Hyperinsulinemia promotes metastasis to the lung in a mouse model of *Her2*-mediated breast cancer

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Abstract

The *Her2* oncogene is expressed in ~25% of human breast cancers and is associated with metastatic progression and poor outcome. Epidemiological studies report that breast cancer incidence and mortality rates are higher in women with type 2 diabetes. Here, we use a mouse model of *Her2*-mediated breast cancer on a background of hyperinsulinemia to determine how elevated circulating insulin levels affect *Her2*-mediated primary tumor growth and lung metastasis. Hyperinsulinemic (MKR^{+/+}) mice were crossed with doxycycline-inducible *Neu-NT* (MTB/TAN) mice to produce the MTB/TAN/MKR^{+/+} mouse model. Both MTB/TAN and MTB/TAN/MKR^{+/+} mice were administered doxycycline in drinking water to induce *Neu-NT* mammary tumor formation. In tumor tissues removed at 2, 4, and 6 weeks of *Neu-NT* overexpression, we observed increased tumor mass and higher phosphorylation of the insulin receptor/IGF1 receptor, suggesting that activation of these receptors in conditions of hyperinsulinemia could contribute to the increased growth of mammary tumors. After 12 weeks on doxycycline, although no further increase in tumor weight was observed in MTB/TAN/MKR^{+/+} compared with MTB/TAN mice, the number of lung metastases was significantly higher in MTB/TAN/MKR^{+/+} mice compared with controls (MTB/TAN/MKR^{+/+} 16.41 ± 4.18 vs MTB/TAN 5.36 ± 2.72). In tumors at the 6-week time point, we observed an increase in vimentin, a cytoskeletal protein and marker of mesenchymal cells, associated with epithelial-to-mesenchymal transition and cancer-associated fibroblasts. We conclude that hyperinsulinemia in MTB/TAN/MKR^{+/+} mice resulted in larger primary tumors, with more mesenchymal cells and therefore more aggressive tumors with more numerous pulmonary metastases.

Key Words

- ▶ type 2 diabetes
- ▶ breast cancer

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Introduction

Over the last three decades, the relationship between type 2 diabetes and breast cancer has been evaluated by numerous epidemiological studies. Most of these support

a positive association between diabetes and breast cancer, with several recent meta-analyses suggesting that women with type 2 diabetes are at significantly greater risk of

developing, relapsing with, and dying from breast cancer than women who do not have diabetes (Larsson *et al.* 2007, Peairs *et al.* 2011, Boyle *et al.* 2012). The relationship between insulin, specifically, and breast cancer has been assessed by several epidemiological studies. Independent of all other confounding factors, hyperinsulinemia was reported to be a significant factor for incident breast cancer (Pisani 2008, Gunter *et al.* 2009), while another study reported an association between raised insulin levels and breast cancer metastasis and mortality (Goodwin *et al.* 2002). Both raised C-peptide (a marker of secreted insulin levels) and insulin resistance have been recently reported to be linked to breast cancer-specific death (Duggan *et al.* 2011, Irwin *et al.* 2011). High circulating insulin levels have also been associated with a risk of breast cancer recurrence (Formica *et al.* 2012). After breast tumor surgery, elevated insulin levels in the circulation have been reported to be associated with an adverse prognosis during the first 5 years after diagnosis (Goodwin *et al.* 2012). While epidemiological studies only suggest a link between hyperinsulinemia and accelerated tumor growth, some proof-of-concept experimental studies indicate that hyperinsulinemia indeed promotes tumor development (Fierz *et al.* 2010, Novosyadlyy *et al.* 2010).

In our previous studies, we have specifically focused on the impact of hyperinsulinemia on breast cancer progression using the MKR mouse model. Female MKR mice are insulin resistant and hyperinsulinemic while the remaining are nonobese and only mildly hyperglycemic. Using this hyperinsulinemic model, we have observed significantly increased growth of transgenic (PyVmT) and orthotopically induced mammary tumors and have demonstrated a mechanistic link between hyperinsulinemia and increased cancer progression through increased activation of the insulin receptor (IR)/IGF1 receptor (IGF1R) and the phosphatidylinositol 3-kinase (PI3-K)/Akt/mTOR pathway (Novosyadlyy *et al.* 2010).

In the clinical setting, the differential influence of type 2 diabetes and its complications on subtype-specific breast cancer warrants further investigation. Around 25% of breast cancers belong to the subtype characterized by amplification of the gene encoding human epidermal growth factor receptor 2 (Her2), which is associated with high risk of metastasis and poor outcome (Slamon *et al.* 1989, Seshadri *et al.* 1993). Recently, a retrospective study has evaluated the benefit of the antidiabetic treatments metformin and thiazolidinediones on women with type 2 diabetes and Her2+ breast cancer. Initial analysis of the study cohort revealed that type 2 diabetes was a significant predictor of reduced overall survival in women with

stage 2 or higher Her2+ breast cancer, independent of other confounding factors such as age, estrogen receptor (ER)/progesterone receptor (PR) expression, and BMI. Furthermore, the antidiabetic therapies significantly increased overall survival and significantly reduced the risk of Her2+ breast cancer-specific mortality (He *et al.* 2012). Although insulin levels in these patients were not reported, both metformin and thiazolidinediones are insulin sensitizers, suggesting that improvements in insulin resistance in type 2 diabetics could have an impact on Her2+ breast cancer progression.

Her2 (also known as ErbB2 or *Neu* in rodents) belongs to the epidermal growth factor receptor family of receptor tyrosine kinases (RTK), which includes Her1 (also known as EGFR or ErbB1), Her3 (or ErbB3), and Her4 (or ErbB4). Unlike the other family members, no specific ligand has been identified for Her2 and its activity is dependent on its dimerization with either ligand-activated EGFR or Her3. At high expression levels resulting from *ERBB2* amplification, homodimers are also activated (Harari & Yarden 2000, Yarden 2001). Despite the lack of kinase activity of Her3, dimers of Her2/Her3 constitute the most potent signaling combination of all EGFR family dimers (Pinkas-Kramarski *et al.* 1996, Holbro *et al.* 2003), and a prevention of Her2/Her3 heterodimerization provides a significant clinical benefit in patients with Her2+ breast cancer (Baselga & Swain 2010). Activation of Her2/Her3 dimers leads to upregulation of multiple downstream pathways including the canonical PI3-K/Akt/mTOR signaling cascade and the mitogen-activated protein kinase (MAPK) pathway (Jin & Esteva 2008). Importantly, these two pathways are also the principal signaling pathways involved in the growth-promoting effects of the activated IR/IGF1R in tumorigenesis. Indeed, Her2 also dimerizes with the IGF1R leading to the emergence of resistance to Her2 pharmacotherapies (Lu *et al.* 2001).

Transgenic mouse models of Her2 (*Neu*)-mediated mammary carcinogenesis include those with constitutive activation of wild-type *Neu* (*c-Neu*) or oncogenic (activated) *Neu* (*Neu-NT*) under control of the MMTV promoter. Both *c-Neu* and *Neu-NT* overexpression result in invasive mammary carcinomas with latency periods of around 7 and 3 months respectively (Ursini-Siegel *et al.* 2007). A conditionally activated model of mammary-specific *Neu-NT* has also been engineered by crossing MMTV-reverse tetracycline transactivator (rtTA) (MTB) transgenic mice with mice bearing the *TetO-Neu-NT* transgene (TAN) to generate MTB/TAN offspring (Moody *et al.* 2002). Tumor latency in this model is short, with development of multiple mammary tumors with 100%

penetrance within a few weeks and further progression to spontaneous lung metastasis (Moody *et al.* 2002).

Given that in mammary tumors from *Neu-NT* transgenic mice, the PI3-K/Akt/mTOR signaling pathway should already be active, an interesting question that arises is whether the induction of systemic hyperinsulinemia in these mice could enhance *Neu-NT*-mediated tumor growth via IR/IGF1R activation. To address this issue, we crossed homozygous *MKR*^{+/+} mice with MTB/TAN mice to yield MTB/TAN/*MKR*^{+/+} offspring, thus generating hyperinsulinemic mice expressing a doxycycline-inducible *Neu-NT* transgene. When induced with doxycycline, MTB/TAN/*MKR*^{+/+} mice develop early *Neu-NT*-mediated mammary gland hyperplastic changes more rapidly than controls and go on to develop larger mammary tumors. Furthermore, MTB/TAN/*MKR*^{+/+} mice exhibit higher numbers of lung macrometastases, suggesting that chronic hyperinsulinemia can augment *Neu-NT*-mediated primary tumor growth as well as the progression to lung metastasis.

Materials and methods

Animal studies

Animal care and maintenance were provided by the Mount Sinai School of Medicine AAALAC Accredited Animal Facility. All procedures were approved by the Institutional Animal Care and Use Committee of the Mount Sinai School of Medicine according to the National Institute of Health Guidelines. All mice used in this study were on Friend Virus B (National Institute of health) (FVB/N) genetic background. Mice were housed four per cage in a clean mouse facility and fed a standard mouse chow (PicoLab Rodent Diet 20, 5053; LabDiet, Brentwood, MO, USA) *ad libitum* on a 12 h light:12 h darkness cycle. Plasma insulin levels were measured by the sensitive rat insulin RIA kit (Millipore, St Charles, MO, USA). An insulin tolerance test was performed on animals previously fasted for 4 h. Insulin (0.75 units/kg of body weight) was injected intraperitoneally and blood glucose values were measured immediately before and 15, 30, and 60 min after insulin injection. For induction of *Neu-NT*, mice were administered 1.5 mg/ml doxycycline (Sigma–Aldrich) in drinking water for the duration of the period of tumor growth from 2 weeks up to 12 weeks. Doxycycline water was changed twice per week. Mice were followed on a daily basis and body score conditions were recorded. To determine tumor mass, each animal was killed and mammary tumors from all thoracic and inguinal glands

were carefully dissected and weighed. For analysis of pulmonary metastases, mice were killed and lungs were inflated via the trachea with 10% formalin, removed, and examined for macrometastatic lesions.

Mammary gland whole mount analysis

Inguinal mammary glands were removed, placed on a glass slide, and fixed for 4 h in Carnoy's fixative (60% ethanol (100%), 30% chloroform, and 10% glacial acetic acid). Glands were serially hydrated in 100, 95, 70, 50, and 30% ethanol for 15 min each, rinsed in water for 5 min, and stained overnight with carmine alum. Glands were then serially dehydrated in 30, 50, 70, 95, and 100% ethanol for 15 min each and cleared overnight in xylene. Glands were then covered by Mount-Quick mounting medium (Daido Sangyo, Tokyo, Japan), before a glass coverslip was placed on top. Photographs were carried out using a stereomicroscope (Zeiss, Thornwood, NY, USA) at 4× magnification. Quantification of the relative area of end buds was performed using Image J by measuring the mean end bud area of four random images at 4× objective taken from each whole mount.

Histology and immunofluorescence

Lungs were fixed in 10% formalin before being embedded in paraffin and sectioned and stained using hematoxylin and eosin (H&E). For immunofluorescence studies, mammary tumors were cut in cross section at the time of killing, fixed in 10% formalin before being embedded in paraffin, and sectioned. Five-micron sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Primary antibodies used were a rabbit polyclonal antibody to vimentin (Cell Signaling Technologies, Danvers, MA, USA) and a mouse MAB to Neu (Abcam, Cambridge, MA, USA). Secondary antibodies used were AlexaFluor-568-conjugated goat anti-rabbit IgG and Alexa-Fluor-488-conjugated goat anti-mouse IgG (Invitrogen, Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 0.2 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma–Aldrich).

Western blotting

Tumor tissues were lysed in chilled lysis buffer (pH 7.4) containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1.25% CHAPS, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 8 mM B-glycerophosphate, and Complete Protease Inhibitor Cocktail tablet. Protein

concentration of samples was measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). Protein samples were resuspended in $3\times$ loading buffer containing DTT (Cell Signaling Technologies) and denatured by boiling for 5 min at 96°C . Samples were then subjected to SDS-PAGE (8 or 8–16% Tris–glycine gel; Life Technologies) and transferred to a nitrocellulose membrane. Membranes were probed with the appropriate primary antibodies: anti-phospho IR- $\beta^{(\text{Y1150/51})}$ /IGF1R $\beta^{(\text{Y1135/36})}$, anti-phospho Akt $^{\text{Ser473}}$, anti-total Akt, and anti-vimentin (Cell Signaling Technologies) and then reblotted with B-actin (Sigma–Aldrich) or anti-IR β (Santa Cruz Biotechnology) before being incubated with secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) and being exposed to the LI-COR infrared detection system (LI-COR Biosciences).

Statistical analysis

Statistical analyses were conducted using the Student's *t*-test. Results are expressed as means $\pm$ S.E.M.

Results

MTB/TAN/MKR $^{+/+}$ mice express the same metabolic phenotype as the parental MKR $^{+/+}$ mouse strain

Transgenic MMTV-rtTA (MTB) and TetO-*Neu-NT* (TAN) mice were previously crossed to yield bitransgenic MTB/TAN offspring demonstrating doxycycline-dependent *Neu-NT* expression in luminal mammary epithelial cells and subsequent development of gross mammary tumors and pulmonary macrometastases (Moody *et al.* 2002). We crossed MTB/TAN with homozygous MKR mice to yield MTB/TAN/MKR $^{+/+}$ mice. Female MTB/TAN/MKR $^{+/+}$ mice showed the same metabolic abnormalities as the homozygous female MKR $^{+/+}$ mice (Novosyadlyy *et al.* 2010) namely lowered body weight, mild hyperglycemia, increased systemic insulin, and severe insulin resistance (Fig. 1A, B, C, and D).

MTB/TAN/MKR $^{+/+}$ mice show augmented *Neu-NT*-induced abnormalities of the mammary gland at 8 weeks compared with MTB/TAN controls

Others have shown that doxycycline-induced *Neu-NT* expression results in noticeable hyperplastic abnormalities in the mouse mammary gland such as the presence of cellular masses along the length of ducts, as well as terminal end bud enlargement due to the presence of

Figure 1

Metabolic characterization of the MTB/TAN/MKR $^{+/+}$ mouse model. (A) Body weight (MTB/TAN, $n=14$; MTB/TAN/MKR $^{+/+}$, $n=10$), (B) blood glucose (MTB/TAN, $n=30$; MTB/TAN/MKR $^{+/+}$, $n=22$), and (C) serum insulin (MTB/TAN, $n=13$; MTB/TAN/MKR $^{+/+}$, $n=20$) of MTB/TAN and MTB/TAN/MKR $^{+/+}$ mice at 8 weeks of age. (D) An insulin tolerance test was performed on fasted 8-week-old MTB/TAN/MKR $^{+/+}$ ($n=5$) and MTB/TAN mice ($n=5$) after i.p. injection of insulin (0.75 units/kg). Blood samples were obtained from the tail vein and glucose concentrations were determined at the indicated time points. Graphs represent mean values of each group, error bars represent the S.E.M. * P value < 0.05 .

acinar-like structures (Moody *et al.* 2002). We investigated whether hyperinsulinemia would have an additive effect on these early *Neu-NT*-mediated hyperplastic abnormalities by induced *Neu-NT* expression for 3 days in 8-week-old MTB/TAN/MKR^{+/+} and MTB/TAN mice, killing the animals and comparing mammary gland morphology by whole mount analysis. As MKR^{+/+} mice have precocious mammary gland growth and differentiation compared with control mice at both 3 and 15 weeks (Novosyadlyy & LeRoith 2010), we also performed whole mount analysis and H&E staining of mammary glands from 8-week-old MTB/TAN/MKR^{+/+} and MTB/TAN mice, which had not been administered doxycycline (Fig. 2A and B, upper panels). In MTB/TAN/MKR^{+/+} mice, we found *Neu-NT*-mediated abnormalities to be enhanced compared with MTB/TAN controls, with larger cellular masses growing on ducts, greater terminal end bud enlargement, and precocious lobular development (Fig. 2A, lower panels and C). This finding was further confirmed by histological evaluation of H&E-stained

sections of the mammary gland (Fig. 2B, lower panels) where we observed advanced ductal hyperplasia in MTB/TAN/MKR^{+/+} mice compared with MTB/TAN controls.

***Neu-NT*-induced mammary tumor burden is greater in MTB/TAN/MKR^{+/+} mice than in MTB/TAN mice and is regulated through IR/IGF1R signaling pathways**

When doxycycline was administered for several weeks, MTB/TAN mice developed multiple invasive mammary adenocarcinomas with tumors arising in all inguinal and thoracic glands (Moody *et al.* 2002). We administered doxycycline to 8-week-old MTB/TAN and MTB/TAN/MKR^{+/+} mice for 2, 4, 6, or 12 weeks. Mice were killed at each time point and the combined tumor weight from all inguinal and thoracic glands from each mouse was recorded. As shown in Fig. 3A, MTB/TAN/MKR^{+/+} mice exhibited significantly higher total tumor mass than MTB/TAN controls at all time points with the exception of the 12-week time point, suggesting

Figure 2

Effect of hyperinsulinemia on *Neu-NT*-mediated mammary gland hyperplasia. (A) Whole mount analysis of mammary glands obtained from 8-week-old female MTB/TAN and MTB/TAN/MKR^{+/+} mice without doxycycline (–Dox) and after 3 days of doxycycline treatment (+Dox). (B) Histological analysis (H&E staining) of mammary glands after 3 days of doxycycline treatment. (C) Quantification of percentage area of mammary

gland whole mounts composed of end buds (MTB/TAN, *n*=3; MTB/TAN/MKR, *n*=3). Arrows, End buds (A) and ductal hyperplasia. (C) At least five animals per group were analyzed and the representative images are shown. Original magnification, $\times 4$ (A) and $\times 40$ (B). Graph represents mean for each group, error bars represent S.E.M. **P* value < 0.05.

Figure 3

Tumor growth, IR β /IGF1R, and Akt signaling are enhanced in tumors from MTB/TAN/MKR^{+/+} mice compared with MTB/TAN controls. (A) MTB/TAN and MTB/TAN/MKR^{+/+} mice were maintained on doxycycline for 2, 4, 6, or 12 weeks. Mice were killed and the combined tumor mass from all mammary glands was recorded. For the 2-week time point, MTB/TAN, $n=11$; MTB/TAN/MKR^{+/+}, $n=4$. For the 4-week time point, MTB/TAN, $n=11$; MTB/TAN/MKR^{+/+}, $n=7$. For the 6-week time point, MTB/TAN, $n=16$; MTB/TAN/MKR^{+/+}, $n=6$. For the 12-week time point, MTB/TAN, $n=12$; MTB/TAN/MKR^{+/+}, $n=11$. (B) Tumor tissue was subjected to western blot

analysis and probed with antibodies to phosphorylated IR β (Y1150/51)/IGF1R β (Y1135/36) and then reblotted with antibodies to IR β . (D) Western blot analysis of tumor tissue from mice maintained on doxycycline for 2 weeks probed with antibodies to phosphorylated Akt^{Ser473} and total Akt. Using densitometry, protein expression was quantified (C and E). Error bars represent s.e.m. * $P<0.05$. For the 2-week time point, western blot is representative of MTB/TAN, $n=9$; MTB/TAN/MKR^{+/+}, $n=4$ samples. For 4- and 6-week time points, MTB/TAN, $n=7$; MTB/TAN/MKR^{+/+}, $n=7$ samples. For 12-week time point MTB/TAN, $n=10$; MTB/TAN/MKR^{+/+}, $n=10$.

that hyperinsulinemia augments *Neu-NT*-driven mammary tumor growth. We extracted proteins from tumor tissues to examine whether IR/IGF1R activation was upregulated in MTB/TAN/MKR^{+/+} mice compared with MTB/TAN controls. As shown in Fig. 3B and C, mammary tumor tissues from MTB/TAN/MKR^{+/+} mice demonstrated higher levels of phosphorylated IR/IGF1R (IR β /IGF1R β) after 2, 4, and 6 weeks of *Neu-NT* upregulation, suggesting that these receptors may be involved in mediating the additional tumor growth present in MTB/TAN/MKR^{+/+} mice. In MTB/TAN/MKR^{+/+} and MTB/TAN mice that had been administered doxycycline for a period of 12 weeks, we observed an increase in IR/IGF1R phosphorylation in mammary tissues of MTB/TAN/MKR^{+/+} mice, which was not statistically significant (Fig. 3B and C). There was no significant difference in tumor weights after 12 weeks

of doxycycline administration, possibly due to tumors reaching their physiologically maximal size after this time period (Fig. 3A). We also compared activation of Akt, which lies downstream of both the IR/IGF1R and Her2 and found that at 2 weeks there was a significant upregulation of phosphorylated Akt in tumors from MTB/TAN/MKR^{+/+} mice compared with MTB/TAN mice (Fig. 3D and E). At later stages of tumor development, levels of activation of Akt in the tumors appeared to be similar in the two groups.

***Neu-NT*-induced spontaneous lung macrometastases are enhanced in MTB/TAN/MKR^{+/+} mice after 12 weeks of tumor growth**

MTB/TAN mice were reported to harbor *Neu-NT*-dependent spontaneous lung macrometastases (Moody *et al.* 2002).

We removed the lungs of killed MTB/TAN/MKR^{+/+} and MTB/TAN mice after 2, 4, 6, or 12 weeks of doxycycline-induced *Neu-NT* expression in mammary epithelium and recorded the numbers of visible macrometastases. We also examined H&E-stained sections of lung at all time points for both micrometastases and macrometastases. After 2, 4, or 6 weeks of *Neu* expression and mammary tumor growth, no visible macrometastases were observed in either MTB/TAN/MKR^{+/+} or MTB/TAN mice. However, H&E staining revealed the presence of micrometastases in lungs of both groups of mice after 6 weeks of *Neu* expression (data not shown). Although there was a trend for the number of micrometastases to be increased in MTB/TAN/MKR^{+/+} mice (0.3 micromets/lung section in MTB/TAN vs 0.8 micromets/lung section in MTB/TAN/MKR^{+/+}), this did not reach statistical

significance. In contrast, after 12 weeks of *Neu* expression in mammary tissue, macrometastases could be clearly observed in the lungs, and these were significantly increased in MTB/TAN/MKR^{+/+} compared with MTB/TAN mice (Fig. 4A and B), suggesting that hyperinsulinemia increases the metastatic potential of *Neu*-driven mammary carcinogenesis.

***Neu-NT*-induced mammary tumors in MTB/TAN/MKR^{+/+} mice express higher levels of vimentin**

To determine whether hyperinsulinemia enhances the progression of *Neu-NT*-mediated primary tumors to lung metastases, we analyzed tumor tissue for the expression of vimentin protein, a marker of mesenchymal cells. As shown in Fig. 5A and B, western blot analysis of tumor

Figure 4

Metastatic progression is enhanced in MTB/TAN/MKR^{+/+} mice. After 12 weeks on doxycycline, MTB/TAN and MTB/TAN/MKR^{+/+} mice were killed, lungs were removed and inflated, and (A) number of lung macrometastases were recorded. Graph represents the mean for each group, error bars

represent S.E.M. **P* value < 0.05. (B) Lungs were paraffin embedded, sectioned, and stained with H&E to reveal macrometastases. Original magnification 4×. Arrows indicate metastatic cells.

lysates demonstrated increased vimentin expression in tumors from 6-week-old MTB/TAN/MKR^{+/+} mice compared with MTB/TAN mice. We additionally analyzed vimentin expression in 6-week tumor tissues by immunofluorescent staining of paraffin-embedded sections. As shown in Fig. 5B and C, vimentin levels as measured by immunofluorescence were significantly elevated, indicating that more mesenchymal cells were present in MTB/TAN/MKR^{+/+} mice at this time point. The vimentin-positive cells generally did not stain positive for Neu, indicating that these were either cells that had undergone epithelial-to-mesenchymal transition (EMT) and lost Neu expression, or were cancer-associated fibroblasts. At 4 and 12 weeks of tumor development, we did not observe significant differences in vimentin expression (data not shown).

Discussion

Epidemiological evidence suggests a causal relationship between type 2 diabetes and the incidence, recurrence, and mortality from breast cancer. Hyperinsulinemia has been identified as a specific factor that may drive breast cancer cell proliferation, but data on human patients that focus on how specific subtypes of breast cancer respond to elevated circulating insulin are limited. Overexpression of ErbB2/Her2 is responsible for around 25% of all human breast cancers and results in aggressive primary tumors, which commonly metastasize to the lungs. In this study, we have employed the MKR mouse model with a conditionally activated *Neu-NT* to represent this subtype of breast cancer. We have demonstrated for the first time

Figure 5

Vimentin expression is enhanced in MTB/TAN/MKR^{+/+} mice. (A). After 6-weeks *Neu-NT* upregulation, mammary tumors were removed from MTB/TAN and MTB/TAN/MKR^{+/+} mice, tumor lysates were subjected to western blot analysis, and probed with anti-vimentin antibodies. Using densitometry, protein expression was quantified (B). Tumor tissue was paraffin embedded, sectioned, and analyzed by immunofluorescence microscopy for the expression of vimentin (red) and Neu (green) proteins,

nuclei were stained with DAPI (blue) (C). Vimentin expression was analyzed using 'Image J' program to quantify expression of vimentin protein in 6-week tumors from MTB/TAN and MTB/TAN/MKR^{+/+} mice (D). Photographs are representative of at least four mice from each group. Five high-power fields were photographed on every slide and each image was quantified. Original magnification 40×. Graphs represent the mean for each group, error bars represent s.e.m. **P* value < 0.05.

in an animal model that hyperinsulinemia significantly affects the rate of *Neu-NT*-mediated mammary tumor development as well as the progression of breast cancer metastasis to the lungs.

Previously, we have employed the MKR mouse model to demonstrate that PyVmT-driven mammary gland hyperplasia at 3 and 15 weeks' postnatal development is more advanced under conditions of hyperinsulinemia (Novosyadlyy et al. 2010). Hyperplasia of the mammary gland has previously been shown to occur rapidly (3–4 days) after conditionally activated *Neu-NT* expression (Moody et al. 2002). In MTB/TAN/MKR^{+/+} mice, we observed that in the presence of elevated insulin, mammary gland hyperplasia was more advanced after 3 days, suggesting that hyperinsulinemia can enhance the mitogenic effect of *Neu* oncogenic transformation on terminal end buds during mammary growth and development. The MMTV-c-*Neu* mouse model has been previously used to determine the effect of a high-fat diet (HFD) on mammary gland hyperplasia. Although this is a different model from ours, having a much longer tumor latency, it is interesting to note that either 5 or 10 weeks HFD treatment in FVB/N mice had no effect on mammary gland hyperplasia in MMTV-c-*Neu* mice compared with controls, suggesting that at very early stages of tumor development, insulin may have a more potent effect than dietary fat in promoting terminal end bud hyperplasia. In the same model, it was shown that an HFD based on corn oil resulted in a shorter tumor latency than an HFD based on fish oils, suggesting that at later stages of tumor development, fats may play a role in tumor development but individual dietary components have different effects (Luijten et al. 2007).

We found that chronic (2–6 weeks) elevations of *Neu-NT* led to significantly larger mammary tumors in MTB/TAN/MKR^{+/+} mice compared with controls, suggesting that hyperinsulinemia enhances *Neu-NT*-mediated tumor formation and growth. This result is in agreement with our previous finding that orthotopic inoculation of three different mammary tumor cell lines (one over-expressing *Neu*) each resulted in larger tumor formation in MKR mice compared with controls (Novosyadlyy et al. 2010, Ferguson et al. 2012) and demonstrates that *Neu-NT*-mediated mammary tumor growth can be specifically enhanced by hyperinsulinemia. The mechanism by which insulin could increase tumor growth in the *Neu-NT* model may be complex. Our results demonstrate that MTB/TAN/MKR^{+/+} mice demonstrate higher levels of phosphorylated IR/IGF1R than MTB/TAN control mice, suggesting that insulin is acting through its cognate

receptor and/or the highly homologous IGF1R to promote mammary tumor growth. In human breast cancer tissues, phosphorylated IR/IGF1R has been reported to be a prognostic marker of poor outcome for breast cancer, regardless of subtype (Law et al. 2008). The ratio of IR isoform A (IR-A) to isoform B (IR-B) is also important in breast cancer, and recently, a higher IR-A:IR-B ratio has been observed in the luminal B subtype of breast cancer (Huang et al. 2011). Breast cancer cells in culture have also been reported to express higher levels of the IR (Osborne et al. 1978) and proliferate directly in response to insulin (Bollig-Fischer et al. 2011). The signaling pathways activated by the *Neu-NT* tyrosine kinase significantly overlap with those of the IR/IGF1R and include the canonical PI3-K/Akt/mTOR and MAPK signaling pathways. It is thus possible that activation of the IR/IGF1R causes amplification of the canonical *Neu* signaling pathways, which could possibly lead to increased tumor cell proliferation and/or survival.

Under conditions of hyperinsulinemia, IGF binding proteins (IGFBP)-1 and -2 are repressed (Calle & Kaaks 2004), which may lead to an increase in circulating 'free' IGF1. In the MTB/TAN/MKR^{+/+} mouse model, increased IGF1 levels at the level of the target tissue could lead to increased activation of the IGF1R, thus directly increasing tumor growth. The *Neu* RTK has no known ligand, depending on its activation upon dimerization with either ErbB3 or ErbB1 (Cho et al. 2003, Garrett et al. 2003). Interestingly, *Neu* also dimerizes with IGF1R, an occurrence that provides a significant source of resistance to *Her2*-mediated therapies in humans (Lu et al. 2001, 2004). Thus, in our MTB/TAN/MKR^{+/+} mouse model, it is possible that elevations of either insulin or 'free' IGF1 could promote tumor growth through increased activation of *Neu*/IGF1R hybrids (Nahta et al. 2006).

Our finding of greater numbers of lung metastases in MTB/TAN/MKR^{+/+} mice suggests that, as well as promoting primary tumor growth, insulin may also enhance primary tumor progression and/or circulating tumor cell survival in the lung. Our previous work has demonstrated that murine mammary tumor cell line Mvt1 is able to form more lung metastases in MKR mice as a result of whether an orthotopic cell inoculation or an intravenous injection (Ferguson et al. 2012). Evidence that the IR specifically is involved in either homing to or survival in the lung has come from a report by Zhang et al. (2010), who showed that shRNA knockdown of the IR in breast cancer cell line LCC6 resulted in reduced ability of these cells to form lung metastases following intravenous injection into nude mice. The EMT permits tumor cells to gain the plasticity

required to extravasate from the primary tumor site. During extravasation cells become increasingly mesenchymal in nature due to numerous changes in cytoskeletal scaffolding protein structures. Vimentin belongs to the intermediate filament (IF) family of proteins and has recently been shown to be an important marker of the EMT in epithelial cells that normally express only cytokeratin-type IFs (Zeisberg & Neilson 2009, Satelli & Li 2011). Vimentin is not specific for EMT and is also a marker of cancer-associated fibroblasts (Sugimoto et al. 2006). Additionally, recent studies have demonstrated that cancer-associated fibroblasts induce EMT in breast cancer cells (Soon et al. 2013). In breast cancer cells, vimentin expression has been shown to be correlated with increased migration and invasion (Gilles et al. 2003, Korsching et al. 2005), and in human breast cancer specimens, several studies have reported overexpression of vimentin as a marker of poor prognosis (Kokkinos et al. 2007). In this study, we observed elevated expression of vimentin as early as 6 weeks of Neu-NT upregulation. The majority of the vimentin-positive cells did not co-stain with Neu. This suggests the MTB/TAN/MKR^{+/+} mice have a greater desmoplastic reaction to the tumors, with more numerous cancer-associated fibroblasts. Another explanation that we cannot exclude is that these vimentin-positive cells are tumor cells that have undergone EMT and have lost Neu expression. Our finding of hyperinsulinemia leading to greater lung metastasis could thus be due to an increase in the dissemination of tumor cells from the primary tumor associated with increase in the number of cancer-associated fibroblasts, or due to a direct effect of insulin on the tumors, inducing EMT. It is also possible that hyperinsulinemia may lead to greater cell survival, intravasation, and proliferation in lung tissue.

In summary, we have shown that hyperinsulinemia promotes advanced mammary gland hyperplasia, primary tumor growth, and lung metastasis in a Her2/Neu model of breast cancer. Further studies are required to determine whether, in promoting lung metastases, hyperinsulinemia enhances cell dissemination from the primary tumor, cell survival/adhesion in the lung, or both of these processes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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