

The Wnt-5a – Derived Hexapeptide Foxy-5 Inhibits Breast Cancer Metastasis *In vivo* by Targeting Cell Motility

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Abstract Purpose: An inherent problem in breast cancer treatment is that current therapeutic approaches fail to specifically target the dissemination of breast cancer cells from the primary tumor. Clinical findings show that the loss of Wnt-5a protein expression in the primary breast tumor predicts a faster tumor spread, and *in vitro* analyses reveal that it does so by inhibiting tumor cell migration. Therefore, we hypothesized that the reconstitution of Wnt-5a signaling could be a novel therapeutic strategy to inhibit breast cancer metastasis.

Experimental Design: We used *in vitro* techniques to show that 4T1 mouse breast cancer cells responded to the reconstitution of Wnt-5a signaling using our novel Wnt-5a mimicking hexapeptide, Foxy-5, in the same way as human breast cancer cells. Therefore, we could subsequently study its effect *in vivo* on the metastatic spread of cancer following the inoculation of 4T1 cells into mice.

Results: *In vitro* analyses revealed that both recombinant Wnt-5a and the Wnt-5a – derived Foxy-5 peptide impaired migration and invasion without affecting apoptosis or proliferation of 4T1 breast cancer cells. The *in vivo* experiments show that i.p. injections of Foxy-5 inhibited metastasis of inoculated 4T1 breast cancer cells from the mammary fat pad to the lungs and liver by 70% to 90%.

Conclusions: These data provide proof of principle that the reconstitution of Wnt-5a signaling in breast cancer cells is a novel approach to impair breast tumor metastasis by targeting cell motility. In combination with existing therapies, this approach represents a potential novel therapeutic strategy for the treatment of breast cancer patients.

Breast cancer is the most common malignancy among women, and it is predicted to afflict 1 in 10 females during their lifetime. The development of improved diagnostics and therapeutics for breast cancer had led to a reduction in overall mortality rates (1). However, tumor metastasis is the predominant cause of death among patients, and the 5-year survival remains less than 30% in patients with distal spread of their tumor (1).

In this context, the nontransforming Wnt-5a protein seems to be a promising therapeutic candidate, as the lack of Wnt-5a protein expression in the primary tumor of breast cancer patients strongly correlates with an increased risk of metastasis and reduced survival (2, 3). This inhibitory effect of Wnt-5a on metastasis may be due to the ability of Wnt-5a to increase adhesion and thereby decrease migration of breast epithelial cells (4, 5), as well as to its ability to counteract activation of NFAT (6), a transcription factor that has been implicated in promoting breast cancer cell invasiveness (7, 8). Indirect support for a suppressive effect of Wnt-5a on breast cancer cell migration comes from a recent and elegant study of mammary gland development (9). In this study the investigators clearly show that Wnt-5a signaling is capable of inhibiting ductal extension and lateral branching in the mammary gland most likely via an increased cell adhesion. A tumor-suppressing effect of Wnt-5a has also been found in B lymphomas (10, 11), thyroid carcinoma (12), and colon cancer (13, 14). In contrast, Wnt-5a has been shown to induce migration and invasion in gastric cancer (15) and melanoma (16, 17).

The Wnt proteins function in an auto or paracrine fashion. This mechanism of action is promoted by the fact that immediately following their release from a cell, they rapidly bind to heparan sulfate proteoglycans on the same or neighboring cell surface, which functions to effectively present Wnt proteins to their respective cell surface receptor (18). Consequently, the heparan sulfate-binding domain on Wnt

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Translational Relevance

The primary tumor is rarely the cause of death for cancer patients. In fact, in the majority of cases cancer-associated mortality is the result of cancer metastasis. The progression of most primary tumors leads to an increase in tumor cell motility. A low-level expression of Wnt-5a protein in primary invasive breast carcinomas is associated with breast cancer cells with increased motility and in shortened recurrence-free survival. Consequently, an attractive antimetastatic approach would be to reconstitute Wnt-5a signaling in breast cancers that have a reduced expression of this protein (50% of all cases). Based on sequence analysis of Wnt-5a, we identified a hexapeptide fragment that after modification (formylation) mimicked the inhibitory effect of Wnt-5a on breast cancer cell migration. In the present study we show that *i.p.* injection of this peptide, Foxy-5, causes a significant reduction (70–90%) of liver and lung metastases in immunodeficient as well as in immunocompetent mice. These results indicate that the reconstitution of Wnt-5a signaling is a valid approach to impair breast cancer metastasis. It is reasonable to presume that the development and use of a specific antimetastatic drug, based on Foxy-5, will increase the quality of life and the overall survival of breast cancer patients.

proteins limits the distribution of a released Wnt protein to the area closest to the cell from which it originated. This implies that administration of a Wnt protein is likely to result in a very restricted dispersal from the site of deposition, thus presenting difficulty in using an intact Wnt protein *in vivo*.

Wnt proteins bind to G-protein coupled receptors of the Frizzled family (19, 20). The functional effect of a particular Wnt protein is not only determined by the Frizzled receptor it binds to, but also by the presence of low-density lipoprotein receptor-related proteins, LRP5/6, and the tyrosine kinase receptor Ror2 that have been shown to function as coreceptors or complementary receptors (21–23). Regarding their function as coreceptors in Wnt-5a signaling, Ror2 has been shown to affect the migratory response to Wnt-5a in human melanoma and mouse fibroblasts (23), whereas LRP5/6 has not been reported to affect the migratory response to Wnt-5a.

In the present study, we have taken advantage of the newly characterized formylated Wnt-5a–derived hexapeptide Foxy-5, which lacks a heparan sulfate-binding domain but mimics the Wnt-5a–induced inhibition of human breast cancer cell migration *in vitro* (5), to investigate the potential *in vivo* effect of reconstituting Wnt-5a signaling. Although it is possible that degradation of such a peptide can occur *in vivo*, small formylated peptides released from bacteria at an infection-induced inflammatory site can potently activate cells of the innate immune system despite the low pH and high protease activities in this milieu (24). Consequently, the fact that the Foxy-5 peptide was designed with a formyl group on its NH₂-terminal methionine residue means that it is potentially similarly protected from degradation *in vivo*.

In this study, we investigated the mechanisms whereby recombinant Wnt-5a (rWnt-5a) and the Foxy-5 peptide affect mouse 4T1 breast tumor cells *in vitro* as a means to interpret

the effects of Foxy-5 administration on metastasis of 4T1 breast tumors in BALB/c mice.

Materials and Methods

Antibodies and peptides. The following antibodies and peptides were used: β -actin monoclonal AC-15 antibody and formyl-Nle-Leu-Phe-Nle-Tyr-Lys (fcp) peptide (Sigma Aldrich Corporation); Ki67 polyclonal antibody (Affinity BioReagents); phospho-p44/42 MAPK Thr202/Tyr204 polyclonal antibody and total p44/42 MAPK polyclonal antibody (Cell Signaling Technology, Inc.); poly(ADP)ribose polymerase monoclonal antibody (Biomol International); rWnt-5a and Ror2 goat polyclonal antibody (R&D Systems); and formyl-Met-Asp-Gly-Cys-Glu-Leu (Foxy-5), Met-Asp-Gly-Cys-Glu-Leu (nfp), and formyl-Met-Ser-Ala-Asp-Val-Gly (RnF) peptides (Pepscan Systems). The synthesized peptides (>95% pure) were quality-controlled by reversed phase–high performance liquid chromatography and mass spectrometry and synthesized on four different occasions. The polyclonal antibodies toward Wnt-5a, Frizzled-2, and Frizzled-5 were produced in our laboratory, as previously described (4, 5). The polyclonal antibody against amino acids 202–220 in the ectodomain of the Frizzled-2 receptor was used as a control antibody. The polyclonal anti–Wnt-5a antibody was raised against amino acids 275–290 of the Wnt-5a molecule (4), a segment that exhibits 100% homology between human and mouse Wnt-5a.

Cell culture. The human mammary carcinoma cell lines MDA-MB-468 (negative control for Wnt-5a expression) and T47D (positive control for Wnt-5a expression) were grown in DMEM D5796 supplemented to contain 10% fetal bovine serum, 5 U/mL penicillin, 0.5 U/mL streptomycin, and 2 mmol/L glutamine. The mouse mammary epithelial cell line 4T1 was grown in RPMI 1640 supplemented to contain 10% fetal bovine serum, 1.5 g/L sodium bicarbonate, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4.5 g/L glucose, 5 U/mL penicillin, 0.5 U/mL streptomycin, and 2 mmol/L glutamine. The mouse macrophage cell lines J774 and Raw264.7 were grown in DMEM D5796 supplemented to contain 10% fetal bovine serum and 2 mmol/L glutamine, and serum-starved in Macrophage SFM serum-free medium 12065. All cells were incubated at 37°C in 5% CO₂.

Western blot. The cells were lysed with a lysis buffer (5, 13) and centrifuged at 15,000 rpm for 8 min at 4°C. The protein content in each sample was determined and adjusted to ensure equal loading of protein in each lane. Thereafter, 50 mmol/L DTT and 5× concentrated Laemmli buffer were added, and the lysates were boiled for 5 min and separated by SDS gel electrophoresis. The proteins were subsequently transferred to polyvinylidene difluoride membranes, blocked in 5% nonfat milk or 3% bovine serum albumin for 45 min, and incubated for 1 h with the indicated primary antibody [1:15,000 for actin; 1:5,000 for Frizzled-5; 1:2,000 for Wnt-5a and Ror2; and 1:1,000 for phospho p44/42 MAPK, p44/42 MAPK, Frizzled-2, and poly(ADP)ribose polymerase] in 1% nonfat milk or bovine serum albumin. After incubating for 1 h with a horseradish peroxidase-conjugated secondary antibody and extensive washing, the antibody-antigen complexes were detected using an enhanced chemiluminescence kit from Amersham Pharmacia Biotech. For reprobing, the membranes were stripped with a Reblot Strong solution from Chemicon International. The Western blots shown are representative of at least five independent experiments.

Cell migration. Cell migration (Fig. 1B and C) was analyzed in a modified Boyden chamber (Transwell; Costar). At the onset of each experiment, the cells were detached with Versene and resuspended as single cells in serum-free RPMI medium supplemented with 0.5% bovine serum albumin. A 0.2-mL aliquot of the cell suspension containing 25,000 4T1 cells was added to the upper transwell chamber together with the indicated peptide or rWnt-5a. In Fig. 1C, 5 μ g/mL Frizzled-2 antibody, Frizzled-5 antibody, or rabbit immunoglobulin

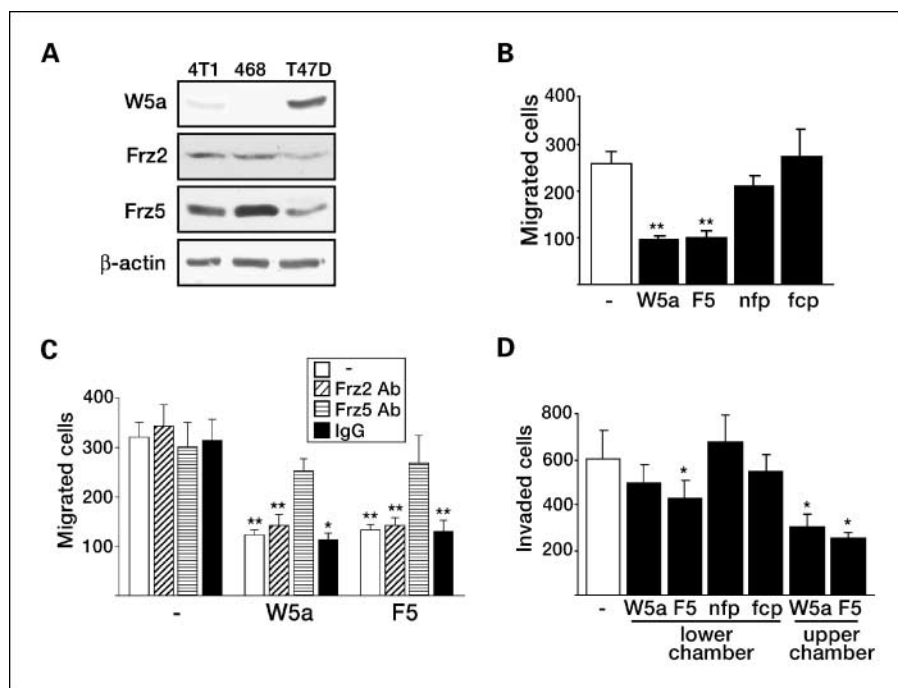


Fig. 1. *In vitro* restoration of Wnt-5a signaling inhibits the migration and invasion of 4T1 breast cancer cells. **A**, Wnt-5a (W5a), Frizzled-2 (Frz2), and Frizzled-5 (Frz5) in 4T1, MDA-MB-468, and T47D breast cancer cell lines. β -Actin served as loading control. **B**, migration of 4T1 cells (18 h) in the presence of 0.4 μ g/mL rWnt-5a or 10 μ mol/L of the described peptides. **C**, migration as in **B**, in the absence or presence of a Frizzled-2 antibody (Frz2 Ab), a blocking Frizzled-5 antibody (Frz5 Ab), or an IgG fraction. **D**, invasion of 4T1 cells through a Matrigel for 48 h in the presence of 0.4 μ g/mL rWnt-5a or 100 μ mol/L of the indicated peptides placed in the lower chamber or together with the cells in the upper chamber (two columns to the right). Columns, mean ($n = 5-7$); bars, SE.

fraction was added to the upper chamber. The lower chamber was filled with serum-free, bovine serum albumin-containing medium supplemented with the chemoattractant IGF-I (1 ng/mL). The cells were allowed to migrate in the Boyden chamber at 37°C in a humidified atmosphere containing 5% CO₂. After discarding the medium and removing the nonmigratory cells from the membrane, the membrane was cut out of the chamber. The cells that had migrated through the membrane and were located on its lower surface were stained for 20 min with 0.5% crystal violet in 20% methanol. The membrane was subsequently washed, and the stained cells were counted (number per membrane). The result of each experiment is presented as the median value from three identical samples done in separate wells. The experiments were independently repeated 5 to 6 times.

Cell invasion. Cell invasion (Fig. 1D) was analyzed in the BD Matrigel invasion chamber (BD Biosciences). At the onset of each experiment, the cells were detached with Versene and resuspended as single cells in serum-free RPMI medium. An aliquot of the cell suspension containing 25,000 4T1 cells was added to the upper transwell chamber, and the lower chamber was filled with serum-containing (10%) medium and supplemented with the indicated peptide or rWnt-5a. In the data presented by the two right-hand columns in Fig. 1D, Foxy-5 or rWnt-5a was added to the upper chamber together with the cells. The cells were allowed to invade at 37°C in a humidified atmosphere of 5% CO₂. The experiment was terminated by discarding the medium and fixing the cells for 10 min in 4% paraformaldehyde. After staining with 0.5% crystal violet in 20% methanol and removing the noninvading cells, the membrane was washed and cut out of the chamber, and the stained cells were counted (number per membrane). The experiment was repeated independently seven times.

Tumor metastasis animal model. All animal experiments were carried out at the central animal laboratory of Turku University. In the first series of animal experiments, 25 female athymic BALB/C mice (8 wk old; Harlan) were randomly divided into 3 groups. The 4T1 mouse breast cancer cells (2.5×10^4 in 0.1 mL PBS) were inoculated into the mammary fat pads under isoflurane anesthesia (1.5-3%, air flow 200 mL/min, Univentor 400 anesthesia unit). The first dose of PBS alone (100 μ L) or PBS (100 μ L) containing 5 μ g of formylated control peptide (fcp) or Foxy-5 was injected on the day of 4T1 cell inoculation.

Thereafter, the mice were i.p. injected with the same amount of peptide or PBS alone every 4th day, with the last dose administered the day before the experiment was terminated on day 25. The primary tumor diameters (d_1 and d_2) were measured using a caliper, and the tumor volumes were calculated every 4th day throughout the experiment (Fig. 2A), according to the formula, $V = (\pi/6)(d_1 \times d_2)^{3/2}$ (25). The mice were also weighed every 4th day throughout the experiment (Fig. 2B). The experiments in Figs. 2 and 3 were done with 9 PBS-treated, 8 fcp-treated, and 8 Foxy-5-treated mice.

In Fig. 4, 26 female normal BALB/c mice (8 wk old; Harlan) were randomly grouped and inoculated with 1×10^4 4T1 mouse breast cancer cells. The cells were suspended in 0.5 mL PBS and injected into the mammary pads. The animals were treated exactly as above with PBS alone, 20 μ g fcp or Foxy-5. The primary tumor volume was calculated as previously described, and the mice were weighed every 4th day throughout the experiment and showed no significant differences (Fig. 4A). The experiment was done with 8 PBS-treated, 9 fcp-treated, and 9 Foxy-5-treated mice. The dose-response study of Foxy-5 (Supplementary Fig. S3) included 52 female normal BALB/c mice (8 wk old). All animals were inoculated with 1×10^4 4T1 mouse breast cancer cells, as described above. The animals were treated exactly as above with PBS alone or the indicated concentration of Foxy-5. The data were calculated as percentage of the PBS-treated controls and given as means from 17 mice treated with PBS alone (9 of those obtained from the second series of experiment; Fig. 4), 9 mice treated with 5 μ g Foxy-5, 9 mice treated with 20 μ g Foxy-5 (obtained from the second series of experiments; Fig. 4), 6 mice treated with 40 μ g Foxy-5, and 7 mice treated with 160 μ g Foxy-5.

In Fig. 5, 24 female normal BALB/c mice (8 wk old; Harlan) were randomly grouped and injected with 1×10^4 4T1 mouse breast cancer cells, as described above. The first i.p. injection of PBS alone (100 μ L) or PBS (100 μ L) containing 40 μ g of Foxy-5 was done on the day of 4T1 cell inoculation. Thereafter, the mice were i.p. injected with the same amount of peptide or PBS alone every 4th day, with the last dose administered the day before the experiment was terminated on day 25. The primary tumor volume was calculated as previously described, and the mice were weighed every 4th day throughout the experiment (Fig. 5A and B). The data are given as means from 11 PBS-treated and 11 Foxy-5-treated mice.

The animal experiment procedures were reviewed by the local ethics committee on animal experimentation at the University of Turku and approved by the local provincial state office of Western Finland (number 1494/05 decision number LSLH-2005-3322/Ym-23, 4.4.2005).

Histomorphometric examination. The mice were euthanized in a CO₂ chamber. The lungs and liver were excised, fixed in 4% buffered formalin for 2 d, and embedded in paraffin. In the first experiment (Fig. 2), the lungs were cut through and 12 horizontal sections (5 μ m) were taken at 250- μ m intervals for H&E staining. Four horizontal sections (5 μ m) were taken from the center area of the liver with a 500- μ m interval. In later experiments (Figs. 4 and 5; Supplementary Fig. S3), sections from only six levels of lungs and two levels of liver were taken because in a control evaluation of the first experiment they gave the same result as the double number of sections. The H&E sections were evaluated and photographed with an Olympus BX51 microscope controlled by the IrfanView 3.97 software (Irfan Skiljan, Graduate of Vienna University of Technology). The relative area of metastases (total metastatic area/total area of section) was measured using the ImageJ 1.37o software (NIH). All metastasis analyses (numbers and areas) were done on blinded samples. The numbers and areas of liver metastases throughout this study are calculated per histologic liver or lung section.

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling staining. To evaluate the degree of apoptosis in the lung and liver metastases, an *In situ* Cell Death Detection Fluorescein kit (Roche Diagnostics GmbH) based on the terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method was used. Tissue

sections were deparaffinized, incubated in the dark with the TUNEL reaction mixture for 1 h at 37°C, and then counterstained with 4',6-diamidino-2-phenylindole. Samples treated with 30 U/mL DNase I for 10 min (Invitrogen) were used as positive controls, whereas samples incubated without the terminal transferase were used as negative controls. No staining was detected in any of the negative controls (data not shown). All metastases in two lung and two liver sections from each mouse were analyzed.

Ki67-staining. To investigate the amount of proliferation, lung and liver sections were stained with a Ki67 antibody. All metastases in two lung and two liver sections from each mouse were analyzed.

Statistical analysis. Student's *t* test for paired samples was used to analyze for statistically significant differences in all the *in vitro* experiments. The Kruskal Wallis test was used to determine if an overall difference was present in the animal experiments; this was complemented with the Mann-Whitney's test to determine between which treatments the difference existed and its corresponding *P* value (**P* < 0.05; ***P* < 0.01; ****P* < 0.001).

Results

The effect of Foxy-5 and rWnt-5a on migration, invasion, apoptosis and proliferation in vitro. To investigate the role of the Wnt-5a–derived peptide Foxy-5 in breast cancer metastasis we chose the 4T1 cancer cells originally derived from a spontaneous breast cancer in a BALB/c mouse (26), because following inoculation into the mammary fat pad, the resultant 4T1 breast tumors give rise to distal metastases in both athymic and normal BALB/c mice (27). 4T1 cells lack endogenous expression of the Wnt-5a protein but still express the proposed Wnt-5a receptors Frizzled-2 and Frizzled-5 (Fig. 1A). Furthermore, similar to human MDA-MB-468 breast cancer cells, murine 4T1 breast cancer cells also lack expression of the coreceptor Ror2 at both the mRNA and protein levels (Supplementary Fig. S1). We therefore believed that this murine cell line was an appropriate model for human breast cancer cells lacking or having a reduced endogenous expression of Wnt-5a (2, 3) and thus suitable for testing the *in vivo* effects of Wnt-5a reconstitution. We first evaluated the mechanism(s) of action of rWnt-5a and the Foxy-5 peptide in 4T1 breast cancer cells.

Both rWnt-5a and Foxy-5 significantly inhibited the migration of 4T1 cells in a modified Boyden chamber assay (Fig. 1B). A nonformylated version of Foxy-5 (nfp) and a formylated control peptide (fcp) were used as controls, and neither of them had any detectable effect on migration (Fig. 1B). Next, we found that the previously described Frizzled-5 receptor-blocking antibody (16, 28) effectively abolished the effects of rWnt-5a and Foxy-5 on 4T1 cell migration (Fig. 1C). As rWnt-5a was previously suggested to have a chemotactic effect on the human breast cancer cell line MCF-7 in an invasion assay (29), we tested the effect of rWnt-5a and Foxy-5 on 4T1 cells in a similar commercially available invasion assay. We found no chemotactic effect of either rWnt-5a or Foxy-5 on 4T1 cells when added to the lower chamber of the Matrigel invasion system (Fig. 1D). On the contrary, we observed an inhibitory effect of Foxy-5 on 4T1 cell invasion even when added to the lower chamber (Fig. 1D). This effect of Foxy-5 can possibly be explained by its more rapid diffusion across the Matrigel layer compared with that of the much larger rWnt-5a molecule. In support of inhibition of 4T1 cell migration by Wnt-5a signaling, we observed that when either rWnt-5a or Foxy5 was added to 4T1 cells in the upper chamber of the Matrigel

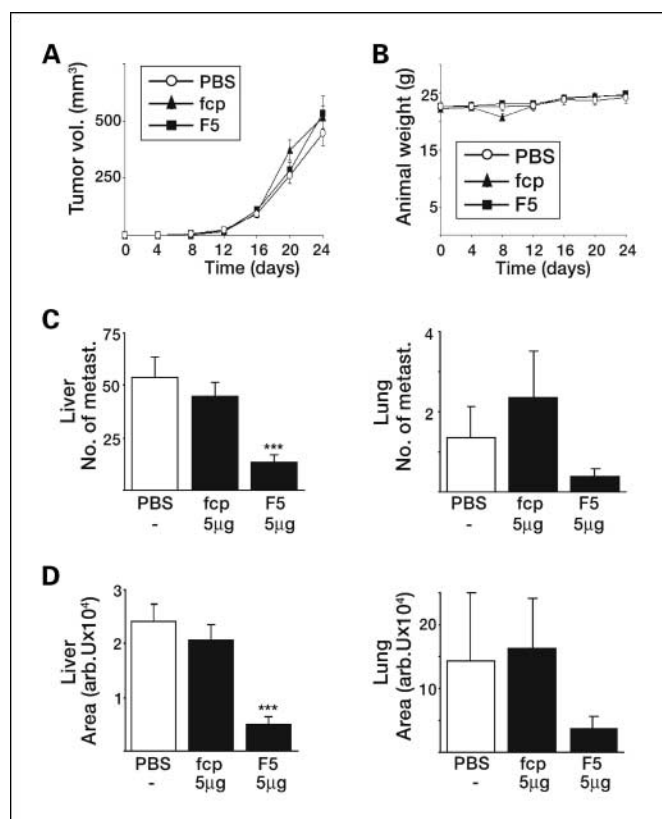


Fig. 2. Athymic BALB/c mice treated with Foxy-5 (F5) developed less liver and lung metastases compared with mice treated with PBS or a formylated control peptide (fcp). *A*, primary tumor growth. *B*, weight of the mice throughout the study. *C*, number of metastases per histologic liver and lung section. *D*, total area of metastases per histologic liver and lung section. Twelve sections/lungs and four sections/liver were analyzed from each animal. Points and columns, mean (9 PBS-treated, 8 fcp-treated and 8 F5-treated animals); bars, SE.

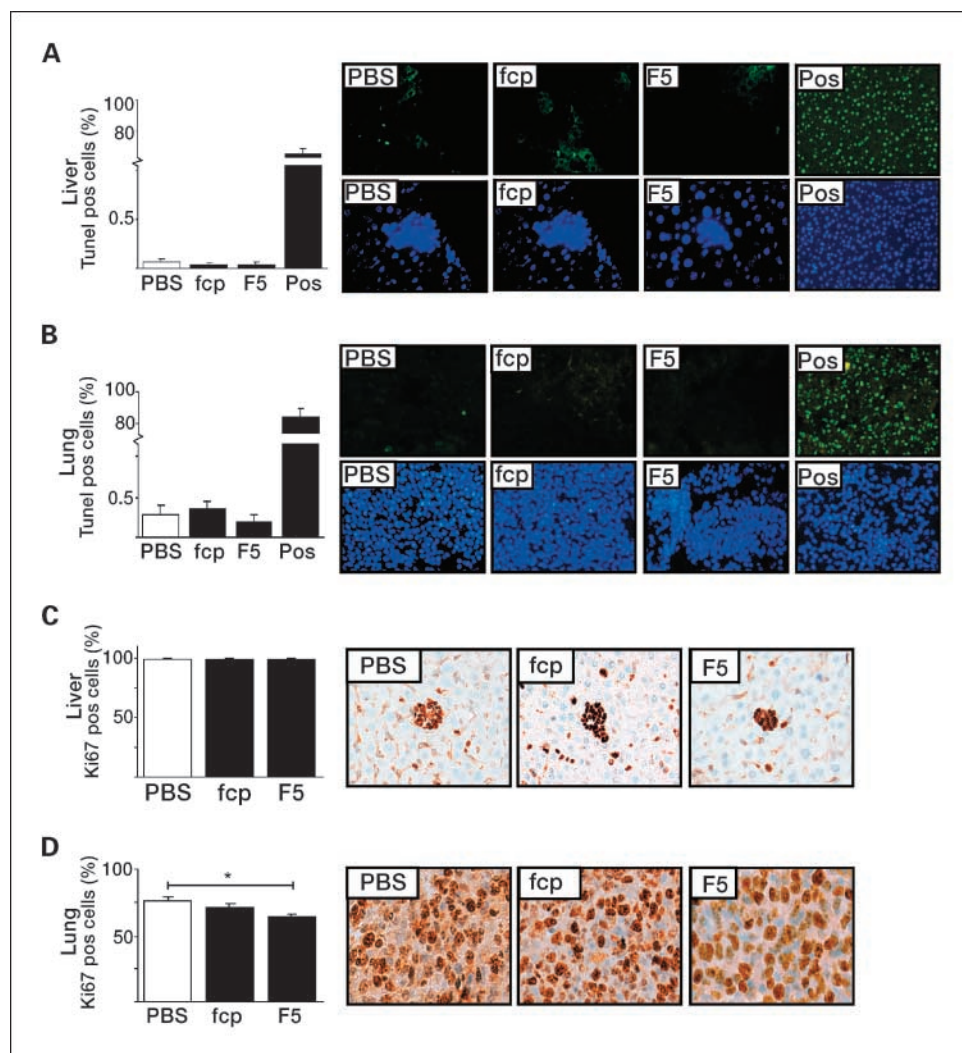


Fig. 3. No effects on apoptosis or proliferation of 4T1 tumor cells in liver and lung metastases from athymic BALB/c mice treated with PBS, a formylated control peptide, or Foxy-5. Percent of TUNEL-positive cells in liver (A) and lung (B) metastases as well as representative TUNEL- (top) and 4',6-diamidino-2-phenylindole-stained (bottom) micrographs. DNase-I-treated sections were used as positive controls (Pos). Percent of Ki67-positive cells in liver (C) and lung (D) metastases as well as representative micrographs. Columns, mean from 9 PBS-treated, 8 fcp-treated, and 8 F5-treated animals; bars, SE.

invasion system, the invasion of these cells was significantly inhibited (the two columns to the right in Fig. 1D).

As shown in Supplementary Fig. S2, neither rWnt-5a nor Foxy-5 or either of the two control hexapeptides had any effect on apoptosis of 4T1 cells *in vitro*. In addition, neither rWnt-5a nor Foxy-5 or either of the two control hexapeptides had any effect on the proliferation of 4T1 cells *in vitro*. Thus, Wnt-5a and Foxy-5 lack an effect on apoptosis and proliferation of 4T1 breast cancer cells, although they readily inhibit migration and invasion *in vitro*.

The effect of Foxy-5 on breast cancer metastasis in athymic BALB/c mice. We first tested if Foxy-5 could inhibit metastasis of 4T1 cells inoculated into the mammary fat pad of athymic BALB/c mice. Following i.p. administration of Foxy-5 (5 µg) every 4th day, our data revealed that Foxy-5 had no significant effect on the growth of the primary tumors or on the weight gain of the animals when compared with animals injected with a formylated control hexapeptide (chemotactic for neutrophils and macrophages), or saline alone (Fig. 2A and B). However, evaluation of the amount of metastases in histologic tissue sections stained with H&E revealed that treatment with Foxy-5 significantly inhibited the number of liver and lung metastases

by 70% compared with the control animals (Fig. 2C). This inhibition was even more pronounced when the total area of tumor metastases was analyzed (Fig. 2D). The effect on lungs was not quite statistically significant ($P = 0.055$ for lung area and $P = 0.074$ for lung number), which is readily explained by the heterogeneous sizes of the lung metastasis.

The effect of Foxy-5 on apoptosis and proliferation *in vivo*. To evaluate the possible effect of Foxy-5 on proliferation and apoptosis in the 4T1 cells *in vivo*, we did a TUNEL assay and stained lung and liver tissue sections with an antibody directed against the proliferative marker Ki67. TUNEL staining of liver and lung sections revealed that neither the Foxy-5 peptide nor the control peptide had any effect on the number of apoptotic cells in the tumor metastases (Fig. 3A and B). Immunohistochemical analysis of the proliferative marker Ki67 in liver sections showed no effect of Foxy-5, whereas we noted a small inhibitory effect of Foxy-5 on the number of Ki67-positive cells in the lung metastases but not in the liver (Fig. 3C and D). The fact that Foxy-5 has no effect on 4T1 cell proliferation *in vitro* (Supplementary Fig. S2D and E) even when incubated over a period of 1 week (Supplementary Fig. S2F) and that the Wnt-5a protein expression in primary human breast tumors was

unrelated to the expression of the proliferate marker Ki67 (3) suggests that the limited effect of Foxy-5 on 4T1 tumor cell proliferation in the lungs is indirect. These data support the concept that Wnt-5a does not act via inhibition of β -catenin signaling and a subsequent inhibition of proliferation. This is in contrast to the situation in colon cancer where Wnt-5a signaling inhibits β -catenin signaling and subsequently tumor cell proliferation (14). A reasonable explanation for this difference relates to the well-known elevated β -catenin signaling in colon cancer cells not present in breast cancer cells. Dolled-Filhart and coworkers have recently shown in a clinical cohort of 688 ductal breast cancers that nuclear β -catenin staining was detected in less than 10 tumors (30). Furthermore, Wnt-5a does not affect the low level of active β -catenin in human breast cancer cells (5). One therefore has to conclude that the effect of Wnt-5a depends on the receptor, coreceptor,

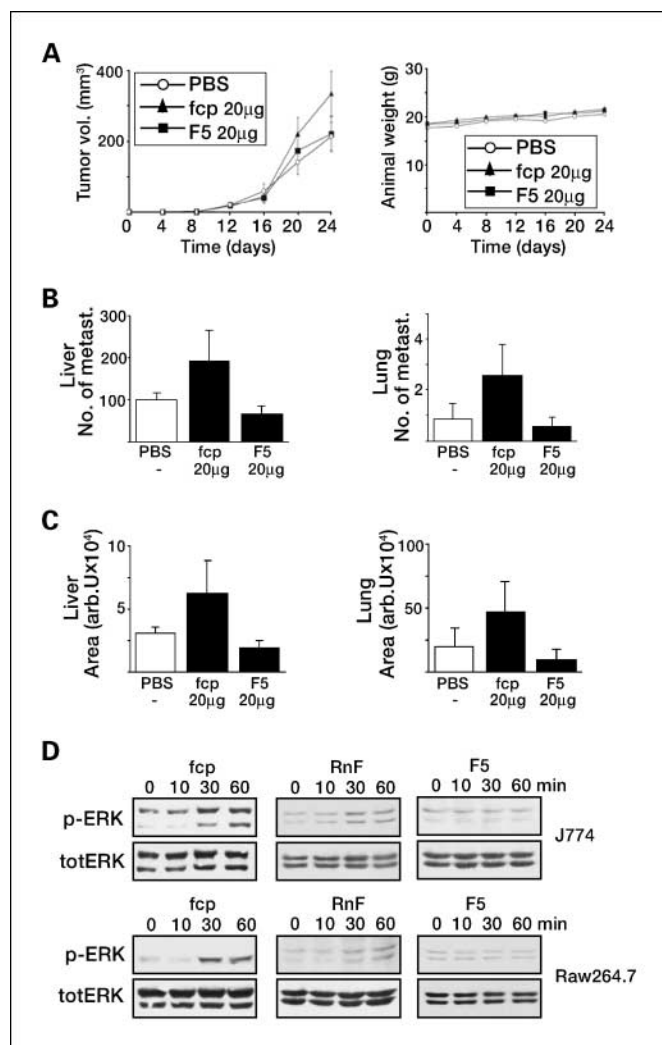


Fig. 4. Different effects of the formylated control peptide and Foxy-5 on liver and lung metastases in normal BALB/c mice. *A*, primary tumor growth and weight of the mice throughout the study. *B*, number of metastases per histologic liver and lung section. *C*, total area of metastases per histologic liver and lung sections. Six sections/lungs and two sections/liver were analyzed from each animal. Points and columns, mean from 8 PBS-treated, 9 fcp-treated, and 9 F5-treated animals; bars, SE. *D*, extracellular signal-regulated kinases (ERK) phosphorylation in J774 and Raw264.7 resting macrophages exposed to 1 μ mol/L fcp, 1 μ mol/L formylated random peptide (RnF), or 1 μ mol/L F5 for the indicated periods of time. The blots are representative of at least five independent experiments.

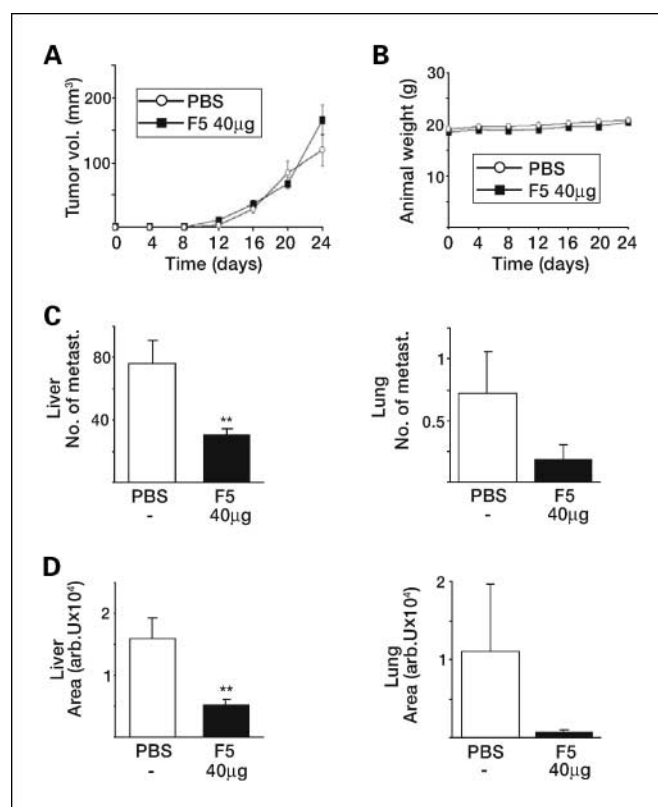


Fig. 5. Normal BALB/c mice treated with Foxy-5 developed less liver and lung metastases compared with PBS-treated mice. *A*, primary tumor growth. *B*, weight of the mice throughout the study. *C*, number of metastases per histologic liver and lung section. *D*, total area of metastases per histologic liver and lung section. Six sections/lungs and two sections/liver were analyzed from each animal. Points and columns, mean from 11 PBS-treated and 11 F5-treated animals; bars, SE.

and signaling context of the cell, and that Wnt-5a inhibits breast cancer cell migration via a β -catenin independent signaling pathway possibly relating to its effect on adhesion receptors (4), NFAT activity (6), and cytoskeletal changes (5).

The effect of Foxy-5 on breast cancer metastasis in normal BALB/c mice. Athymic mice are often used in experimental cancer research, but a defective immune response might influence, possibly reduce, tumor progression and thus the results of an antimetastatic substance could be overestimated. Inoculation in athymic BALB/c mice has been shown to lead to the development of less metastases than in normal syngeneic BALB/c mice (31). Therefore, we took advantage of the present mouse model enabling us to test the antimetastatic effect of Foxy-5 in normal BALB/c mice. We initially chose a 4-fold higher dose of Foxy-5 than that used in the immunocompromised BALB/c mice. This dose of Foxy-5 had no, as in Fig. 2, statistically significant effect on the growth of the primary tumor or on the weight gain of the animals (Fig. 4A). We noted that the effects of Foxy-5 on the development of liver and lung metastases were qualitatively similar to that seen in athymic mice but less pronounced and statistically not significant. The Kruskal Wallis analysis revealed that $P = 0.473$ in Fig. 4B (left), $P = 0.376$ in Fig. 4B (right), $P = 0.541$ in Fig. 4C (left), and $P = 0.396$ in Fig. 4C (right). In accordance with the findings of increased development of tumor metastases in normal syngeneic BALB/c mice (31), these data indicate the need of a higher dose of Foxy-5 to obtain

inhibitory effects similar to that found in athymic mice. We therefore initiated a limited dose-response study (0-160 μg) on the effect of Foxy-5 on the metastasis to the lungs. The data revealed that 40 μg Foxy-5 exhibited a near maximal inhibitory effect on 4T1 cancer cell metastasis in normal BALB/c mice (Supplementary Fig. S3).

Interestingly, administration of the control, non-Foxy-5 formylated hexapeptide could not be used as a control peptide in the experiments in normal BALB/c mice, because it led to increased metastasis (Fig. 4B and C). The fact that this "control" peptide had no effect on migration, invasion, proliferation or apoptosis of 4T1 cells *in vitro* (Fig. 1; Supplementary Fig. S2), suggests that its effect is related to nontumor cells, for example tumor-associated macrophages. This possibility was supported by the findings that both the formylated control hexapeptide and to a lesser extent a formylated random hexapeptide, but not Foxy-5, triggered extracellular signal-regulated kinases activation in two different murine macrophage-like cell lines, J774 and Raw264.7 (Fig. 4D). Extracellular signal-regulated kinases activation was chosen as a representative downstream signal, as it has been shown to be essential for both proliferation and migration of murine macrophages (32, 33).

Based on the above results, we tested how i.p. injection of 40 μg Foxy-5 in normal BALB/c mice affected the progression of 4T1 cancer development and metastasis. Our observation that even the formylated random hexapeptide stimulated murine macrophages and 4T1 cell metastasis led us to use i.p. injection of saline alone as a control to Foxy-5. Clearly, if we had used any of the peptides that stimulated murine macrophages as a control we would incorrectly have overestimated the effect of Foxy-5. The dose of Foxy-5 used in this series of experiments had no statistically significant effect on the growth of the primary tumor or on the weight gain of the animals (Fig. 5A and B). It should be mentioned that the volumes of the primary tumors in this series of experiments (Fig. 5A) were smaller than those in the previous series of experiments (Fig. 4A). This reduced growth of the primary tumor is a plausible explanation for the reduced number of metastases and smaller areas of the metastatic lesions observed in this series of experiments (Fig. 5C and D). Despite this difference, Foxy-5 inhibited the number of liver and lung metastases by 60% to 70% (Fig. 5C). These inhibitions were even more pronounced (70% and 90%) if the total area of tumor metastases were analyzed as an indicator of metastasis (Fig. 5D).

Discussion

In the present investigation we have shown that the Foxy-5 peptide reduced 4T1 breast cancer metastasis to the lungs and liver. The clinical relevance of this finding is strengthened by the fact that we used an *in vivo* model in which 4T1 breast cancer cells, derived from a spontaneous breast cancer in BALB/c mouse (26), were inoculated into the mammary fat pad of immunocompromised (nude) or normal BALB/c mice. Orthotopically implanted syngeneic tumors are preferable when evaluating the response of a possible antimetastatic treatment, because the importance of the interaction of tumor cells with the surrounding tissue has become increasingly evident (31). In support of a metastasis-suppressing role of Wnt-5a, the Wnt-5a mimicking peptide Foxy-5 inhibited breast cancer metastasis to both the lungs and the liver in normal BALB/c mice. As could be

expected, a higher dose of the Foxy-5 peptide was required in the normal BALB/c mice to attain a similar level of liver metastasis suppression as in the immunocompromised BALB/c mice. Despite the increased amount of Foxy-5 (40 μg , i.e., 1.6 mg/kg body weight) needed to obtain a 70% to 90% inhibition of tumor metastasis in normal mice, this amount of Foxy-5 is still relatively low when compared with similar studies using other types of tumor suppressors (34–36). The increased metastasis in normal BALB/c mice is most readily explained by an intact immune system of this mouse strain, as has been previously observed (31). Activation of immune cells in such animals, and in particular tumor-associated macrophages, can promote metastasis formation (37). The increased metastasis to the lungs and liver in the normal BALB/c mice treated with the formylated control peptide relative to those given PBS alone support this idea because this peptide is known for its ability to activate murine macrophages, as also shown in our *in vitro* experiments. Importantly, the Foxy-5 peptide had no such effect on murine macrophage activation *in vitro*. Consequently, the finding that Foxy-5 has the ability to impair 4T1 breast cancer metastasis in BALB/c mice with a normal immune response, i.e., animals with a high metastatic spread, ensures that the antimetastatic effect shown by the Foxy-5-induced reconstitution of Wnt-5a signaling is not overestimated as could have been the case if only immunocompromised mice would have been used.

In this study, we used histomorphometrical analyses of lung and liver metastases as an end point to establish the effects of Foxy-5 on breast cancer cell metastasis. Although it was not possible to quantitatively assay tumor cell migration *in vivo*, the accumulated *in vitro* and *in vivo* data suggest that the main mechanism by which Foxy-5 suppresses the metastatic process is by impairing tumor cell migration and invasion. Previous investigations on how Wnt-5a signaling inhibits breast epithelial cell migration have indicated the involvement of several different mechanisms, including increased DDR1-dependent adhesion (4, 38), impaired NFAT activity (6), and an increased amount of actin stress fibers (5, 13). Consequently, to ascertain that all these mechanisms and also yet unidentified ones are activated, it makes more sense to use a receptor agonist than an approach that triggers activation of a specific downstream signaling event in the cascades of Wnt-5a signaling. In future studies it will be interesting to apply *in vivo* imaging techniques that enable longitudinal follow-up of the metastatic process in live animals (39, 40). The *in vivo* imaging will probably also enable studies on how Foxy-5 affects the dynamics of cancer cell migration and invasion at the cellular level (41).

In contrast to our previous findings, it has been suggested that Wnt-5a released from tumor-associated macrophages could have a chemotactic effect on breast cancer cells and thereby increase metastasis (29). Because breast cancer cells that exhibit an endogenous expression of Wnt-5a have a lower metastatic potential, this would mean that Wnt-5a must affect the tumor cells differently when released from tumor-associated macrophages than when released from breast cancer cells. However, we did not in the present study find a chemotactic effect of Wnt-5a or Foxy-5 on 4T1 breast cancer cell invasion when using a commercially available invasion assay, similar to that previously used (29). Thus, the correlation between reduced Wnt-5a expression in clinical breast cancer samples with reduced disease-free survival, the present and previous *in vitro* data, and

the present *in vivo* data strongly support the concept that endogenous Wnt-5a expression in breast cancer cells and the reconstitution of Wnt-5a signaling in breast cancer cells with reduced endogenous Wnt-5a expression act to significantly reduce breast cancer metastasis. In principle, reconstitution of Wnt-5a signaling might have a similar therapeutic potential in other forms of cancer in which Wnt-5a has been shown to have a tumor suppressor function (12–14, 42).

In conclusion, reconstitution of Wnt-5a signaling by the administration of Foxy-5 significantly inhibits the metastasis of 4T1 breast cancer cells. Importantly, this Wnt-5a–derived peptide ligand can serve as a model substance for the development of a novel therapeutic approach to specifically target the dissemination process in breast cancer metastasis, in

particular for the ~50% of human breast cancer patients that exhibit a reduced endogenous expression of Wnt-5a in their primary tumor (2).

Disclosure of Potential Conflicts of Interest

T. Andersson has filed a patent for the use of the Foxy-5 peptide in anti-metastatic treatment.

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