of the disease, where peripheral insulin resistance results in a compensatory increase in insulin secretion by the pancreatic β-cells to meet the higher insulin demand. Thus, before the onset of clinically overt type 2 diabetes, patients are often hyperinsulinemic but euglycemic, and hence unaware of their disease for many years. There is growing evidence that the risk for the development of breast cancer is substantially increased in patients with early stage type 2 diabetes (6,7).

Pharmacological treatment of type 2 diabetes may have an impact on cancer risk and mortality. Early stage type 2 diabetes is treated by two main approaches: insulin secretagogues (e.g. sulfonylureas) stimulate insulin secretion from the pancreatic β-cells and thus increase insulin levels. Conversely, insulin sensitizing agents (e.g. metformin and thiazolidinediones (TZDs)) improve insulin action in peripheral tissues and, as a consequence, reduce hyperinsulinemia. There is growing evidence that anti-diabetic therapy elevating insulin levels increases cancer risk as well cancer-related mortality (8,9), whereas insulin sensitizing drugs may reduce cancer risk, morbidity and mortality (8-14) in patients with type 2 diabetes. However, it is as yet unclear whether the anti-neoplastic effects of the two mainly used insulin sensitizing agents (metformin and TZDs) are a result of their direct action on tumor cells (15-23) or an indirect effect via a reduction of insulin levels.

Our study was aimed to explore whether lowering insulin levels in type 2 diabetes would mitigate mammary tumor progression, independent of any direct effect of the applied drug. To address this question, we utilized the insulin sensitizing drug CL-316243 (24), a potent β_3-adrenergic receptor (β_3-AR) agonist with no known direct effects on breast cancer, in a non-obese mouse model of type 2 diabetes ($MKR^{+/+}$ mice). $MKR^{+/+}$ mice develop severe insulin resistance and hyperinsulinemia at an early age due to overexpression of muscle creatine kinase-driven dominant-negative IGF-I receptors (IGF-IR), and subsequent abrogation of IGF-I and insulin signaling in skeletal muscle (25). Female $MKR^{+/+}$ mice develop only mild dysglycemia but display marked insulin resistance and hyperinsulinemia, similar to early stages of type 2 diabetes in humans (5). The non-obese hyperinsulinemic phenotype of these mice make them an ideal model to specifically study the effect of insulin reduction on mammary tumor progression, independent of numerous confounding factors originating from obesity or overt type 2 diabetes (e.g. adipokines, proinflammatory cytokines, adipocyte-derived sex steroids, hyperglycemia) (26). To initiate mammary tumors, we employed three different approaches: Polyoma Virus middle T (PyVmT) transgenic mice (27) served as a model for early stages of cancer development. To study solid tumor formation, PyVmT- and Neu/ErbB2-expressing tumor cells (28,29) were used in syngeneic orthotopic cell injection experiments.

Herein we demonstrate that chronic CL-316243 treatment is capable of reducing insulin levels in female $MKR^{+/+}$ mice, leading to an abrogation of the accelerated mammary tumor progression in all three cancer models tested. Furthermore, we show that this effect is accompanied by a reduced activation of the insulin receptor (IR) and the IGF-IR in transformed mammary tissue. Our findings indicate that insulin sensitizing therapy is

sufficient to abrogate the tumor promoting activity of early stage type 2 diabetes. Thus, we propose that early treatment of hyperinsulinemia might contribute to lower breast cancer risk, morbidity and mortality in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Animals: All mice were on the FVB/N background. The generation and characterization of $MKR^{+/+}$ mice (5,25) as well as MMTV-$PyVmT^{+/-}$ mice (27) was described previously. The mice were housed in a clean mouse facility, had free access to a standard mouse chow (Picolab rodent diet 5053, LabDiet, St. Louis, MO, USA) and fresh water *ad libitum* and were kept on a 12-h light/dark cycle. Animal care and maintenance were provided through the Mount Sinai School of Medicine AAALAC Accredited Animal Facility. All procedures were approved by the Animal Care and Use Committee of Mount Sinai School of Medicine.

Metabolic assays: Body weight and food intake were measured twice a week. Food intake was normalized to body weight and expressed as grams of food per grams of body $weight^{0.75}$ per day. Blood glucose levels were measured weekly in the non-fasting state between 9AM and noon_with an automated glucometer (Elite, Bayer, IN, USA). Plasma and serum were obtained in the non-fasting state and collected in heparinized and non-heparinized capillary tubes, respectively. Plasma insulin levels were measured by a Radio-Immunoassay (RIA) according to the manufacturer's instructions (Linco, St. Charles, MO, USA). Serum leptin, adiponectin, TNFα and IL6 levels were measured by ELISA (Millipore, Billerica, MA, USA (leptin); R&D systems, Minneapolis, MN, USA (adiponectin, TNFα and IL6)). Serum free fatty acids (FFA) and serum triglycerides were measured by a colorimetric assay (Roche Applied Science, Indianapolis, IN, USA (FFA) and BioVision, Mountain View, CA, USA (triglycerides)). Body composition was determined in non-anesthetized mice using an EchoMRI 3-in-1™ NMR system (Echo Medical Systems, Houston, TX, USA).

Transgenic mammary tumor model: $PyVmT^{+/-}$ male mice were interbred with $MKR^{+/+}$ or wild type (WT) female mice to generate cohorts of $PyVmT^{+/-}$ and $PyVmT^{+/-}/MKR^{+/+}$ female mice. CL-316243 (Sigma Aldrich, St. Louis, MO, USA) was dissolved in sterile saline and administered daily intraperitoneally (i.p.) at a dose of 1 mg/kg body weight (30) from 3-6 weeks of age. Control mice received an equal amount of vehicle (sterile saline). Following euthanasia, inguinal mammary glands (#4) were subjected to whole mount analysis or immediately snap-frozen in liquid nitrogen for further studies.

Syngeneic orthotopic tumor models: Met-1 and MCNeuA mouse carcinoma cells were derived from MMTV-PyVmT (FVB/N) and MMTV-Neu (FVB/N) transgenic mice, respectively (28,29). The cells were allowed to grow until confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, were detached by a non-enzymatic cell dissociation solution and Met-1 cells ($0.5x10^6$) or MCNeuA cells (10^6) were injected into the left inguinal mammary fat pad (#4) of 8 week old female $MKR^{+/+}$ and WT mice. One week following tumor cell inoculation, CL-316243 (1 mg/kg body weight/d i.p.) was administered for 21 days. Tumor growth was monitored by palpation and tumor volume was measured in a three-coordinate system using calipers. Tumor volume was calculated by the formula: $4/3 \times \pi \times r_1 \times r_2 \times r_3$ (r = radius).

Protein extraction and Western blot analysis: Tissues were lysed in buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS (Roche, Indianapolis, IN, USA), 1 mM sodium

orthovanadate, 2 mM sodium fluoride, 10 mM sodium pyrophosphate (Sigma-Aldrich, St. Louis, MO, USA), 8 mM β-glycerophosphate (VWR, West Chester, PA, USA) and Complete® Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). After denaturation, the proteins were subjected to SDS-PAGE (8% Tris-glycine gel, Invitrogen, Carlsbad, CA, USA) and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was sequentially blocked and probed with primary and secondary antibodies, and then analyzed by direct infrared fluorescence detection using an Odyssey® Infrared Imaging System (Li-cor, Lincoln, NE, USA). Densitometric analysis was performed using MacBAS V2.52 software. The antibodies were purchased from the following sources: Phospho-IRβ$^{Tyr1150/51}$/IGF-IRβ$^{Tyr1135/36}$ (Cell Signaling Technology, Danvers, MA, USA), IRβ (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Whole mount analysis of mammary glands: The #4 inguinal mammary glands were carefully excised, spread out on a glass slide, and fixed for 2-4 hours in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). The fixed glands were hydrated in decreasing concentrations of ethanol (100%, 95%, 70%, 50% and 30% for 15 minutes each), rinsed in double distilled water and stained overnight in carmine alum staining. After dehydration in increasing ethanol concentrations (30%, 50%, 70%, 95%, and 100% for 15 minutes each) and clearing in xylene overnight (Fisher Scientific, Pittsburgh, PA, USA), the glands were covered by Mount-Quick mounting medium (Daido Sangyo Co., Tokyo, Japan) and photographic documentation was performed using a stereomicroscope and MicroSuite FIVE imaging software (Olympus, Center Valley, PA, USA). Quantification of the hyperplastic mammary lesion as a ratio of the total glandular area was performed using Image J software (National Institutes of Health, Bethesda, MD, USA).

Determination of β_3-AR expression: RNA was extracted from Met-1, MCNeuA and mammary epithelial cells (MEC) derived from FVB/N mice using the NucleoSpin RNA II kit (Clontech Laboratories, Mountain View, CA, USA). After reverse transcription (RT), the resulting cDNA was amplified by PCR using the following primers (Operon, Huntsville, AL, USA): β_3-AR: forward: 5' ATGGCTCCGTGGCCTCAC 3' and reverse: 5' CTGGCTCATGATGGGCGC 3' (31); 18S rRNA: forward: 5' TTGACGGAAGGGCACCACCAG 3' and reverse: 5' GCACCACCACCCACGGAATCG 3'.

Statistical analysis: Results are expressed as mean ± SEM. Statistical analyses were conducted using ANOVA followed by a Fisher's test, with $p \leq 0.05$ considered significant. All analyses were performed using STATVIEW Version 5.0., SAS Institute Inc. (Cary, NC, USA).

RESULTS

Mammary epithelial cells, mammary tumor cells and mammary gland development are not affected by CL-316243: CL-316243 has potent anti-obesity and anti-diabetic effects in various rodent models (30,32-34). The action of CL-316243 is highly specific to β_3-AR expressing cells which are predominantly the white and brown adipocytes (35,36). Chronic activation of the β_3-AR increases fatty acid oxidation and energy expenditure, thereby reducing adiposity. Subsequently, chronic β_3-AR stimulation leads to improved insulin sensitivity and reduced insulin levels (30,33,34).

To address whether CL-316243 has direct effects on the mammary epithelium or tumor cells we determined the expression levels of the β_3-AR. As shown in Fig. 1*A*, RT-PCR revealed undetectable levels of β_3-AR transcripts in normal mouse mammary

epithelial cells (MEC) and in the two mammary tumor cell lines: Met-1 and MCNeuA cells.

Chronic β_3-AR activation with CL-316243 leads to an increased formation of brown adipose tissue in the mammary gland (37). Thus, we assessed whether treatment with CL-316243 affects mammary gland development *in vivo* through alteration of the mammary fat pad. Four week-old female wild type (WT) mice were subjected to intraperitoneal (i.p.) injections of CL-316243 (1 mg/kg body weight/d) or an equal volume of vehicle for 3 weeks. Whole mount analyses of mammary glands obtained from these animals at the age of 7 weeks demonstrated no significant changes in mammary ductal outgrowth and side branching (Fig. 1*B*).

These data suggest that CL-316243 has a minimal effect on mammary gland development and that a direct effect of CL-316243 on mammary tumor cells via activation of the β_3-AR is unlikely.

CL-316243 treatment affects food intake and body composition in female WT and MKR$^{+/+}$ mice and reduces hyperinsulinemia in female MKR$^{+/+}$ mice: We have previously shown that chronic treatment with CL-316243 effectively reverses the diabetic phenotype in male MKR$^{+/+}$ mice, which, in contrast to female MKR$^{+/+}$ mice, develop overt type 2 diabetes (30). To test whether the predominantly hyperinsulinemic phenotype in female MKR$^{+/+}$ mice would respond in a similar manner, we treated 8 week old female MKR$^{+/+}$ and WT mice chronically with CL-316243 (1 mg/kg body weight/d) or vehicle for 3 weeks. Female MKR$^{+/+}$ mice exhibit reduced body weight and body adiposity compared to WT mice (5). As observed in our previous study in male WT and MKR$^{+/+}$ mice (30), we found that CL-316243 treatment did not affect body weight (Fig. 2*A*), but increased food intake (Fig. 2*B*) and decreased body adiposity (Fig. 2*C*), predominantly affecting the gonadal fat pads (Fig. S1 in the online appendix at http://diabetes.diabetesjournals.org).

Additionally, we found that after 1 week of CL-316243 treatment, the moderately elevated blood glucose levels (~ 20%) in MKR$^{+/+}$ mice were significantly lowered (Fig. 2*D*). Importantly, we show that female MKR$^{+/+}$ mice display severely elevated plasma insulin levels, which were significantly reduced upon CL-316243-treatment, while no effect of the drug on insulin levels was seen in WT mice (WT, vehicle: 0.42 ± 0.01 vs. WT, CL: 0.43 ± 0.02; MKR$^{+/+}$, vehicle: 13.12 ± 3.74 vs. MKR$^{+/+}$, CL: 3.28 ± 0.85 ng/ml) (Fig. 2*E*).

As chronic CL-316243 treatment lowers body adiposity in both WT and MKR$^{+/+}$ mice, serum levels of circulating lipids, adipokines and proinflammatory cytokines were determined (Table 1). Serum levels of free fatty acids (FFAs) were unchanged in female MKR$^{+/+}$ mice compared to WT mice, whereas there was a moderate (~30%) increase in triglyceride levels. Upon chronic treatment with CL-316243, FFAs and triglycerides were significantly reduced to a similar level in both MKR$^{+/+}$ and WT mice. Serum leptin levels were lower in MKR$^{+/+}$ mice compared to WT mice, and CL-316243 treatment significantly reduced leptin levels in both MKR$^{+/+}$ and WT mice. The levels of adiponectin and the proinflammatory cytokines TNFα and IL-6 were not affected by CL-316243 treatment.

Taken together, these data show that chronic treatment with CL-316243 effectively reduces insulin levels in female MKR$^{+/+}$ mice alone while having a comparable effect on food intake, relative body adiposity, circulating lipids and leptin levels in both WT and MKR$^{+/+}$ mice. This allows us to study the effect of insulin reduction on mammary tumor progression in MKR$^{+/+}$ mice.

CL-316243 treatment abrogates the accelerated formation of hyperplastic mammary lesions in PyVmT$^{+/-}$/MKR$^{+/+}$ mice: To investigate the effect of insulin sensitizing therapy on early stages of mammary tumor

development, we used a PyVmT-induced transgenic tumor model (27). PyVmT$^{+/-}$ transgenic mice express the PyVmT oncogene exclusively in the mammary epithelium under the control of the Mouse Mammary Tumor Virus (MMTV) promoter. PyVmT-induced mammary tumors recapitulate many morphological and pathophysiological processes found in human breast cancer, and their development begins with the formation of a single hyperplastic focus in the lateral part of the mammary gland at 3-4 weeks of age (38).

To assess the effect of anti-diabetic therapy on mammary tumor development, we treated PyVmT$^{+/-}$/MKR$^{+/+}$ and PyVmT$^{+/-}$ mice with CL-316243 (1 mg/kg body weight/d) or vehicle from 3-6 weeks of age. The metabolic effects of CL-316243 treatment were similar to those observed in adult mice (Fig. S2 in the online appendix). The inguinal mammary glands (#4) were dissected following the treatment at 6 weeks of age. Whole mount analyses of vehicle-treated PyVmT$^{+/-}$/MKR$^{+/+}$ mice revealed a larger area of hyperplastic mammary lesions (increase of 57%) compared to the PyVmT$^{+/-}$ mice (Fig. 3*A* and *B*) due to accelerated formation of hyperplasia in a hyperinsulinemic milieu. In contrast, pharmacological correction of hyperinsulinemia in PyVmT$^{+/-}$/MKR$^{+/+}$ mice resulted in a tumor phenotype similar to non-diabetic PyVmT$^{+/-}$ mice (Fig. 3*A* and *B*). Note that the CL-316243-treated non-diabetic PyVmT$^{+/-}$ mice show no changes in mammary transformation. To determine whether the attenuation in tumor progression in the CL-316243-treated mice resulted from an inhibition of insulin signaling, we analyzed the phosphorylation of the IR/IGF-IR in the hyperplastic mammary tissue of vehicle- and CL-316243-treated mice (Fig. 3*C* and *D* and Fig. S3 in the online appendix). We found that hyperinsulinemia led to an increased activation of the IR/IGF-IR in transformed mammary tissue of PyVmT$^{+/-}$/MKR$^{+/+}$ mice whereas pharmacological correction of insulin levels by CL-316243 treatment significantly reduced this effect. Taken together, we demonstrate that lowering insulin levels in type 2 diabetic PyVmT$^{+/-}$/MKR$^{+/+}$ mice reduces IR/IGF-IR activation and abrogates the accelerated formation of hyperplastic mammary lesions.

Treatment with CL-316243 abrogates the accelerated growth of advanced mammary tumors in MKR$^{+/+}$ mice: To investigate whether CL-316243 treatment would also affect growth of fully transformed tumor cells, we injected estrogen receptor-negative PyVmT-transformed mammary tumor cells (Met-1 cells (28)) orthotopically into the #4 mammary fat pad ($0.5x10^6$ cells) of 8 week old female MKR$^{+/+}$ or WT mice. Starting seven days after tumor cell inoculation, when tumors were palpable, the animals were treated with CL-316243 (1 mg/kg body weight/d) for 3 weeks. As shown in Fig. 4*A*, tumor volume increased in vehicle-treated MKR$^{+/+}$ mice, whereas MKR$^{+/+}$ mice treated with CL-316243 displayed a significant reduction in tumor growth and were similar in size to WT controls. Importantly, CL-316243 treatment had no effect on tumor growth in the WT mice. These findings were confirmed by measuring wet tumor weight at the end of the study (Fig 4*B*).

To ascertain that our findings are not limited to PyVmT-induced mammary carcinogenesis, we also studied estrogen receptor-negative Neu- transformed mammary tumor cells (MCNeuA cells (29)) in a similar manner as the Met-1 cells. Neu is the rodent analogue of the *ERBB2* gene, which is amplified and/or overexpressed in 30% of human breast carcinomas (39). MCNeuA cells were inoculated orthotopically into the #4 mammary fat pad (10^6 cells) and the treatment protocol and follow-up of tumor growth were performed as described above. As shown in Fig. 5*A* and *B*, tumor volume and weight were increased in vehicle-treated MKR$^{+/+}$ mice compared to WT controls, however, following CL-316243 treatment and subsequent decreases in insulin levels, we observed a significantly

reduced tumor size in $MKR^{+/+}$ mice. These data suggest that insulin sensitizing therapy can abrogate insulin-mediated tumor progression independent of the tumor-inducing oncogene.

DISCUSSION

There is an increasing interest in the effect of insulin sensitizing therapy on breast cancer risk and mortality. The current study demonstrates that treatment with the insulin sensitizing drug_CL-316243, a highly selective β_3-AR agonist, reverses diabetes-induced mammary tumor progression in hyperinsulinemic, type 2 diabetic $MKR^{+/+}$ mice. Our findings corroborate a major role of insulin in type 2 diabetes-mediated mammary tumor progression, which is in concert with our previous findings (5).

High concentrations of insulin are known to activate the IGF-IR (40) and our previous findings demonstrate that the IR, and to lesser extent the IGF-IR, are activated in hyperplastic mammary tumor tissue extracted from type 2 diabetic mice (5). Moreover, pharmacological blockade of the IR/IGF-IR using BMS-536924, a small-molecule tyrosine kinase inhibitor, led to abrogation of diabetes-induced mammary tumor progression in $MKR^{+/+}$ mice; however, it also worsened insulin resistance and hyperinsulinemia (5). Herein we demonstrate that insulin sensitizing treatment has a comparable effect on tumor progression but simultaneously exerts anti-diabetic activity. Our results imply that elevated insulin levels should be targeted pharmacologically at early stages of the disease to lower breast cancer risk and progression in patients with type 2 diabetes.

The effect of anti-diabetic medications on cancer development is poorly understood and no prior experimental studies have investigated thoroughly if pharmacological reduction of insulin levels reduces mammary tumor growth in a type 2 diabetic organism. The reason for this is that the most widely used insulin sensitizing drugs (metformin and TZDs) are known to exert direct, predominantly anti-neoplastic, effects on cancer cells, both *in vitro* and *in vivo* in non-diabetic animals (15-17,19-23,41). Thus, we decided to use CL-316243, a highly selective β_3-AR agonist with no known direct effect on mammary tumors, to study the consequence of lowering insulin levels on diabetes-mediated mammary tumor progression. However, it should be noted that β_3-AR agonists (unlike metformin and TZDs) have not yet been developed for clinical use in humans due to various problems including a lack of selectivity and poor pharmacokinetics (42).

Algire *et al.* have reported that metformin attenuates the effect of high energy diet-induced insulin resistance on growth of lewis lung LLC1 carcinoma cells (18). Both, the insulin sensitizing action as well as the direct anti-neoplastic activity of metformin may be involved in this finding. However, in the setting of excess energy intake and obesity other factors besides hyperinsulinemia can promote tumor growth, such as adipokines, proinflammatory cytokines, adipocyte-derived sex steroids or altered levels of circulating carbohydrates and lipids (43), some of which may be affected by metformin treatment (44,45).

Thus, as an experimental model for insulin resistance independent of obesity, we employed a lean, hyperinsulinemic mouse model of type 2 diabetes, the female $MKR^{+/+}$ mice. These mice develop severe insulin resistance and hyperinsulinemia but have only mild dysglycemia and thus recapitulate the early stages of type 2 diabetes. As these mice display reduced body adiposity and show no increase in serum FFAs, leptin or the proinflammatory cytokines TNFα or IL-6 and only a moderate elevation in serum triglycerides, they serve as an ideal model to study mammary tumor progression uncoupled from obesity related tumor promoting factors.

Apart from reducing insulin levels, chronic treatment with CL-316243 also decreases blood glucose levels in $MKR^{+/+}$ mice.

Hyperglycemia has been proposed to promote tumor growth through the increased flux of glucose, which fuels tumor cells (46). However, a major pathophysiological role of elevated blood glucose levels in the tumor promoting action of type 2 diabetes in $MKR^{+/+}$ mice is unlikely due to the following reasons: (i) female $MKR^{+/+}$ mice display only mild dysglycemia (~20% increase in blood glucose levels compared to WT mice), (ii) in animals with type 1 diabetes, which are insulin-deficient due to immune destruction of the pancreatic β-cells, tumors regress despite severe hyperglycemia (47,48), (iii) although some epidemiologic studies show a correlation between elevated blood glucose levels and cancer risk, they may be confounded by pre-existing hyperinsulinemia (49). Chronic CL-316243 treatment reduces serum FFAs, triglyceride and leptin levels in both $MKR^{+/+}$ and WT mice. As this decrease is observed in both genotypes and the anti-neoplastic effect of CL-316243 is specific to $MKR^{+/+}$ mice, these factors are most likely not major players in the tumor-reducing effect of CL-316243. Nevertheless, we cannot fully exclude that changes in lipid or leptin levels may, at least in part, have an impact on breast tumor progression in $MKR^{+/+}$ mice. Taken together, our experimental approach allowed us to test the effect of insulin sensitizing therapy on tumor progression in a setting where obesity-associated confounding factors are minimal.

Our findings demonstrate that hyperinsulinemia in mice with type 2 diabetes significantly increases IR/IGF-IR activation in transformed mammary tissue, whereas pharmacological reduction of insulin levels attenuates this effect. Law *et al.* reported that phosphorylation of the IR/IGF-IR was present in nearly 50% of all analyzed human primary breast tumors and was predictive for a poor outcome (50). However, the authors did not obtain information on the insulin levels in the patients studied. It remains to be evaluated whether the extent of IR/IGF-IR activation in breast cancer specimen correlates with underlying type 2 diabetes and hyperinsulinemia in breast cancer patients. If this is the case, then insulin lowering therapy could reduce breast cancer risk and mortality.

In conclusion, we demonstrate that the administration of insulin sensitizing therapy using a β_3-AR agonist abrogates the accelerated mammary tumor progression in a non-obese mouse model of type 2 diabetes. This effect is accompanied by a reduction of insulin levels leading to a decrease in the phosphorylation of the IR/IGF-IR in transformed mammary tissue. These results demonstrate that insulin lowering therapy is an important modifier of breast cancer progression. Our findings thus provide a rationale for early administration of insulin sensitizing therapy in patients with hyperinsulinemia and/or type 2 diabetes, as such treatment may have a significant impact on breast cancer risk, morbidity and mortality.

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Table 1: Effect of chronic treatment with CL-316243 on serum lipids, adipokines and proinflammatory cytokines in female WT and $MKR^{+/+}$ mice

	Wild type mice		$MKR^{+/+}$ mice	
	vehicle	CL-316243	vehicle	CL-316243
FFA, mM	0.34 ± 0.04	0.16 ± 0.03 *	0.29 ± 0.05	0.17 ± 0.03 *
Triglycerides, mM	1.40 ± 0.09	1.01 ± 0.08 *	1.83 ± 0.14 #	1.13 ± 0.15 *
Leptin, ng/ml	3.11 ± 0.44	2.04 ± 0.19 *	2.02 ± 0.28 #	1.00 ± 0.06 *#
Adiponectin, µg/ml	8.39 ± 0.58	9.61 ± 0.34	9.74 ± 0.38 #	9.16 ± 0.42
TNFα, pg/ml	20.95 ± 1.23	19.54 ± 0.81	18.48 ± 0.47 #	18.27 ± 0.66
IL-6, pg/ml	4.26 ± 0.51	5.03 ± 1.82	6.75 ± 1.96	7.32 ± 3.27

Serum was obtained in the non-fasting state at the end of the study, 21 days after the onset of treatment with CL-316243 (1mg/kg/d). Data are expressed as mean ± SEM. * $p \leq 0.05$ for CL-316243 vs. vehicle group; # $p \leq 0.05$ for WT vs. $MKR^{+/+}$ from the same treatment group. FFA and triglycerides: n = 10-16 mice/group; adiponectin, leptin, IL-6 and TNFα: n = 5-7 mice/group.

Figure legends

Figure 1: Normal mammary epithelial cells, mammary tumor cells and mammary gland development are not affected by CL-316243 (A) Determination of β_3-AR expression in mammary epithelial cells (MEC, derived from FVB/N mice), Met-1 and MCNeuA tumor cells analyzed by PCR followed by agarose gel electrophoresis (for primers see *Research design and methods*). Negative control: NIH/3T3 fibroblasts; positive control: mouse white adipose tissue. 18S rRNA was used as a loading control. (**B)** Representative whole-mount analysis of the #4 mammary gland obtained from 7 week old wild type (WT) mice. The mice were treated from 4-7 weeks of age with CL-316243 (1mg/kgBW/d) i.p. or a vehicle control. n = 4-5 mice/group. LN: Lymph node. Original magnification x 4.

Figure 2: Chronic CL-316243 treatment abrogates the diabetic phenotype in female MKR$^{+/+}$ mice. MKR$^{+/+}$ and WT mice were treated with CL-316243 (CL, closed bars) or vehicle (V, open bars) from 9-12 weeks of age. (**A**) Body weight and (**B**) food intake were measured twice a week. The average food intake over 3 weeks of treatment is presented. (**C**) Body adiposity was measured 10 days after the onset of treatment in non-anesthetized mice using an EchoMRI 3-in-1™ NMR system; (**D**) glucose and (**E**) insulin levels were measured in whole blood and plasma, respectively, 1 week after initiation of treatment in the non-fasting state. Data are expressed as mean ± SEM. * $p \leq 0.05$ for CL-316243 vs. vehicle group; # $p \leq 0.05$ for WT vs. MKR$^{+/+}$ from the same treatment group. n = 6-8 mice/group. These data have been reproduced in 3 independent experiments.

Figure 3: Treatment with CL-316243 prevents the accelerated formation of hyperplastic mammary lesions in diabetic mice and attenuates IR/IGF-IR activation.
(**A**) Whole-mount analysis of the #4 mammary gland of 6 week old virgin PyVmT$^{+/-}$/MKR$^{+/+}$ mice and PyVmT$^{+/-}$ mice. Both groups were treated with CL-316243 or vehicle from 3-6 weeks of age. LN: Lymph node; PF: primary focus; Arrows indicate secondary foci. Original magnification x10. Representative whole mounts are presented. (**B**) Quantification of the area of hyperplastic lesions is presented as percentage compared to the total gland area (n = 6-8 mice/group). CL-316243: closed bars, vehicle: open bars (**C**) Proteins extracted from hyperplastic mammary tissue of 6 week old PyVmT$^{+/-}$/MKR$^{+/+}$ and PyVmT$^{+/-}$ mice treated with either CL-316243 (CL) or vehicle were size fractioned by SDS page and immunoblotted against phospho-IR$\beta^{Y1150/51}$/IGF-IR$\beta^{Y1135/36}$ and the IRβ. Representative western blot analysis is shown. (**D**) Densitometric analysis of the relative IR/IGF-IR phosphorylation (normalized to the IR) is presented as a fold change compared to the vehicle treated control group (PyVmT$^{+/-}$, vehicle) (n = 6-8 mice/group). CL-316243: closed bars, vehicle: open bars. Data are expressed as mean ± SEM. * $p \leq 0.05$ for CL-316243 vs. vehicle group; # $p \leq 0.05$ for WT vs. MKR$^{+/+}$ from the same treatment group.

Figure 4: Treatment with CL-316243 reverses the accelerated growth of advanced PyVmT-induced mammary tumors. Met-1 cells ($0.5x10^6$) were orthotopically injected into the #4 mammary fat pad of 8 week old virgin WT or MKR$^{+/+}$ mice. Seven days after cell injection, treatment with either CL-316243 (CL) or vehicle (V) was initiated. (**A**) Tumor growth was followed by measuring tumor volume weekly and was calculated as indicated in *Research design and methods*. Arrow indicates the beginning of treatment. (**B**) Wet tumor weight was determined

at the end of the study. CL-316243: closed bars, vehicle: open bars. Data are expressed as mean ± SEM. * $p \leq 0.05$ for CL-316243 vs. vehicle group; # $p \leq 0.05$ for WT vs. $MKR^{+/+}$ from the same treatment group. n = 7-8 mice/group.

Figure 5: Treatment with CL-316243 reverses the accelerated growth of advanced Neu/ErbB2 induced mammary tumors. MCNeuA cells (10^6) were orthotopically injected into the #4 mammary fat pad of 8 week old virgin WT or $MKR^{+/+}$ mice. Seven days after cell injection, treatment with either CL-316243 (CL) or vehicle (V) was initiated. (**A**) Tumor growth was followed by measuring tumor volume weekly and was calculated as indicated in *Research design and methods*. Arrow indicates the beginning of treatment. (**B**) Wet tumor weight was determined at the end of the study. CL-316243: closed bars, vehicle: open bars. Data are expressed as mean ± SEM. * $p \leq 0.05$ for CL-316243 vs. vehicle group; # $p \leq 0.05$ for WT vs. $MKR^{+/+}$ from the same treatment group. n = 7-8 mice/group.

Figure 1

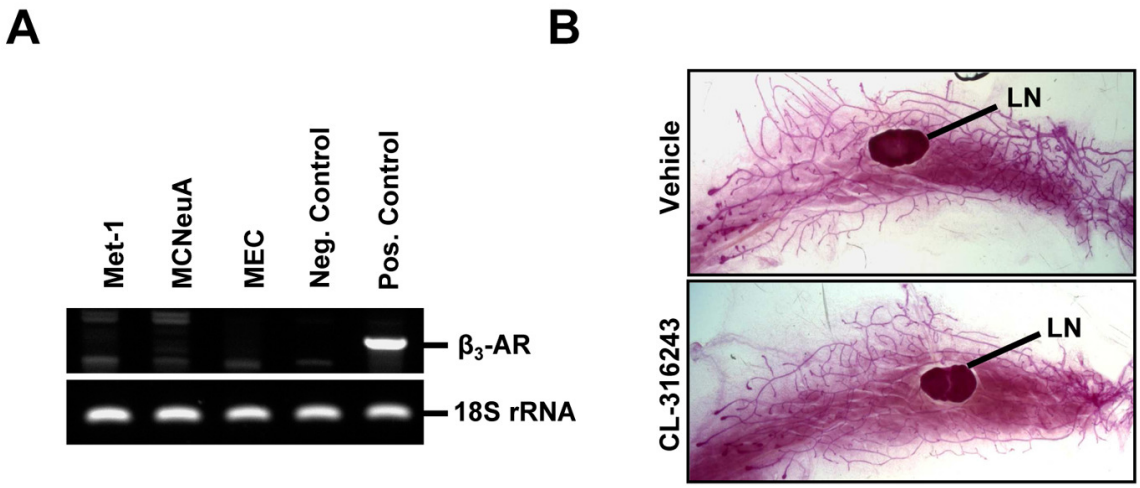


Figure 2

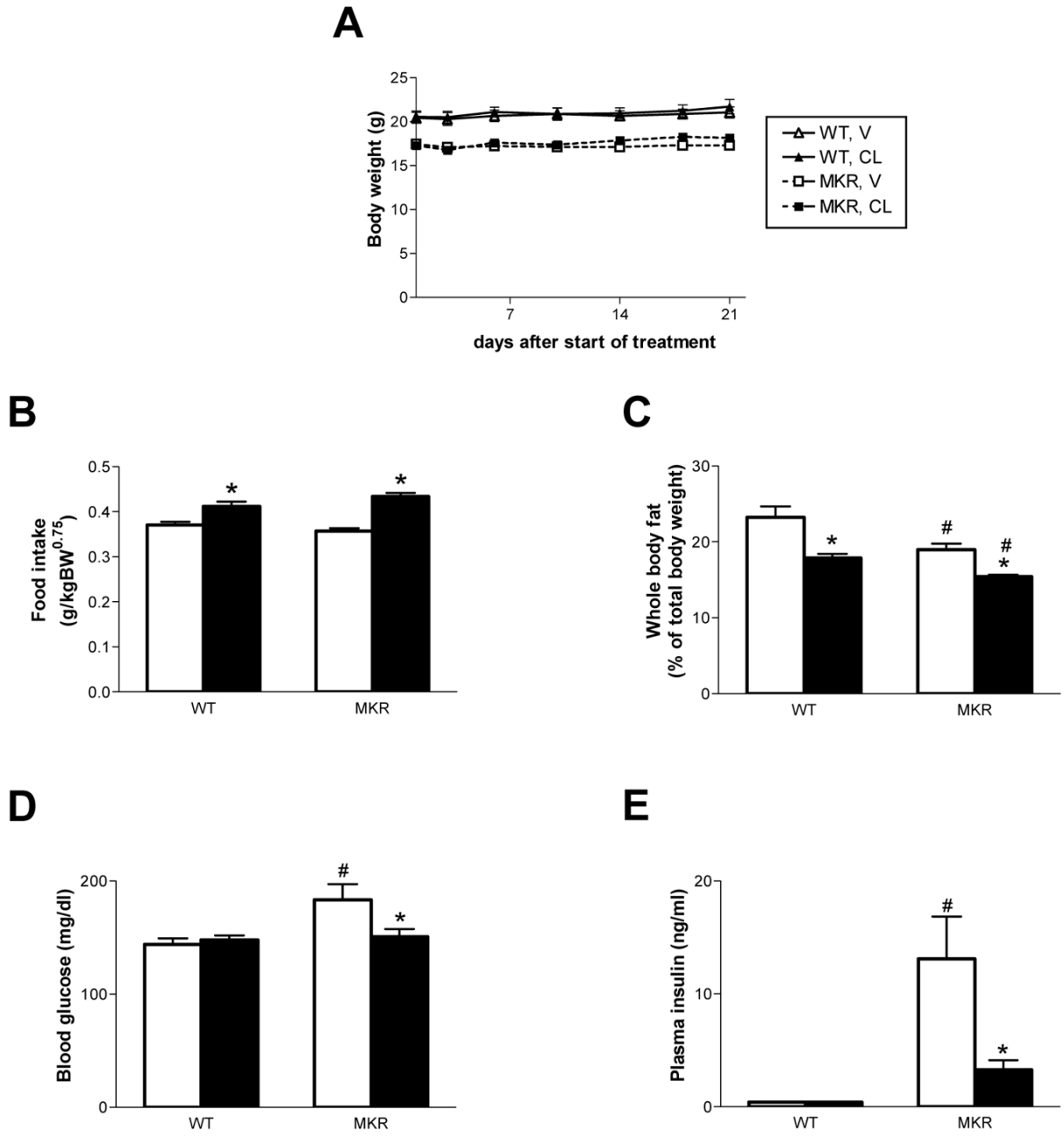

A
Body weight (g)
days after start of treatment
WT, V
WT, CL
MKR, V
MKR, CL
B
Food intake (g/kgBW^0.75)
C
Whole body fat (% of total body weight)
D
Blood glucose (mg/dl)
E
Plasma insulin (ng/ml)
WT
MKR

Figure 3

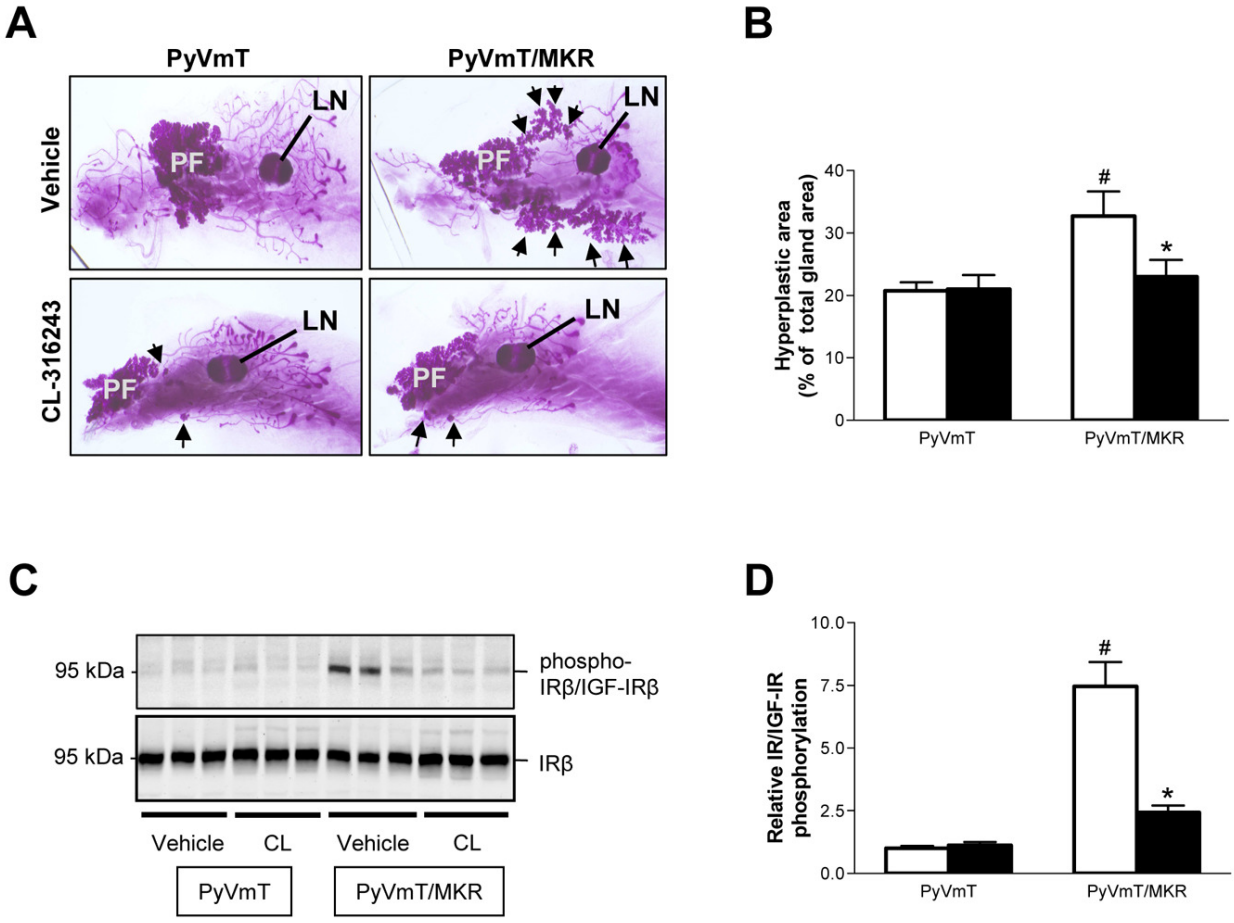

A
PyVmT
PyVmT/MKR
Vehicle
CL-316243
LN
PF
B
Hyperplastic area
(% of total gland area)
40
30
20
10
0
#
*
PyVmT
PyVmT/MKR
C
95 kDa
phospho-
IRβ/IGF-IRβ
95 kDa
IRβ
Vehicle
CL
Vehicle
CL
PyVmT
PyVmT/MKR
D
Relative IR/IGF-IR
phosphorylation
10.0
7.5
5.0
2.5
0.0
#
*
PyVmT
PyVmT/MKR

Figure 4

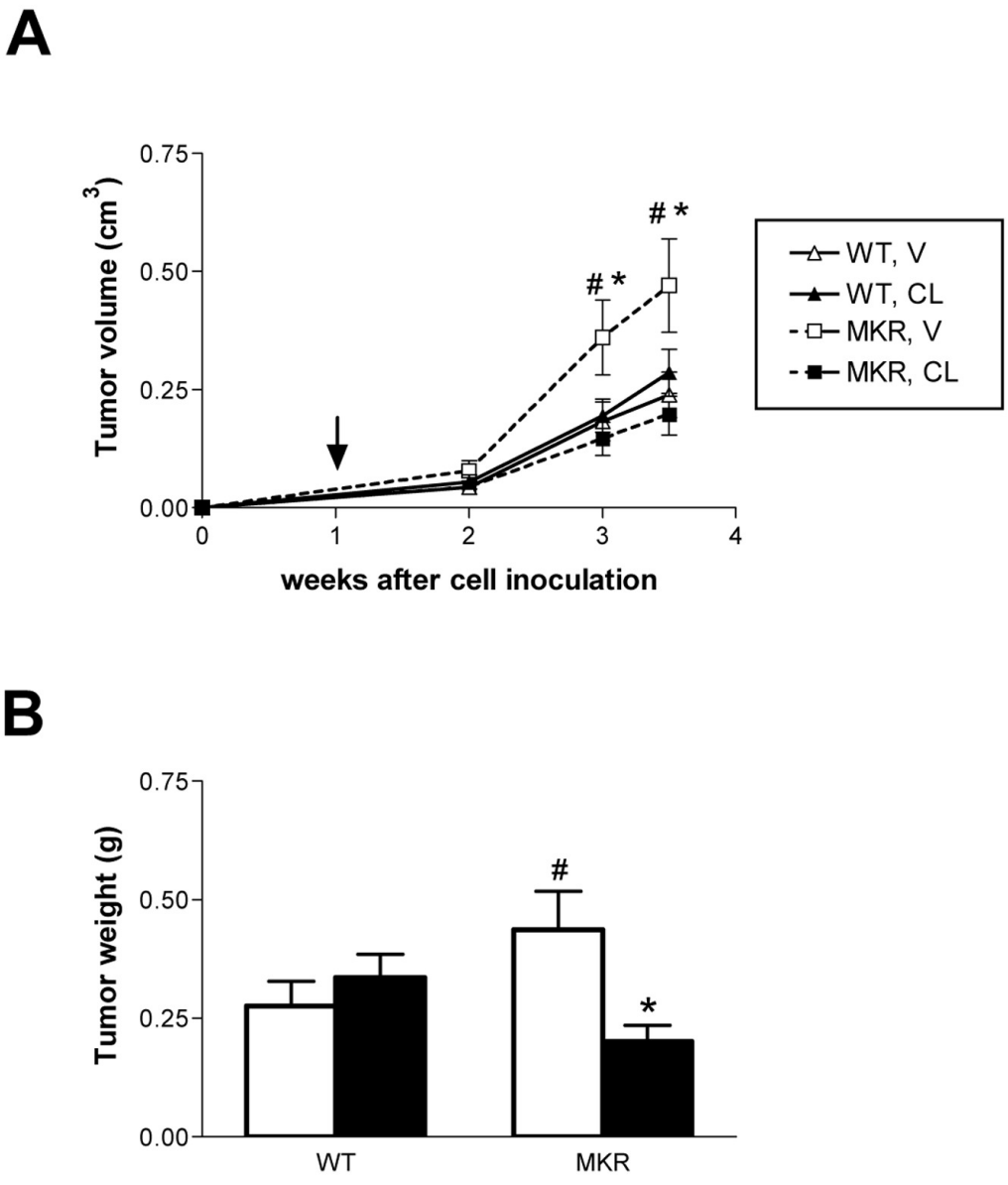
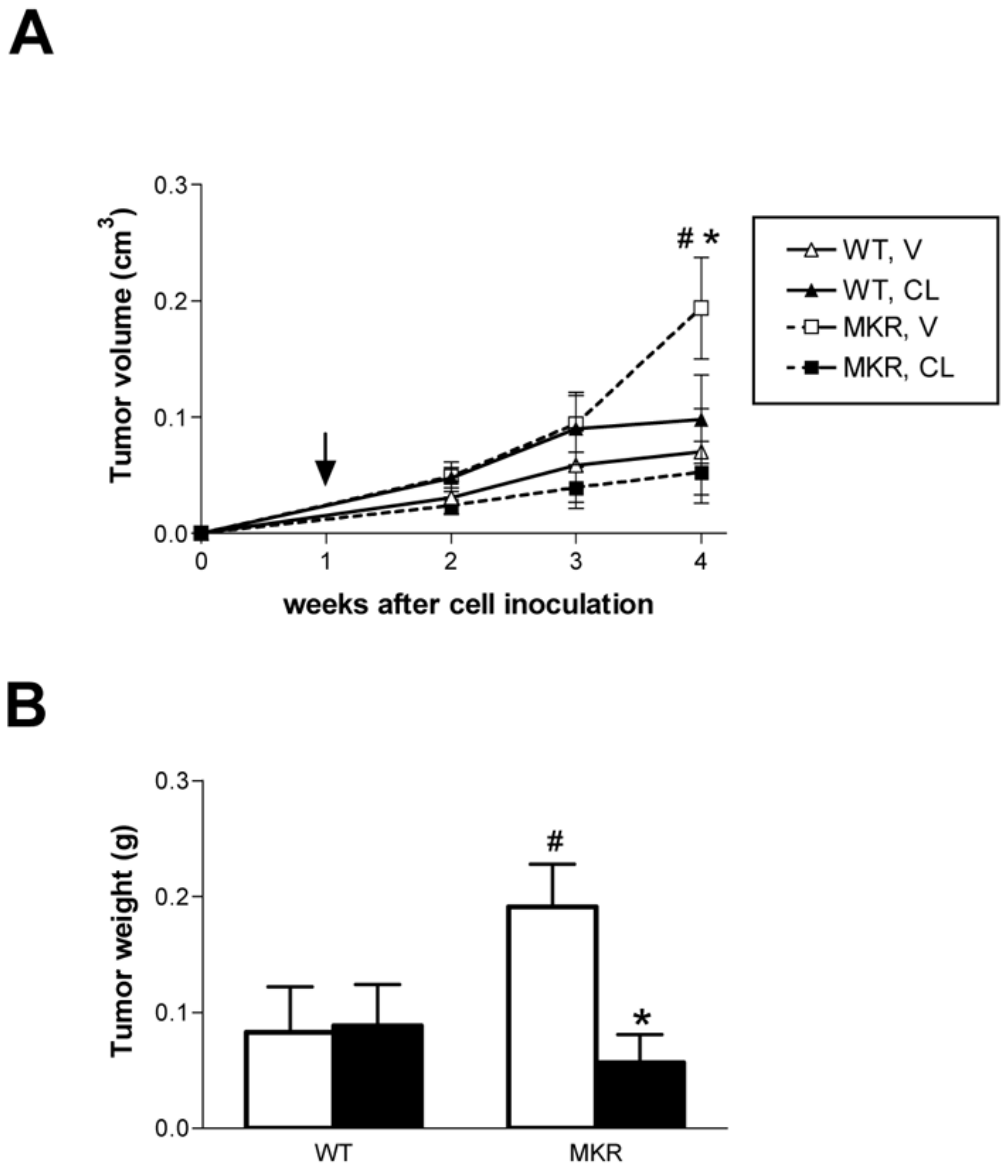

A
Tumor volume (cm3)
0.75
0.50
0.25
0.00
0
1
2
3
4
weeks after cell inoculation
#*
#*
WT, V
WT, CL
MKR, V
MKR, CL
B
Tumor weight (g)
0.75
0.50
0.25
0.00
#
*
WT
MKR

Figure 5


A
Tumor volume (cm3)
0.3
0.2
0.1
0.0
0
1
2
3
4
weeks after cell inoculation
*
WT, V
WT, CL
MKR, V
MKR, CL
B
Tumor weight (g)
0.3
0.2
0.1
0.0
#
*
WT
MKR