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Ribozyme targeting of HER-2 inhibits pancreatic cancer cell growth *in vivo*

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Abstract

We have analysed HER-2 expression and function in pancreatic cancer cells to determine whether HER-2 has a rate-limiting role for pancreatic cancer cell growth *in vitro* and *in vivo*. To specifically assess HER-2 function, we used HER-2-targeted ribozymes expressed under the control of the tet-off promoter system. Six out of 11 human pancreatic cancer cell lines expressed all four epidermal growth factor (EGF)-receptor family members (HER-1 (EGF-R), HER-2, HER-3, and HER-4), including Panc89 cells. Expression of the ribozymes quenched endogenous *HER-2* mRNA levels in Panc89 cells by approximately 40–60% which was reflected by a 40–50% reduction of the HER-2 surface glycoprotein. HER-2 depletion inhibited the *in vitro* proliferation rate by approximately 40% and decreased *in vivo* tumour growth by approximately 60% ($P<0.05$). Our study demonstrates for the first time a rate-limiting role for HER-2 in pancreatic cancer cell proliferation and suggests HER-2 targeting as a potential approach in pancreatic cancer therapy.

Keywords: Pancreatic cancer; EGF-receptor family; Ribozymes; Tet-off promoter system

1. Introduction

28 000 pancreatic cancer cases are diagnosed in the United States (US) every year. A chance of cure exists only for a minority of the patients with a locally limited and surgically resectable tumour [1]. However, 70–80% of patients whose tumour can be surgically exstirpated will suffer an incurable local relapse, distant metastases or peritoneal carcinosis [2]. Pancreatic cancer is one of the most aggressive tumours and has an overall 5-year survival rate of only 2% [1]. It is hoped that understanding the molecular basis of pancreatic cancer may provide new therapeutic targets and improved clinical outcomes.

The *HER-2* proto-oncogene belongs to the epidermal growth factor (EGF) receptor family and has been implicated in malignant transformation [3]. HER-2 can be activated by at least three different genetic mechanisms including point mutation [4], gene amplification [5] and overexpression [6]. Amplification and/or overexpression is observed in 20–30% of adenocarcinomas such as breast, ovary, lung, stomach and pancreatic cancer [7,8]. So far, the vast majority of experimental studies regarding HER-2 function have been performed in breast cancer [11] and show that HER-2 overexpression is correlated with an unfavourable prognosis in these patients [5]. In pancreatic cancer, however, the assessment of the prognostic influence of HER-2 overexpression has produced conflicting data [9,10] and studies evaluating HER-2 function in pancreatic cancer have not been reported.

Analysis of HER-2 function becomes difficult because no ligand for HER-2 has been found and HER-2 is now viewed merely as a signal transducing subunit of EGF- and NDF/heregulin-receptors, i.e. HER-3 and HER-4 [12]. Depending on the cellular context, HER-2 targeted antibodies can thus cause activating or inhibitory effects, making it difficult to determine the precise role of HER-2. In this study, we utilise specific ribozymes, expressed under the control of a tet-off promoter system, to selectively cleave *HER-2* mRNA and thus deplete the cells of the endogenous gene product. With this approach, the effects of a functional knockout can be studied in model cell lines and thus the contribution

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of a particular gene product can be delineated [13–18]. The tet-off promoter system provides a model for studying HER-2-mediated effects in identical clones by switching on and off the ribozyme, and subsequently, HER-2 expression. This eliminates the potential risk of clonal variations which may otherwise contribute to phenotypic differences.

2. Materials and methods

2.1. Plasmids and generation of constructs

Plasmids expressing the tetracycline transactivating (tTA)/VP16 fusion protein (pUHG15-1 [19]) and the tTA/heptameric operator binding site (tet-O; pUHC13-3 [19]) were obtained from Bujard (Heidelberg, Germany). The ribozyme expression plasmid (pTET) was derived from pUHC13-3 and modified as described in Ref. [20]. The *HER-2*-targeted hammerhead ribozyme was designed and cloned as described in Ref. [18].

In brief, the following ribozyme coding sense and antisense oligonucleotides (sense: 5′-agcttCCTGAA-AG*CTGATGAGTCCGTTAGGACGAA*AAAGTCCTa-3′; antisense: 5′-agcttAGGACTTT*TTCGTCCTAACG-GACTCATCAG*CTTTCAGGA-3′; with small letters indicating *HindIII*-restriction site overhangs; bold capital letters showing *HER-2*-specific antisense regions; and italic capital letters indicating the hammerhead ribozyme core sequence) were annealed together and ligated into the *HindIII*-restriction site of pTET. The resulting ribozyme expression plasmid pTET/Rz3 contains *HER-2*-specific antisense flanking regions of eight nucleotides (nt) on both ends of the 22 nt catalytic hammerhead ribozyme core sequence, that target it to a central region in the *HER-2* RNA just downstream of the transmembrane region of the protein. Correct sequences of the ribozymes were verified by DNA sequencing and specific catalytic ribozyme activity was demonstrated in *in vitro* cleavage assays as previously shown in Ref. [18].

2.2. Cell lines and transfections

The human gastric cancer cell line MKN7 (positive control cell line for HER-2 overexpression) and the human pancreatic cancer cell lines BXPC-3, Capan2, HPAF were obtained from ATCC (Rockville, MD), Capan1, Colo357, Panc89, A816-4, Panc-Tu1, SW850, ASPC-1 and PT45P1 were kindly provided by H. Kalthoff, University Hospital Kiel, Germany. Cells were maintained in culture at 37°C/5% CO_2 using IMEM (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with L-glutamine and 10% heat-inactivated fetal bovine serum (FBS). Panc89 cells were transfected using LipofectAmine (Life Technologies). Briefly, cells at 50–70% confluence were incubated for 5 h with plasmid DNA mixed with LipofectAmine in serum-free Opti-MEM medium (Life Technologies) at 37°C in 5% CO_2. The transfection medium was then replaced with normal growth medium and 36 hours later supplemented with the respective drugs for selection of stable integrants. Panc89 cells, stably expressing tetracycline-regulated *HER-2* targeted ribozymes, were generated in a two-step transfection protocol. In a first step, Panc89 cells were transfected with 10 μg of pUHG15-1 plasmid DNA and 1 μg of pcDNA3 plasmid DNA (Invitrogen, San Diego, CA, USA) to provide G418 resistance. After selection for stable integrants in the presence of G418 at 0.7 mg/ml, individual tTA-expressing clones were isolated with cloning rings. To test the clones for tTA-expression and tetracycline regulation, the cells were transiently transfected in the absence and presence of 1 μg/ml tetracycline (Sigma) with pUHC13-3 plasmid DNA that contains a *luciferase* cDNA under the control of the tet-O binding site [19]. Cell lysates were prepared 36 h after the transfections and luciferase activities were measured in a luminometer as described in Ref. [19]. The clone (Panc89/tTA-3) that demonstrated maximal tetracycline regulation of luciferase activity was used for further transfections with the ribozyme expression plasmids. Panc89/tTA-3 cells were then transfected with 10 μg of pTET/Rz3 mixed with 1 μg of pZeo (Invitrogen) to provide Zeocin resistance. Clonal cell lines (Panc89/Rz3) were obtained after selection with 0.4 mg/ml Zeocin and 1 μg/ml tetracycline and screened by fluorescent activated cell sorting (FACS) analysis (FACStarplus, Becton Dickinson) for tetracycline-regulated *HER-2* expression.

2.3. Northern analysis

Total cellular RNA was isolated with the RNA STAT-60 method (Tel-Test, Friedenswood, TX, USA), and 30 μg were separated and blotted as described in Ref. [21]. A *HER-2/neu* cDNA probe (1.5 Kb *EcoRI* fragment) was hybridised, washed and exposed to film for 16 h [18]. To correct for variability in loading, blots were stripped and reprobed with a Glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) cDNA probe (Clontech, Palo Alto, CA, USA). Relative band intensities were measured by densitometry.

2.4. Fluorescence activated cell sorting (FACS)

To quantitate HER-1 (EGF-R), HER-2, HER-3 and HER-4 protein levels by FACS-analysis, cells were trypsinised, washed once with growth medium containing serum, twice with phosphate-buffered solution (PBS) (Sigma) and resuspended in PBS at 5×10^5 cells/100 μl. The cells were incubated for 30 min at 4°C with a 1:100 dilution of the respective primary mouse mono-

clonal antibody (Neomarkers, Fremont, CA, USA). Cells were washed twice with PBS and incubated for 30 min at 4°C in the dark with a 1:200 diluted fluorescein isothiocyanate (FITC)-labelled goat anti-mouse secondary antibody (Boehringer-Mannheim, Mannheim, Germany). After two final washes with PBS, the mean value of fluorescence intensity of 10 000 cells was determined by FACS analysis. Unlabelled cells and cells labelled with secondary antibody alone served as negative controls.

2.5. Proliferation assay

Cells (5×10^3/well) were plated on microtitre plates and cultivated using culture medium with and without 1 μg/ml tetracycline, respectively. After 6 days, WST-1 reagent was added (Boehringer-Mannheim) and incubated for 30 min to determine the number of intact cells. The absorbance were determined at 450 nm using an enzyme-linked immunosorbent assay (ELISA) microreader.

2.6. Tumour growth in animals

10^6 pancreatic cancer cells (100 μl) were injected subcutaneously (s.c.) in both flanks of female athymic nude mice (NCr nu/nu: NCI, Frederick, MD, USA). We compared three groups (each $n=5$ mice): group 1 were injected with Pan89/tTA-3 control cells, groups 2 and 3 with Panc89/Rz3 ribozyme-expressing cells. Mice of group 3 obtained food containing 200 mg/kg doxycyline (Bioserve, Frenchtown, NJ, USA) to block ribozyme expression (doxycycline has the same properties as tetracycline, but can be absorbed in the intestinal tract). Tumour growth was monitored for 16 days and tumour sizes were estimated from the product of the perpendicular diameters of the tumours.

2.7. Data analysis

Mean±standard error of the mean (S.E.M.) are depicted unless indicated otherwise. Student's unpaired *t*-test was used for comparisons between data sets and $P<0.05$ was considered significant.

3. Results

3.1. Screening of pancreatic cancer cell lines for HER-1, HER-2, HER-3 and HER-4 expression

We used the HER-2 overexpressing gastric cancer cell line MKN7 as a standard cell line to determine HER-2 overexpression in the pancreatic cancer cell lines. Overall, HER-2 expression was detectable in all pancreatic cancer cell lines, but low compared with the expression in MKN7 cells (Fig. 1b). While all pancreatic cancer cell lines also expressed HER-1 (Fig. 1a), detectable levels of HER-3 and HER-4 were found in 9/11 and 6/11 cell lines, respectively (Fig. 1c and d).

HER-2 function depends on an interaction with HER-3 and HER-4 which heterodimerise in a ligand-dependent manner with HER-2. In pancreatic cancer tissue, HER-2 coexpression with HER-3 and HER-4 is frequently seen [22]. We chose Panc89 pancreatic cancer cells for our experiments because they show this typical expression pattern and express average HER-2 levels.

3.2. Generation of tetracycline Transactivator (tTA) expressing Panc89 cells

To avoid promoter interference and to generate cells in which tet-O controlled transgene expression can be tightly regulated by tetracycline, a two-step transfection protocol was used as originally described in Ref. [19]. In a first step, Panc89 cells were stable transfected with pUHG15-1 plasmid DNA. Individual clones were screened for tetracycline-regulation of tTA by transient transfection with a pUHC13-3 plasmid DNA which codes for luciferase under the control of the tet-O binding site. Luciferase activity was measured in the absence and presence of tetracycline. In the absence of tetracycline, luciferase activity was high in all clones. Maximal tetracycline regulation was observed in clone Panc89/tTA-3 showing tetracycline-dependent repression of tTA-driven luciferase activity by more than 95% (Fig. 2). This clone was used for further transfections with pTET/ribozyme expression plasmids.

HER-1(EGF-R), HER-2, HER-3 and HER-4 expression as measured by FACS analysis did not vary between the clones, indicating that clonal selection and/or tTA expression had no effect on the expression of the EGF-R family members in Panc89 cells (data not shown).

3.3. Efficacy of HER-2-targeted ribozymes

Panc89/tTA-3 cells were transfected with pTET/Rz3 plasmids. Under Zeocin selection several clones were obtained and screened for tetracycline-regulated HER-2 expression using repetitive (at least three times) FACS analyses. For further studies, we used clone Panc89/Rz3. These cells demonstrated a consistent 40–60% downregulation of *HER-2* on the mRNA level (northern blot). Because for the biological ribozyme effect downregulation of HER-2 protein levels is most relevant, we confirmed the northern blot data by FACS analysis. In correlation with the mRNA levels (Fig. 3, left panel of inset) we found a 40–50% reduction of HER-2 surface glycoprotein (Fig. 3 and right panel of inset). In our FACS analysis, we determined HER-1 (EGF-R) as an internal control and found levels were not affected by tetracycline-mediated modification of

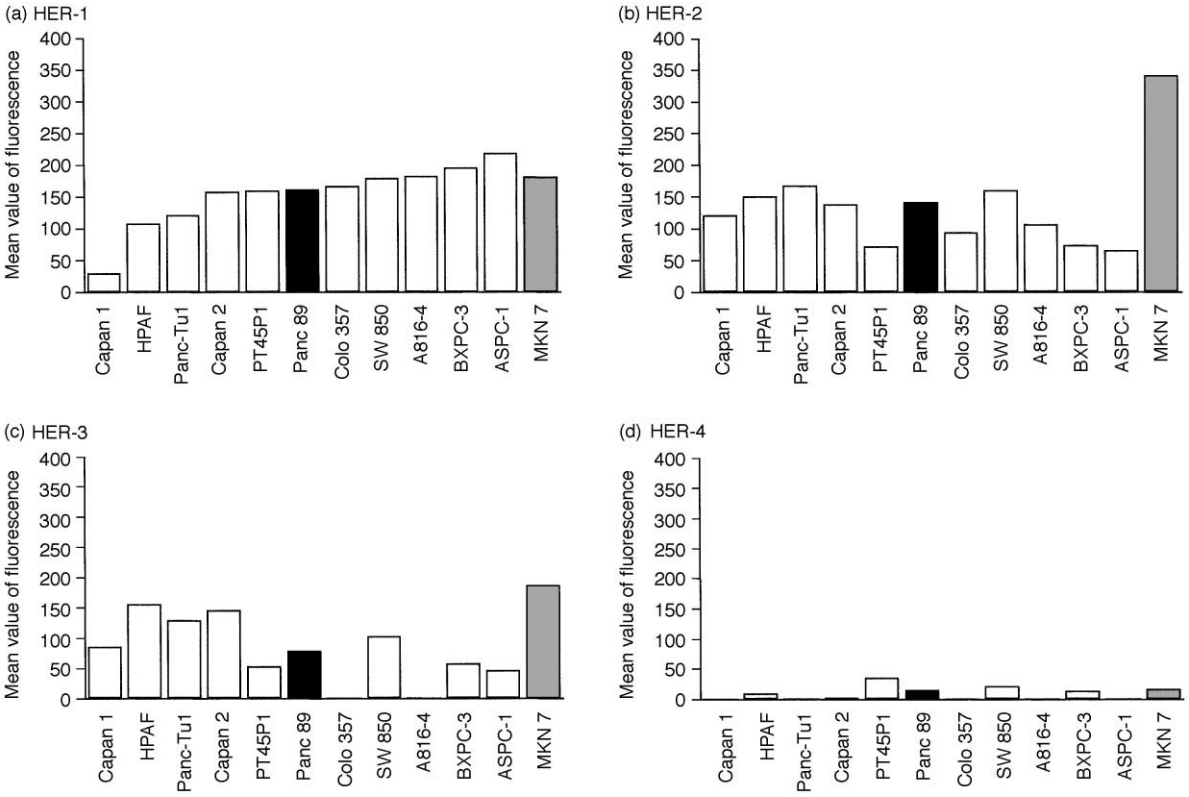

Fig. 1. HER-1 (A), HER-2 (B), HER-3 (C) and HER-4 (D) expression levels in 11 human pancreatic cancer cell lines and the reference gastric cancer cell line MKN7 as determined by fluorescent activated cell sorting (FACS) analysis. Shown are the mean fluorescence values.

ribozyme expression. HER-1 protein expression was comparable with Panc89 wild-type cells (data not shown).

3.4. *HER-2 affects pancreatic cancer cell proliferation* in vitro

As shown in Fig. 4, ribozyme targeting of HER-2 inhibited the *in vitro* proliferation rate of Panc89 cells by 40%. The anti-proliferative ribozyme effect was completely reversed in tetracycline-treated cells. The proliferation rate of Panc89/tTA-3 control cells was not affected by tetracycline as expected.

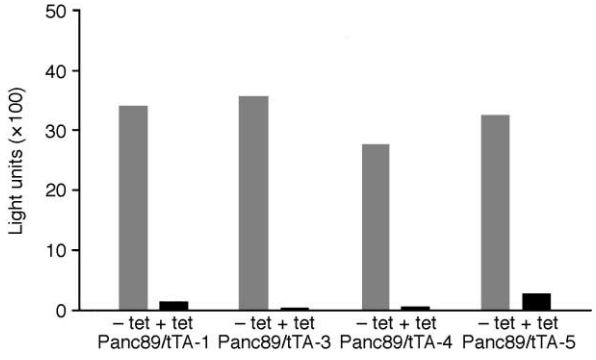

Fig. 2. Luciferase assay to determine tetracycline-dependent regulation of gene expression in stably transfected Panc89 pancreatic cancer cell clones. Panc89 cells expressing the tetracycline Transactivator (tTA) protein were transiently tranfected with the pUHC13-3 plasmid DNA that codes for luciferase under the control of the tet-O binding site. Luciferase activity was measured 36 hours after transient transfection in the absence (−tet) and presence of tetracycline (+ tet).

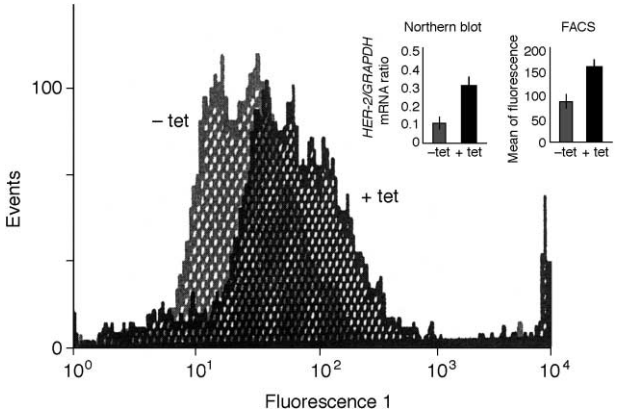

Fig. 3. Fluorescent activated cell sorting (FACS) analysis to determine tetracycline-controlled ribozyme cleavage of HER-2 surface protein in Panc89/Rz3 pancreatic cancer cells. Addition of tetracycline (+tet) maintained HER-2 at baseline levels of Panc89 wild-type cells, while in the absence of tetracycline (−tet) HER-2 levels were reduced by approximately 40%. The inset compares ribozyme-dependent mRNA level of *HER-2* (adjusted to *GAPDH* as a loading control) and HER-2 protein levels (FACS; mean values of fluorescence adjusted to background control).

3.5. *Downregulation of HER-2 expression inhibits tumour growth* in vivo

When injected s.c. into nude mice, Panc89/tTA-3 control cells grew into tumours within a few days (Fig. 5, dashed line). Likewise, injection of Pan89/Rz3 cells resulted in tumours of comparable size (70 mm^2, ± 15 mm^2) when doxycycline was added to the food to abolish ribozyme expression (Fig. 5, bold line). However, when HER-2-targeted ribozymes were expressed (Fig. 5, dotted line), the growth of Panc89 tumours was significantly inhibited by approximately 60% showing a mean tumour size of 25 mm^2 size (± 10 mm^2) after 15 days ($P < 0.05$).

4. Discussion

The significance of HER-2 on tumour growth and its role as a potential target in the development of novel anti-cancer agents was initially deduced from clinical studies in breast cancer which found a significant correlation between HER-2 overexpression and prognosis [5]. Subsequent functional analyses of HER-2 have been almost exclusively performed in breast cancer models and have demonstrated that HER-2 overexpression promotes breast cancer cell growth *in vitro* and *in vivo* [11]. Consequently, HER-2 targeting agents were developed and, recently, the HER-2 blocking antibody Herceptin has been shown to be a valuable anti-cancer drug in the metastatic breast cancer setting due to its growth inhibiting properties [23]. These clinical studies suggested that HER-2, which is also overexpressed in a variety of other cancers including lung, gastric and pancreatic cancer [24] may be a most promising molecular target.

In previous studies, we and others found HER-2 overexpression in approximately 30% of pancreatic cancer specimens [8,22]. However, in contrast to studies in breast cancer, the prognostic impact of HER-2 in pancreatic cancer is not proven and the available data are conflicting [9,10]. This, and the lack of studies concerning the function and significance of HER-2 in pancreatic cancer, prompted us to monitor HER-2 expression levels in pancreatic cancer cells and to perform HER-2 ribozyme targeting experiments *in vitro* and *in vivo*.

We found HER-2 expression in all pancreatic cancer cell lines with HER-2 levels being at least 50% below the level of our reference cell line MKN7. These relatively low expression levels of HER-2 in pancreatic cancer cells are in agreement with earlier studies showing that HER-2 expression in pancreatic cancer tissue is lower overall compared with breast cancers [25].

To analyse the specific role of HER-2 in pancreatic cancer cell growth, we chose human Panc89 pancreatic cancer cells because our FACS analysis (Fig. 1) demonstrated that HER-2 levels in Panc89 represent average values for most pancreatic cancer cell lines. In addition, Panc89 cells express all four EGF-R family members which is consistent with the expression pattern frequently found in pancreatic cancer tissues [22].

To specifically inhibit HER-2 expression we used a ribozyme which has been extensively characterised by *in vitro* and *in vivo* cleavage studies [18]. Furthermore, this ribozyme has no antisense activity as demonstrated

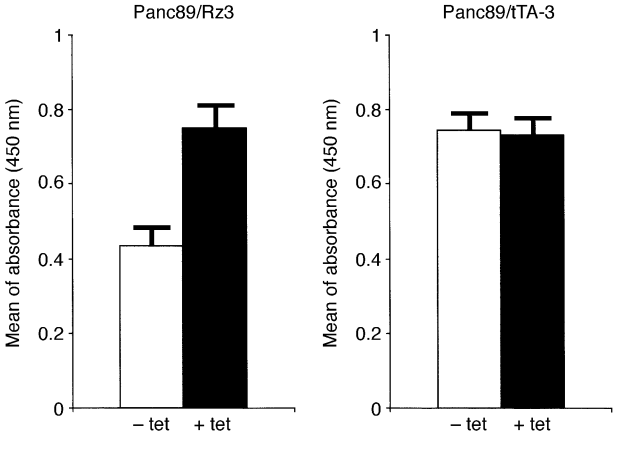

Fig. 4. Analysis of the proliferation rate of Panc89/Rz3 cells (left) and Panc89/tTA-3 control cells (right). Cells were grown for 6 days untreated (−tet) and treated with tetracycline (+tet) which inhibits the expression of HER-2-targeted ribozymes in Panc89/Rz3 cells. Cell numbers were analysed after the addition of WST-1 reagent in an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm.

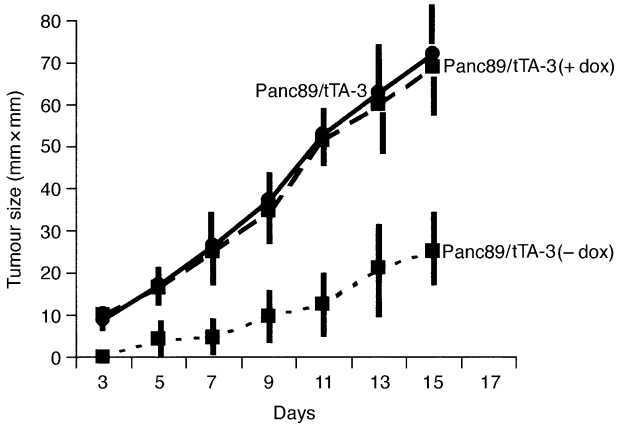

Fig. 5. Tumour growth in nude mice following the subcutaneous (s.c.) injection of 10^6 Panc89/Rz3 pancreatic cancer and Panc89/tTA-3 control cells (bold line) in each flank. One group of Panc89/tTA-3 mice received doxycycline-enriched food (+dox, dashed line) to inhibit ribozyme expression and reconstitute HER-2 levels. Tumour growth was compared with untreated, ribozyme-expressing and HER-2-depleted, cells (−dox, dotted line). The tumour size ($n = 10$ per group) is the product of perpendicular diameters and differed significantly between doxycyline-treated and untreated mice ($P < 0.05$).

using a mutant control, which lacks cleavage activity [18]. Finally, the specific regulation of HER-2 levels is strongly supported by a study from Werner and Uhlenbeck [26] who showed that the presence of only 2 nucleotide mismatches completely diminished ribozyme cleavage. As determined by Blast-search the target sequence of our ribozyme is unique for *HER-2* mRNA.

This ribozyme under control of the tet-off promoter system allowed us to specifically regulate HER-2 levels within the intact pathophysiological context of identical cancer cells. Hence, the observed changes of the phenotype and growth characteristics are directly linked to HER-2. This experimental design excludes pitfalls of other models which may include errors derived from clonal variations, unphysiological HER-2 levels in transfected cell lines, or inconsistent data caused by antibody interference with HER-2 receptor function.

Our study is the first to demonstrate a direct role of HER-2 in pancreatic cancer cell growth suggesting HER-2 may act as a rate-limiting growth factor receptor. It is remarkable that an inhibition of HER-2 protein levels by only 40% substantially affected the *in vitro* proliferation rate, which was reduced by 40%. Even more dramatic was the finding under *in vivo* conditions where targeting HER-2 reduced the tumour growth of Panc89 pancreatic cancer cells by 60%. Assuming that higher depletion of HER-2 in pancreatic cancer cells results in a further reduction of tumour growth as demonstrated in ovarian cancer cells [18], the significance of HER-2 for pancreatic cancer cells is clear.

In summary, for the first time the functional role of HER-2 has been studied in pancreatic cancer cells implicating HER-2 as a rate-limiting growth factor receptor for pancreatic cancer cell growth. The reduction of tumour growth by the inhibition of HER-2 receptor activity is comparable to studies in breast and ovarian cancer and strongly suggests HER-2 may be a novel therapeutic target in pancreatic cancer.

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References

1. Warshaw AL, Fernandez-del Castillo C. Pancreatic cancer. *N Engl J Med* 1992, **26**, 455–465.
2. Henne-Bruns D, Vogel I, Lüttges J, Klöppel G, Kremer B. Ductal adenocarcinoma of the pancreas head: survival after regional versus extended lymphandectomy. *Hepato-Gastroenterol* 1998, **45**, 855–866.
3. Bacus SS, Zelnick CR, Plowman G, Yarden Y. Expression of the erbB-2 family of growth factor receptors and their ligands in breast cancers. Implication for tumor biology and clinical behavior. *Am J Clin Pathol* 1994, **102**, S13–S24.
4. Bargmann CI, Hung MC, Weinberg RA. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. *Cell* 1986, **45**, 649–657.
5. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987, **235**, 177–182.
6. Di Fiore PP, Pierce JH, Kraus MH, Segatto O, King CR, Aaronson SA. erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells. *Science* 1987, **237**, 178–182.
7. Stancovski I, Sela M, Yarden Y. Molecular and clinical aspects of the Neu/ErbB-2 receptor tyrosine kinase. *Cancer Treat Res* 1994, **71**, 161–191.
8. Yamanaka Y, Friess H, Kobrin MS, *et al*. Overexpression of HER2/neu oncogene in human pancreatic carcinoma. *Hum Pathol* 1993, **24**, 1127–1134.
9. Lei S, Appert HE, Nakata B, Domenico DR, Kim K, Howard JM. Overexpression of HER2/neu oncogene in pancreatic cancer correlates with shortened survival. *Int J Pancreatol* 1995, **17.1**, 15–21.
10. Dugan MC, Dergham ST, Kucway R, *et al*. HER-2/neu expression in pancreatic adenocarcinoma: relation to tumor differentiation and survival. *Pancreas* 1997, **14**, 229–236.
11. El-Ashry D, Lippman M. Molecular biology of breast cancer. *World J Surg* 1994, **18**, 12–20.
12. Karunagaran D, Tzahar E, Beerli RR, *et al*. ErbB-2 is a common auxiliary subunit of NDF and EGF receptors: implications for breast cancer. *EMBO J* 1996, **15**, 254–264.
13. Christoffersen RE, Marr JJ. Ribozymes as human therapeutic agents. *J Med Chem* 1995, **38**, 2023–2037.
14. Czubayko F, Schulte AM, Berchem GJ, Wellstein A. Melanoma angiogenesis and metastasis modulated by ribozyme targeting of the secreted growth factor pleiotrophin. *Proc Natl Acad Sci USA* 1996, **93**, 14753–14758.
15. Feng M, Cabrera G, Deshane J, Scanlon KJ, Curiel DT. Neoplastic reversion accomplished by high efficiency adenoviral-mediated delivery of an anti-ras ribozyme. *Cancer Res* 1995, **55**, 2024–2028.
16. Scanlon KJ, Jiao L, Funato T, *et al*. Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. *Proc Natl Acad Sci USA* 1991, **88**, 10591–10595.
17. Jarvis TC, Alby LJ, Beaudry AA, *et al*. Inhibition of vascular smooth muscle cell proliferation by ribozymes that cleave c-myb mRNA. *RNA* 1996, **2**, 419–428.
18. Juhl H, Downing S, Wellstein A, Czubayko F. HER-2/neu rate limiting for ovarian cancer growth: conditional depletion of HER-2/neu by ribozyme-targeting. *J Biol Chem* 1997, **272**, 29482–29486.
19. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 1992, **89**, 5547–5551.
20. Schulte A, Lai S, Kurtz A, Czubayko F, Riegel A, Wellstein A. Human trophoblast and choriocarcinoma expression of the growth factor pleiotrophin attributable to germ-line insertion of an endogeneous retrovirus. *Proc Natl Acad Sci USA* 1996, **93**, 14759–14764.
21. Fang W, Hartmann N, Chow D, Riegel A, Wellstein A. Pleiotrophin stimulates fibroblasts and endothelial and epithelial cells and is expressed in human cancer. *J Biol Chem* 1992, **267**, 25889–25897.
22. Thybusch-Bernhardt A, Beckmann S, Juhl H. Comparative analysis of the EGF-receptor family in pancreatic cancer: expression of HER-4 correlates with a favourable tumor stage. *Int J Surg Investig* 2001, **2**, 393–400.
23. Cobleigh MA, Vogel CL, Tripathy D, *et al*. Multinational study of the efficacy and safty of humanized anti-HER-2 overexpressing

metastatic breast cancer that has progressed after chemotherapy for metastatic disease. *J Clin Oncol* 1999, **9**, 2639–2648.
24. Hung MC, Lau YK. Basic science of HER-2/neu: a review. *Semin Oncol* 1999, **26**, 51–59.
25. Hall PA, Hughes CM, Staddon SL, Richman PI, Gullick WJ, Lemoine NR. The c-erbB-2 proto-oncogene in human pancreatic cancer. *J Pathol* 1990, **161**, 195–200.
26. Werner M, Uhlenbeck O. The effect of base mismatches in the substrate recognition helices of hammerhead ribozymes on binding and catalysis. *Nucleic Acids Res* 1995, **23**, 2092–2096.