SPANDIDOS PUBLICATIONS

ONCOLOGY REPORTS 29: 549-554, 2013

Antiproliferative effects of kisspeptin-10 depend on artificial GPR54 (KISS1R) expression levels

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Received September 6, 2012; Accepted October 18, 2012

DOI: 10.3892/or.2012.2135

Abstract. Kisspeptins are peptides derived from the metastasis suppressor gene KISS1 interacting with GPR54 as their corresponding receptor. The KISS1/GPR54 system is one regulator of cellular motility mechanisms leading to decreased migration and invasion. Its role in cell proliferation processes is not clearly understood. In this study, breast cancer cell lines, T47D, ZR75-1, MDA-MB-231, MDA-MB-435s, MDA-MB-453, HCC 70, HCC 1806, HCC 1937 and MCF-7, were investigated for their endogenous GPR54 expression by immunocytochemistry, RT-PCR and western blot analysis. The effect of kisspeptin-10 on proliferation was measured in MDA-MB-231, MDA-MB-435s, HCC 1806 and MCF-7 cells. Further experiments on proliferation were carried out with cells transfected with GPR54. All of the tested breast cancer cell lines expressed GPR54 in different amounts. No effects on proliferation were detected in the breast cancer cells expressing the receptor endogenously. In transfected neuronal cells overexpressing GPR54, proliferation was significantly inhibited by kisspeptin-10. The results indicate that the antiproliferative action of kisspeptin depends on the nature of GPR54 expression. The effect was detected in an artificial system of cells transfected with GPR54 and not in cells expressing the receptor endogenously. Thus, the antiproliferative action of kisspeptin seems not to be important for pathophysiological processes.

Introduction

KISS1 was originally discovered as metastasis suppressor gene in human melanoma and breast cancer cell lines reducing metastasis *in vivo* (1-6). The KISS1 gene encodes a peptide of 145 amino acids, which is cleaved proteolytically into shorter peptides, the kisspeptins (KP). KP-54, KP-14, KP-13 and KP-10 are active agonists binding to the KISS1 receptor, the Gq-protein coupled receptor GPR54. All kisspeptins share a common C-terminal structure, which is necessary for receptor activation showing the highest potency for KP-10 (7-9).

Based on clinical studies, importance of the interaction of kisspeptin with GPR54 is discussed controversially for the antimetastatic effects of KISS1. In healthy tissues, the receptor is expressed mainly in brain, pancreas and placenta (10). Upregulation of receptor levels from normal to tumor tissue were detected in breast, ovarian, small intestine and colon cancer (7). Others showed no change of GPR54 expression in breast cancer between background and tumor tissue. However, elevated levels of KISS1 expression correlated with poor patient prognosis and outcome (11). Further analysis resulted in differences regarding breast cancer progression and histology. GPR54 and KISS1 were upregulated in invasive tumors compared to non-invasive forms leading to shorter relapse-free survival in patients (12). In contrast, matching of GPR54 and kisspeptin expression and overall survival in ovarian cancer patients showed longer survival concomitant with increased expression levels (13). In pancreatic cancer, high levels of GPR54 and kisspeptin were correlated (14) with a longer overall patient survival (15). Increased receptor and KISS1 levels were observed in hepatocellular carcinoma compared to normal tissue (16). In renal cell carcinoma, GPR54 was increased, but kisspeptin showed no changes in expression (17). In summary, there is no definite correlation between GPR54 and KISS1 expression and cancer progression.

Research with regard to the antimetastatic effects *in vitro* indeed shows an interaction of the KISS1/GPR54 system and cellular motility mechanisms. The KISS1/GPR54 system is involved in reduced migration and invasion, modified adhesion processes, changes in cytoskeleton and chemotactic behavior (7,8,14,17-24). In this context, some studies indicate an antiproliferative action of kisspeptin and induction of apoptosis. In contrast, there are data in literature showing no effect on cell proliferation by the KISS1/GPR54 system. One explanation for the controversial results might be the use of different cell lines and especially differences in their GPR54 expression. Studies, showing an antiproliferative effect of kisspeptin, used cells overexpressing the receptor. Some experiments were carried out by transfecting Chinese hamster ovary CHO cells with GPR54 (8,18). Others used murine fibroblast NIH3T3 cells (19) and human breast cancer MDA-MB-435s cells overexpressing GPR54 (25). Only one study used non-transfected human umbilical vein endothelial cells (HUVECs), which showed an endogenous receptor expression (20). In these studies, an

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Key words: kisspeptin-10, GPR54, KISS1R, proliferation

antiproliferative effect of kisspeptin was found. However, no influence on proliferation was observed in pancreatic cancer cell lines AsPC-1 and PANC-1 (14), in renal cell carcinoma cell lines Caki-1 and ACHN (17), in HUVECs (26) and trophoblast cells (21). Low endogenous GPR54 expression was detected in these cell lines. Further experiments in breast cancer cell lines MDA-MB-231 and MCF-7 showed no effect on proliferation as well, but no information of the receptor status was given (22). No involvement of the KISS1/GPR54 system in antiproliferative actions was detected by transfection of the KISS1 gene into MDA-MB-231 breast cancer cells (11), SKOV3 ovarian cancer cells (23) and C8161 melanoma cells (2). In summary, these results show no antiproliferative action in cancer cells, which express GPR54 endogenously. The antiproliferative effect was only observed in artificial cell models transfected to overexpress the receptor.

In the present study, the effect of the KISS1/GPR54 system on proliferation was studied in the breast cancer cell lines T47D, ZR75-1, MDA-MB-231, MDA-MB-435s, MDA-MB-453, HCC 70, HCC 1806, HCC 1937 and MCF-7. This tumor was chosen based on different information in literature, showing reduced proliferation (25) on the one hand and on the other hand no changes in proliferation after treatment with kisspeptin (11,22). GPR54 expression levels were measured and the effect of kisspeptin on proliferation was compared to neuronal cells transfected to overexpress the receptor. The aim of this study was to investigate the relationship of antiproliferative effects of kisspeptin and the nature of GPR54 expression.

Materials and methods

Cell lines and culture conditions. The human breast cancer cell lines T47D, ZR75-1, MDA-MB-231, MDA-MB-435s, MDA-MB-453, HCC 70, HCC 1806, HCC 1937 and MCF-7 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). In order to guarantee the identity of the cell lines over the years, cells were expanded after purchase and aliquots were stored in liquid nitrogen. Every year a new frozen stock was opened and expanded to carry out the experiments. Murine GPR54 stable transfected neuronal B35 cell clones (rat) were kindly provided by Robert P. Millar (Edinburgh, UK). Cells were cultured as monolayer in medium [MEM, Biochrom, Berlin, Germany; containing insulin (0.05 IU/ml) and transferrin (1 μg/ml) for human breast cancer cell lines; DMEM, Gibco®, Life Technologies, Darmstadt, Germany; containing G418 (1 mg/ml) as transfection media for B35 clones] supplemented with 10% fetal calf serum (Biochrom), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Life Technologies) at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Chemicals. Kisspeptin-10 (KP-10; Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe) was synthesized by Peptide Specialty Laboratories (Heidelberg, Germany). KP-10 was initially dissolved in dimethyl sulfoxide (DMSO) and diluted in water for injection. KP-10 solutions used for experiments contained <0.006% DMSO with no influence on cell viability referring to controls.

Immunocytochemistry. Cells were grown to ~70% confluence on Lab-Tek two-well chamber slides (Nunc, Thermo Fisher Scientific, Langenselbold, Germany). Before each treatment, cells were washed with phosphate buffered saline (PBS). Cells were fixed in 4% paraformaldehyde, treated with hydrogen peroxide (3%) and incubated with blocking solution (Histostain® Bulk kit, Life Technologies). As primary antibody, a polyclonal rabbit anti-human GPR54 (SP4238P, Acris, Herford, Germany) was used diluted 1:250 in PBS. Cells were incubated at 4˚C overnight before they were again treated with Histostain Bulk kit according to the manufacturer's description combined with detection reagent AEC substrate chromogen 3-amino-9-ethylcarbazole (Dako, Carpinteria, CA, USA). Controls were performed by omission of the primary antibody.

Isolation of mRNA and cDNA synthesis. Total RNA was prepared by the RNeasy mini kit protocol (Qiagen, Hilden, Germany). The concentration of RNA in each sample was determined by photospectroscopy. First-strand cDNA was generated by reverse transcription of 1 μg total RNA in a 40 μl reaction volume containing DNase I, dNTPs, Primer $p(dT)_{15}$ primer (Roche Diagnostics, Mannheim, Germany), RNase inhibitor (RNasin®, Promega, Madison, WI, USA), 5X First-strand buffer, DTT and Superscript™ II reverse transcriptase (Life Technologies). Samples were tested for integrity by PCR analysis of the ribosomal housekeeping gene L7.

PCR amplification. cDNA templates were amplified in a 15 μl reaction volume containing 0.3 U KAPA2G™ Fast (2X ReadyMix with Dye; Peqlab, Erlangen, Germany) and 0.5 μM of the appropriate primers (GPR54 in breast cancer cell lines: sense primer 5' CGA CTT CAT GTG CAA GTT CGT C 3', antisense primer 5' CAC ACT CAT GGC GGT CAG AG 3'; GPR54 in transfected B35 cells: sense primer 5' TGA CCG CCA TGA GTG TGG AC 3', antisense primer 5' GCG GAG TGG CTG TAG GAC AT 3'; L7: sense primer 5' AGA TGT ACA GAA CTG AAA TTC 3', antisense primer 5' ATT TAC CAA GAG ATC GAG CAA 3') in a thermal cycler (T3000, Biometra, Goettingen, Germany). PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining and UV photometric detection. Bands were analyzed using the Biometra BioDoc Analyze system (Biometra, Goettingen, Germany). For semi quantitative analysis, amount of amplification product was standardized to the amount of L7 transcripts of each sample using L7 as ribosomal housekeeping gene.

For analysis of GPR54 mRNA, PCR was run up to 35 cycles to get a signal for some of the breast cancer samples, whereas amplification product of transfected B35 clones was found by <25 cycles. For reasons of comparison, cDNA samples of the transfected B35 clones were diluted 1:100 and arranged together with the undiluted breast cancer samples. According to this, the amount of the housekeeping gene L7 used as standard is very low for the clone in contrast to the breast cancer cells (Fig. 2).

Protein extraction and western blot analysis. Cell pellets were washed with PBS and resuspended in CelLytic™ buffer (Sigma, St. Louis, MO, USA) containing protease inhibitor (Sigma). Equal amounts of protein per sample were diluted with 4X LDS sample buffer supplemented with 10X sample reducing agent (NuPAGE®, Life Technologies). After denaturation, samples were separated on SDS-PAGE (ProSieve® 50 Gel

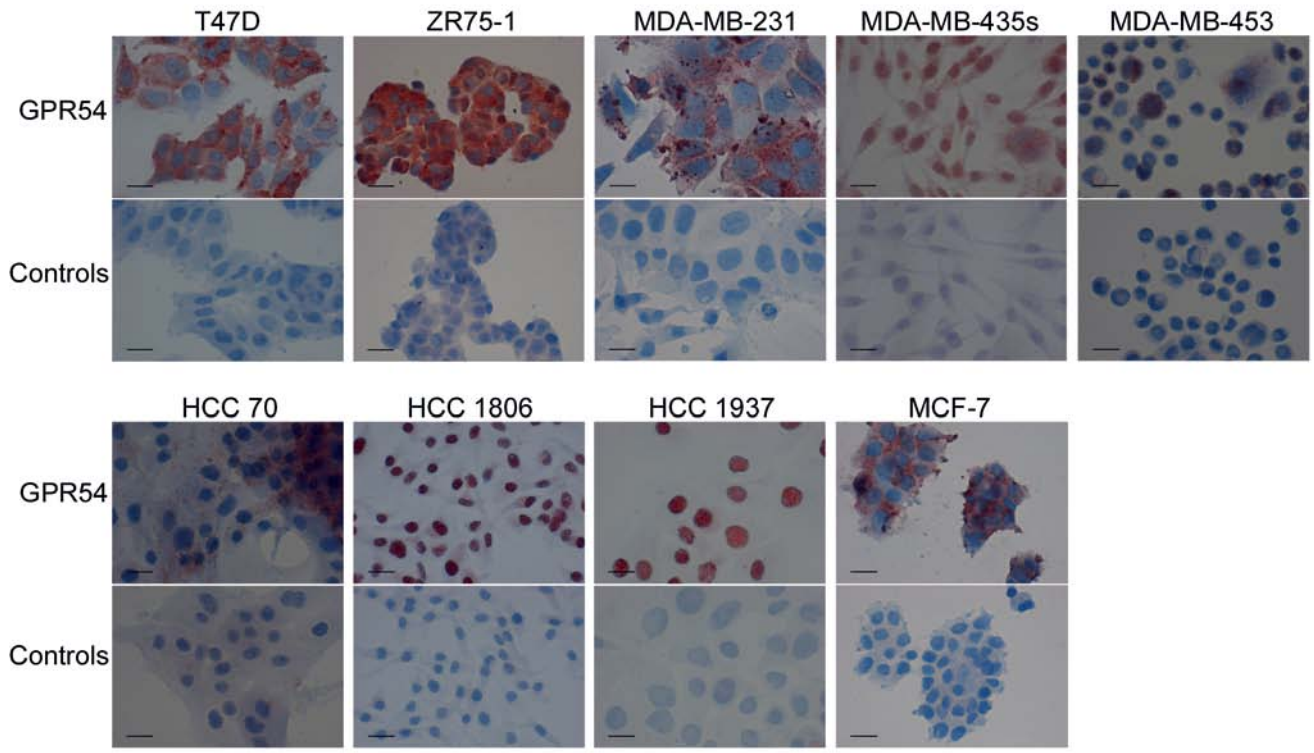

Figure 1. GPR54 in breast cancer cell lines. Immunocytochemical detection of GPR54 in breast cancer cell lines and negative controls without primary antibody; scale bar=40 μm. Images represent the findings in at least three different passages of each cell line.

solution; Lonza, Rockland, ME, USA; 5% for concentrating and 10% for separating) under reducing conditions. Gels were blotted on PVDF membranes (Millipore, Billerica, MA, USA). Membranes were blocked with 5% instant skimmed milk powder, spray-dried (Saliter GmbH, Oberguenzburg, Germany) in TBST (137 mM NaCl, 2.7 mM KCl, 24.8 mM Tris, 0.1% Tween, pH 7.4) for 1 h, washed with TBST and incubated at 4˚C overnight with polyclonal rabbit anti-GPR54 (AKR-001, Alomone Labs, Jerusalem, Israel) in a 1:1000 dilution in TBST. After washing, horseradish peroxidase-linked species-specific whole anti-rabbit IgG (GE Healthcare Europe, Munich, Germany; 1:33000 in TBST) was put on the membranes for 1 h. Membranes were washed and exposed to chemiluminescent HRP substrate (Immobilon™; Millipore) for detection of specifically bound antibody by X-ray film (Biomax MR, Kodak, Rochester, NY, USA). Monoclonal rabbit antibody for actin (Epitomics, Burlingame, CA, USA) in a 1:1000 dilution in TBST was used for standardization.

Proliferation assay. Cell lines were grown (plating density: 1-2x10^4 cells/well in 96-well plates depending on their metabolism) in phenol red-free medium (DMEM, Gibco, Life Technologies) supplemented with 10% charcoal treated fetal calf serum (PAN Biotech, Aidenbach, Germany), L-glutamine (2 μmol/ml) (Biochrom), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco, Life Technologies) at 37˚C in a humidified atmosphere of 5% CO_2 in air overnight. KP-10 solutions and vehicle (control) were added in final concentrations of 10^{-11} M - 10^{-5} M every day up to 72 h. The experimental setting included treatments with KP-10 once daily and twice daily. Experiments were done in six replicates for each sample and proliferation was determined by a colorimetric assay (alamarBlue®, AbD Serotec, Oxford, UK). Changes in viability were used as marker for proliferation. Optical density of the reduced dye was measured at 570 nm vs. 630 nm by a microplate reader (Synergy HT, BioTek, Vermont, USA).

Statistical analysis. All experiments were repeated at least three times with different passages of the respective cell lines. Data were tested for significant differences by one-way analysis of variance followed by Dunnett's multiple comparison test respectively by Tukey's multiple comparison test using GraphPad Prism software (GraphPad Software Inc., La Jolla, CA, USA).

Results

GPR54 expression in breast cancer cell lines. GPR54 expression was analyzed by immune cytochemical staining in breast cancer cell lines T47D, ZR75-1, MDA-MB-231, MDA-MB-435s, MDA-MB-453, HCC 70, HCC 1806, HCC 1937 and MCF-7 (Fig. 1). All cell lines expressed GPR54, visualized by red staining with GPR54 antibody. Cell lines were further investigated on mRNA and protein levels. mRNA analysis was done by RT-PCR for GPR54 (Fig. 2A). T47D, ZR75-1 and MCF-7 showed receptor mRNA expression. In MDA-MB-231, MDA-MB-435s, MDA-MB-453, HCC 70, HCC 1806 and HCC 1937 no GPR54 mRNA was found. GPR54 protein levels were detected in every breast cancer cell line with different quantities by western blot analysis (Fig. 2B).

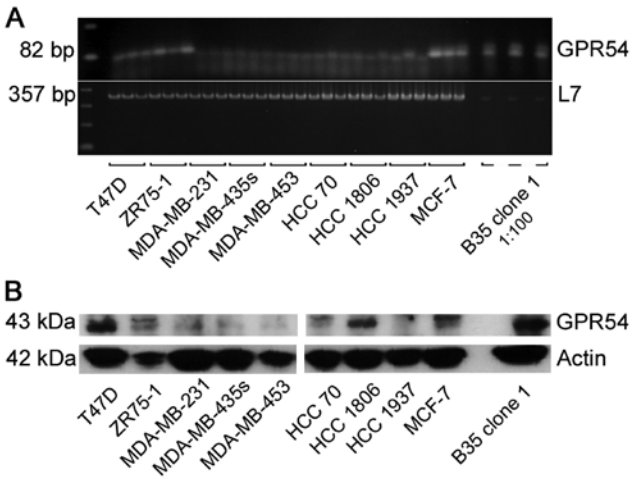

Figure 2. GPR54 expression in breast cancer cell lines. (A) mRNA expression levels of GPR54 and L7 in breast cancer cell lines and B35 clone 1 (cDNA dilution 1:100). (B) Protein expression levels of GPR54 and actin in breast cancer cell lines and B35 clone 1 (30 μg). Results are representative for experiments within three different passages of each cell line.

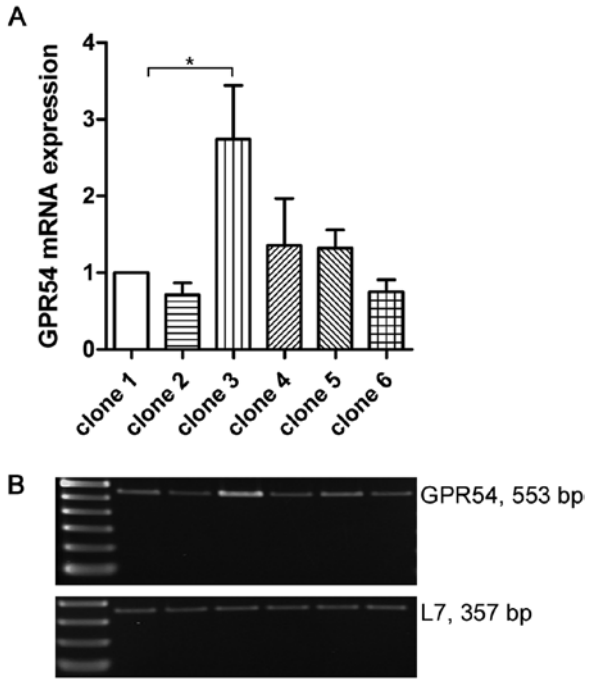

Figure 3. GPR54 mRNA expression in B35 mGPR54 clones. mRNA expression levels of GPR54 and L7 in B35 clones. Receptor expression was standardized to the housekeeping gene L7 in relation to B35 clone 1 [n=3 (mean ± SEM); *p<0.05 between clone 1 and clone 3]. Results and images represent the findings of experiments within three different passages of each cell clone.

GPR54 expression in cells overexpressing the receptor. As an artificial cell model overexpressing GPR54, B35 neuronal rat cells stable transfected with murine GPR54 were chosen. Different clones of transfected B35 cells were tested for their GPR54 expression levels. mRNA of six clones was analyzed by RT-PCR. Results are shown in relation to B35 clone 1 (Fig. 3). Clone 2, 4, 5 and 6 expressed GPR54 in a similar quantity compared to clone 1. GPR54 expression level in clone 3 was

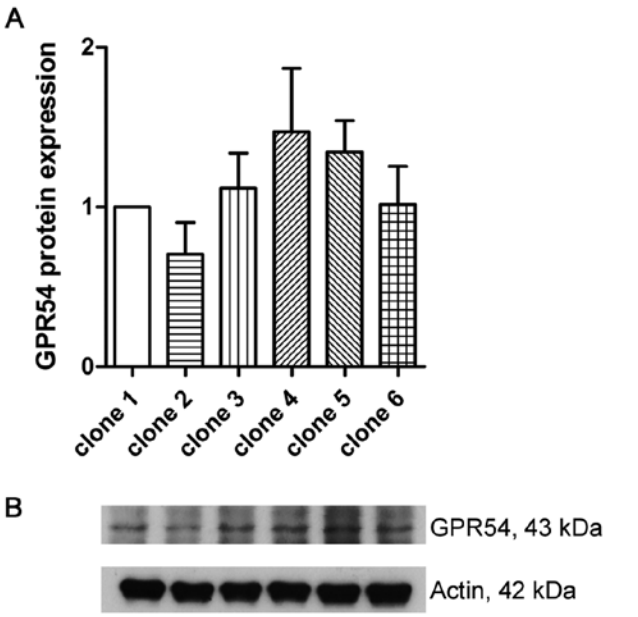

Figure 4. GPR54 protein expression in B35 mGPR54 clones. Protein expression levels of GPR54 and actin in B35 clones. Receptor expression was standardized to actin in relation to B35 clone 1 [n=3 (mean ± SEM)]. Results and images represent the findings of experiments within three different passages of each cell clone.

2.5-fold higher than in clone 1 ($p<0.01$). On protein levels, all of the clones showed no significant difference of GPR54 expression (Fig. 4).

Endogenous and artificial GPR54 expression. mRNA analysis on GPR54 in B35 clone 1 and breast cancer cell lines showed a highly different extent of expression (Fig. 2A). In transfectants, GPR54 levels were extremely increased compared to the breast cancer cells with regard to the used initial cDNA concentration (1:100 for B35 clone 1). This effect was not observed on protein levels for the same amount of protein of each sample (Fig. 2B).

Kisspeptin-10 has no effect on proliferation in breast cancer cells. For proliferation studies, four breast cancer cell lines were chosen. MDA-MB-231, MDA-MB-435s, HCC 1806 and MCF-7 showed different GPR54 expression levels according to the results on mRNA and protein analysis (Fig. 2). Proliferation was measured after treatment with KP-10 in different concentrations. KP-10 was added once daily (Fig. 5A) or twice daily (data not shown) to account for its rapid degradation (27-29). Under both treatments, no effect on proliferation was detected in all of the breast cancer cell lines.

Kisspeptin-10 inhibits proliferation in cells overexpressing GPR54. The effect of KP-10 on proliferation was studied in cells stably transfected with GPR54. B35 cells (rat) overexpressing murine GPR54 were used. Proliferation was analyzed in three B35 clones treated with KP-10 once daily (Fig. 5B) respectively, twice daily (data not shown) in different concentrations. The two treatments showed comparable results. Transfection was stable as controlled by RT-PCR samples of cells growing in experimental or transfection media (data not

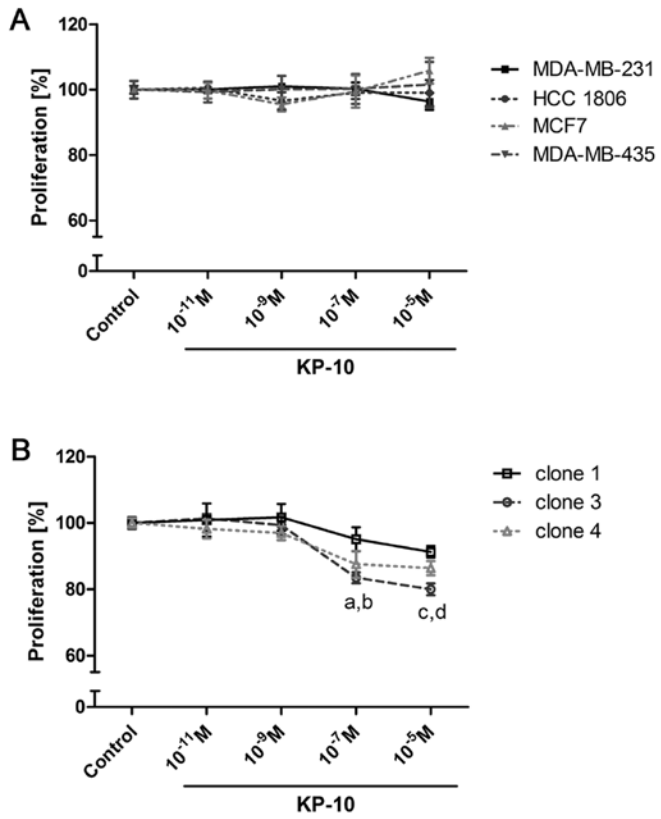

Figure 5. Proliferation of breast cancer cell lines and B35 mGPR54 clones after KP-10 treatment. Proliferation was measured after 72 h of daily KP-10 treatment with increasing concentrations in MDA-MB-231, MDA-MB-435s, HCC 1806, MCF-7 (A) and in B35 clone 1, 3 and 4 (B). Results in (A and B) are representative for at least three experiments with different passages of each cell line [n=6 respectively n=8 (mean ± SEM); a, $p<0.001$, c3 vs. control; b, $p<0.01$, c4 vs. control; c, $p<0.001$, c3 vs. control; d, $p<0.001$, c4 vs. control].

shown). KP-10 showed an antiproliferative effect in transfected B35 cells. Proliferation of clone 1 was inhibited in concentrations of 10^{-5} M KP-10 vs. control (91.3%; not significant). The proliferation of clone 3 and clone 4 was significantly inhibited at concentrations of 10^{-7} M vs. control (83.5%; $p<0.001$, respectively 87.5%; $p<0.01$) and 10^{-5} M KP-10 vs. control (80.0% respectively 86.4%; $p<0.001$).

Discussion

The antimetastatic effect of kisspeptin was investigated and validated in a large number of studies showing reduced migration and invasion, modified adhesion processes, changes in cytoskeleton and chemotactic behavior (7,8,14,17-24). However, the results on the influence of kisspeptin on cell proliferation differed. In cell lines transfected with GPR54, an antiproliferative effect of kisspeptin was shown, e.g. in Chinese hamster ovary CHO cells (8,18), murine fibroblast NIH3T3 cells (19) and human breast cancer MDA-MB-435s cells (25). HUVECs with endogenous receptor expression also showed reduced proliferation by kisspeptin treatment (20). In contrast, another study in HUVECs did not detect an influence of kisspeptin on proliferation (26). No changes in proliferation were shown in pancreatic cancer cell lines AsPC-1 and PANC-1 (14), in renal cell carcinoma Caki-1 and ACHN cells (17) and trophoblasts (21). These cell lines showed low endogenous GPR54 expression. Similar results were detected in MDA-MB-231 and MCF-7 breast cancer cells, but information was not given on GPR54 receptor levels (22). No effect on proliferation was shown in MDA-MB-231 breast cancer cells (11), SKOV3 ovarian cancer cells (23) and C8161 melanoma cells (2) transfected with the KISS1 gene. In summary, no antiproliferative effects of kisspeptin were shown in cancer cells expressing GPR54 endogenously. In contrast, cells artificially overexpressing the receptor were reduced in their proliferation by kisspeptin. The present study offers evidence for a connection between the antiproliferative effect of kisspeptin and the nature of GPR54 expression.

Breast cancer cell lines were used as experimental model. Diverging results have been published using these cells. Reduced proliferation of MDA-MB-435s breast cancer cells treated with kisspeptin was shown (25), while no changes in proliferation of MDA-MB-231 and MCF-7 breast cancer cells were found (11,22). GPR54 expression levels were assessed by immunocytochemistry, on mRNA and protein levels because of controversial findings in literature. T47D and ZR75-1 were described as GPR54-positive (12). GPR54 was found in MDA-MB-231 (11,30), but there are also studies showing no receptor expression in this cell line (12). In MDA-MB-435s no GPR54 was detected (12,25,31). MCF-7 cells showed receptor expression (12). In the present study, all of the tested breast cancer cell lines were assessed as GPR54-positive based on the immune cytochemical and western blot results. Compared to the findings by western blot analysis, mRNA of GPR54 was only detected in cells with higher endogenous GPR54 protein levels.

Proliferation studies in breast cancer cell lines with natural GPR54 expression showed no effect of kisspeptin. However, in B35 neuronal rat cells transfected with murine GPR54, inhibition of proliferation was recorded. Murine and human GPR54 proteins are homologous up to 82% within their amino acid structure (10). Gene products of human KISS1 and murine KISS1 share related parts and are much conserved within their active short forms (19). Both, receptors and ligands, were used interchangeable with similar effects (8,32). Regarding to this, experiments carried out in breast cancer cell lines were compared to experiments with transfected B35 cells. The breast cancer cell lines represented cells with endogenous GPR54 expression and the B35 clones were used as an artificial cell model for GPR54 transfected cells. Differences in the amount of cellular GPR54 were observed by mRNA analysis. On protein levels, this trend could not be confirmed. This may be due to differences in the antigen structure and no commercially available antibody for parallel detection of human and murine GPR54. The results showed an antiproliferative effect of kisspeptin only in cells overexpressing GPR54 artificially. This effect did not occur in cells with spontaneous GPR54 expression. These findings are in agreement with another study showing an antiproliferative effect in MDA-MB-435s cells transfected with GPR54 (25). As shown in the present study, no changes in proliferation were detectable in these cells without transfection.

In studies with HUVECs endogenously expressing GPR54, and transfected CHO cells, a dose-dependent receptor activation was measured showing a ten times more sensitive reaction

in the overexpressing cells (26). Thus, there is evidence for GPR54 mediated cellular mechanisms involved in proliferation, that are only detectable in cells with up regulated receptor expression. The results of the present study showed a connection between the antiproliferative effect of kisspeptin and the nature of GPR54 expression. All tested cell lines with endogenous GPR54 expression showed no changes in proliferation by kisspeptin. The effect was only detectable in cells with artificial receptor expression. Based on this, the antiproliferative action of kisspeptin seems to be not relevant in the pathophysiological context.

Acknowledgements

This study was supported by a grant of the Deutsche Krebshilfe, Dr Mildred Scheel Stiftung. We thank Robert P. Millar and Kevin Morgan (Edinburgh, UK) for the gift of the transfected cell lines. We thank Sonja Blume, Renate Dietrich and Matthias Läsche for technical assistance.

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