SUPPLEMENTARY MATERIAL:

The Wnt-5a-derived hexapeptide Foxy-5 inhibits breast cancer metastasis in vivo by targeting cell motility

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Supplementary Methods:

Reverse transcription-polymerase chain reaction (RT-PCR)

Total mRNA from T47D, MDA-MB-468 and 4T1 cells was obtained using TRIzol® Reagent according to the manufacturer's protocol. Isolated total RNA was reversed transcribed using Superscript™ II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA), per the manufacturer's instructions. To control for DNA contamination, the reverse transcription reaction was performed without the reverse transcriptase enzyme. The RT reaction products and 1μg of control genomic DNA extracted from the 4T1 cells was then subjected to PCR using Taq DNA polymerase (Invitrogen, Carlsbad, CA). The following primer sets were used for PCR: β-actin F (5'-TTC AAC ACC CCA GCC ATG TA-3'), β-actin R (5'-TTG CCA ATG GTG ATG ACC TG-3'), corresponding to nucleotides 452-471 and 809-828 of the human β-actin sequence; human Ror2 F (5'-CCC

TGG TGC TTT ACG CAG AA-3') and human Ror2 R (5'-CAT GAA CCT CAC CGC AGA CA-3'), corresponding to nucleotides 1322-1341 and 1608-1627 of the human Ror2 sequence, mouse Ror2 F (5'-CCC TGC GAA CGT GTT CTT TT-3') and mouse Ror2 R (5'-GAA AAC GGC ACA CGG AAA AC-3'), corresponding to nucleotides 3573-3592 and 3910-3929 of the mouse Ror2 sequence. The resulting PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining. The gels shown (Supplementary Fig. 1) are representative of four independent experiments.

Fluorometric assay for caspase-3 activity

Caspase-3 activity was analyzed by fluorescence spectrometry using the fluorogenic peptide DEVD-amc as a substrate (Upstate Biotech. Inc., Lake Placid, NY). The 4T1 cells were allowed to adhere for 24h in 6-well plates. The cells were then incubated for 18h in the presence or absence of 100μM of the indicated peptide, 0.4μg/ml rWnt-5a or 100nM taxol (positive control). To complement the results from the Hoechst staining, both adherent and floating cells were lysed in caspase lysis buffer (10mM Tris-HCl, 10mM NaH_2PO_4/Na_2HPO_4 [pH 7.5], 130mM NaCl, 1% Triton-X-100, and 10mM $NaPP_i$). 50μl lysates were added to the reaction wells, together with 200μl HEPES buffer (20mM HEPES [pH 7.5], 10% glycerol, and 2mM DTT) and 3μl of DEVD-amc (dissolved in DMSO). The reaction mixture was incubated at 37ºC for 1h, and thereafter analyzed for amc fluorescence in a FLUOstar plate reader (BMG Lab Technologies, Germany) at excitation and emission wavelengths of 390 and 460nm, respectively. The protein content of lysates was determined and adjusted for, using the Coomassie Plus Protein Assay. The

fluorescence data were corrected by subtracting background values and duplicates of each sample were used to calculate a mean value for each separate experiment, which was calculated as percent of control. The experiment was independently repeated 5 times, Supplementary Fig. 2A.

Hoechst staining

4T1 cells were plated on cover glasses and incubated for 24h in the presence or absence of 100μM of the indicated peptide, 0.4μg/ml rWnt-5a or 100nM taxol (positive control). The cells were then fixed with 4% paraformaldehyde for 15 min, stained in the dark with Hoechst stain (Hoechst 34580, Molecular Probes, Eugene, OR) for 10min, washed with PBS and mounted with Dako Cytomation fluorescent mounting medium. The samples were examined and photographed in a Nikon Eclipse 800 microscope using a 60X oil immersion objective. 300 cells were counted from each experiment. The experiment was independently repeated 6 times, and the accumulated results are given in Supplementary Fig. 2C.

Viable cell count

We seeded 5,000 4T1 cells and allowed them to adhere in 96-well plates for 24h. The cells were incubated for 18h with serum-free RPMI medium in the presence or absence of 100μM of the indicated peptide or 0.4μg/ml rWnt-5a. The cells were subsequently detached with trypsin and the number of viable cells was determined by trypan blue exclusion. Triplicates of each sample were analyzed and a mean value was calculated. The experiment was repeated independently 6 times, Supplemental Fig. 2D.

Thymidine incorporation

An optimal cell density (data not shown) were seeded and allowed to adhere for 24h in 24-well plates. The cells were incubated for 18h with serum-free RPMI medium containing 0.25μCi tritium-labeled thymidine/well in the presence or absence of 100μM of the indicated peptide or 0.4μg/ml rWnt-5a. Thereafter the cells were washed with PBS, incubated for 30min with 10% trichloroacetic acid and lysed with 1M NaOH for another 30min. The lysates were transferred to tubes containing scintillation liquid and the tritium activities were counted in a beta-counter. Scintillation liquid without lysate was used as a blank. Triplicates of each sample were analyzed and a mean value was calculated. The experiments were independently repeated 6 times, Supplementary Fig. 2E. As a control, the 4T1 cells were also incubated with 100μM Foxy-5 for a week (tritium-labeled thymidine was included during the last 18h), and no difference was found compared to control cells, Supplementary Fig. 2F.

Supplementary Figures:

Supplementary Figure 1. The western blot to the left shows Ror2 protein expression whereas the *Ror2* mRNA expression is shown to the right in 4T1, MDA-MB-468 and T47D breast cancer cell-lines. DNA from 4T1 cells (DNA-4T1) was used as positive control for the mouse Ror2. β-actin served as loading and PCR control. The experiments were repeated independently at least four times.

Supplementary Figure 2. *In vitro* restoration of Wnt-5a signaling has no effect on apoptosis or proliferation of 4T1 breast cancer cells. **A**) Caspase-3 activity in 4T1 cells incubated for 18h with 0.4μg/ml rWnt5a (W5a), 100μM of Foxy-5 (F5), the non-formylated control peptide (nfp) or the formylated control peptide (fcp). 100nM Taxol (Tax) was used as a positive control in Supplementary Fig. 2A-C. **B**) Representative Western blot to detect PARP cleavage in 4T1 cells incubated for 18h with either 100μM of the indicated peptides or 0.4μg/ml rWnt5a. The membrane was reprobed for β-actin to confirm equal loading. **C**) Hoechst staining of 4T1 cells incubated with either 0.4μg/ml rWnt-5a or 100μM of the indicated peptides for 24h. **D**) 4T1 cells were incubated for 18h with 0.4μg/ml rWnt5a or 100μM of the indicated peptides, after which viable cells were counted by trypan blue exclusion. **E**) Thymidine incorporation in 4T1 cells stimulated with 0.4μg/ml rWnt-5a or 100μM of the described peptides for 18h. **F**) Thymidine incorporation in the 4T1 cells incubated for 1 week with 100μM F5 peptide. The results in are given as means ± SEM (n = 5-6).

Supplementary Figure 3. The dose-response of Foxy-5 on lung metastasis in normal BALB/c mice. The mice were treated with PBS alone or with 5, 20, 40 or 160μg Foxy-5. The effects of Foxy-5 are expressed as percent of PBS treated controls of 17 mice treated with PBS alone, 9 mice treated with 5μg F5, 9 mice treated with 20μg F5, 6 mice treated with 40μg F5, and 7 mice treated with 160μg F5.

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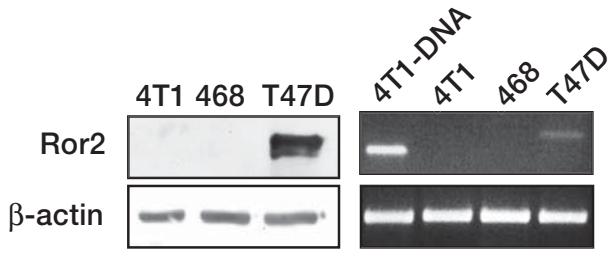
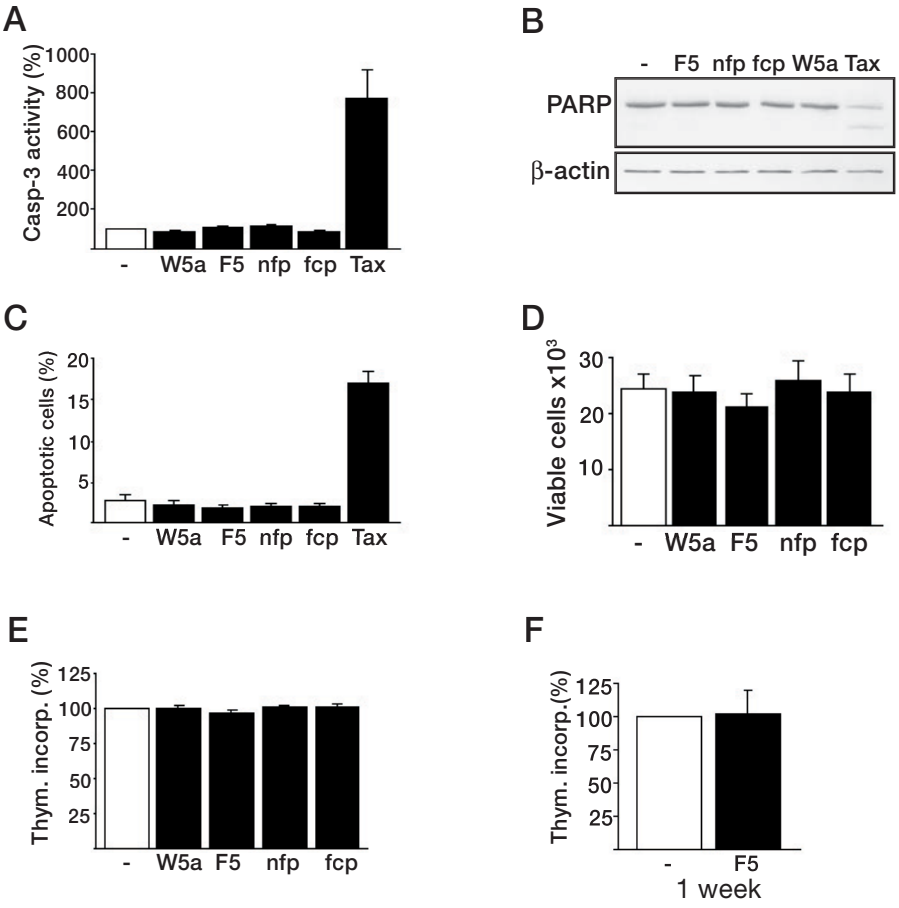
Supplementary figure 1



Supplementary figure 2



Supplementary figure 3