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Targeted Gene Expression Using a 1.1 Kilobase Promoter Fragment of the Tumour-associated Antigen EpCAM

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Abstract. *The epithelial cell adhesion molecule EpCAM is over- or de novo expressed during carcinogenesis. EpCAM expression correlates with increased proliferation and de-differentiation. Recently, we reported the cloning of a 1.1 kilobase fragment upstream of the epcam gene and demonstrated its specific transcriptional activity. Here, we analyzed the potential of this fragment for targeted gene expression. The fragment was used to regulate the expression of the green fluorescent protein (GFP) and HSV-1 thymidine kinase (HSV-TK), as a model therapeutic gene. Transfection of the pEpProm-control or pEpProm-GFP plasmids resulted in the expression of functional GFP and HSV-TK proteins specifically in EpCAM-positive cells. Expression levels of both proteins correlated with the amount of EpCAM. Additionally, the targeted expression of HSV1-TK transferred a marked sensitivity to ganciclovir treatment in EpCAM-positive HEK293-EBNA1 and SkBr3 carcinoma cells. The EpCAM promoter fragment is, thus, a novel tool to allow for the transcription of therapeutic genes, specifically, in EpCAM-positive carcinomas.*

Selective gene, protein or toxin delivery to tumour cells is of great value with respect to therapy. In recent years, several concepts have emerged and proved feasible: (i) targeting of carcinoma cells with retroviruses expressing single chain antibodies to specific cell surface molecules along with toxic substances or genes (1), (ii) treatment of carcinoma cells with recombinant proteins composed of a single chain antibody and immunotoxins (2), and, moreover, (iii) gene delivery using specific promoters (3, 4). All these approaches have in common the necessity for a specific anchor molecule or promoter in order to achieve gene delivery selectively in the cells of interest. In the case of squamous cell carcinoma of the upper aerodigestive tract, few disease-associated proteins are known, which may serve as targets. Among these proteins, the epithelial cell adhesion molecule EpCAM is one promising target for the above-mentioned therapeutic approaches (5). The expression of EpCAM positively correlates with the grade of neoplasia and with the proliferative status of epithelial cells (6, 7). Recent work by our group demonstrated a direct involvement of EpCAM in processes regulating cell cycle, proliferation and metabolism. *De novo* expression of EpCAM induced the transcription of the oncogene c-myc, cyclins and additional targets (8, 9). In an effort to understand the regulation of *epcam* gene transcription, we cloned and characterised a fragment of the promoter encompassing 1.1 kilobases (kb). Two subfragments of the promoter acted synergistically in epithelial cells expressing endogeneous EpCAM. The transcriptional activity correlated directly with the amount of EpCAM present in cells (10, 11).

In the present study, we focussed on the usage of the 1.1kb promoter fragment to express genes based on the presence of factors activating the endogeneous *epcam* promoter. For this purpose, two proteins were selected: the green fluorescent protein (GFP) and the herpes simplex virus 1-derived thymidine kinase protein (HSV1-TK). Both proteins were selectively expressed and functional in EpCAM-positive cells when transcribed under the control of the 1.1kb promoter fragment. Thus, the fragment of the EpCAM promoter has the potential to serve as a tool for gene expression in EpCAM-positive carcinomas.

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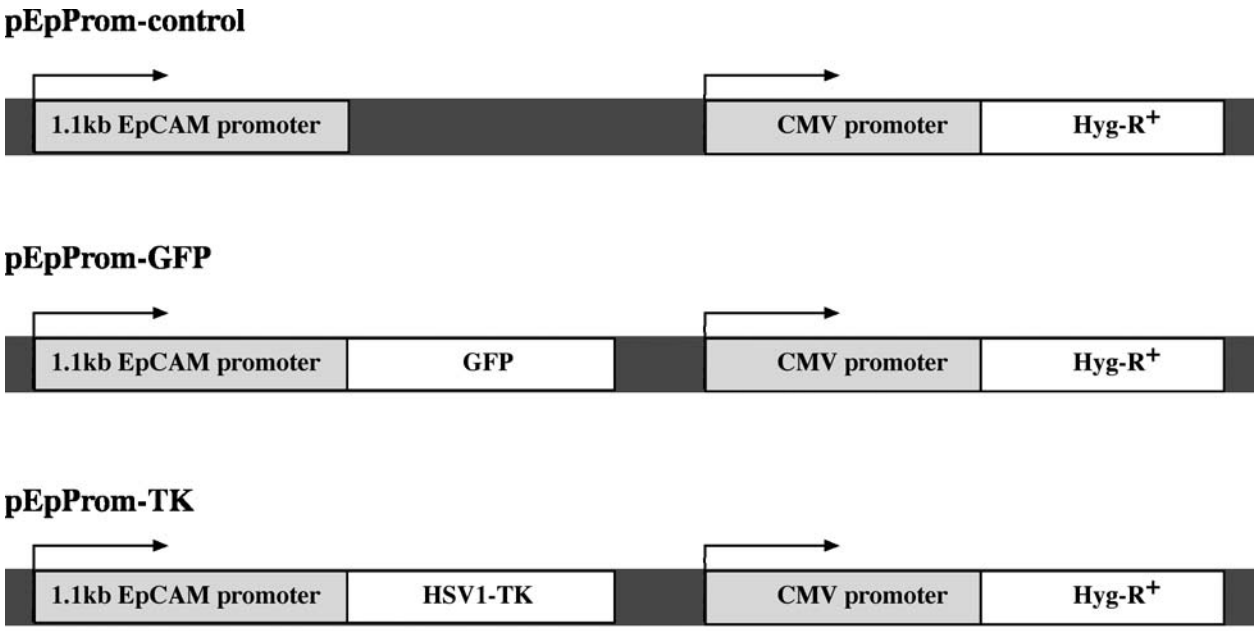

Figure 1. *Schematic representation of EpCAM promoter constructs. The 1.1kb fragment of the EpCAM promoter was inserted into pMEP4 to replace the metallothionein promoter, generating pEpProm-control. The cDNAs encoding GFP and HSV1-TK were inserted into the pEpProm-control to produce pEpProm-GFP and pEpProm-HSV1-TK.*

Materials and Methods

Plasmid cloning. The metallothionein promoter of the pMEP4 vector (Invitrogen, Karlsruhe, Germany) was replaced by an EpCAM promoter fragment of 1.1kb (pEpProm-control). The coding sequences for GFP and HSV1-TK were excised from the expression vectors pEGFP-C1 (BD Clontech, Heidelberg, Germany) and pGEX-6P-2-HSV-TK (kind gift of Dr. Maria Fogli, ETH Zürich, Switzerland), respectively. The coding sequences were cloned into the pEpProm-control to generate the pEpProm-GFP and pEpProm-HSV1-TK vectors.

Cell lines. HEK293 is a human epithelial embryonic kidney cell line (12). HEK293-EBNA1 is a stable transfectant of HEK293, which expresses the Epstein-Barr viral nuclear antigen 1 (EBNA1). SkBr3 is an EpCAM-positive breast cancer cell line (HTB-30, ATCC). Cell lines were grown in standard DMEM culture medium containing 10% fetal calf serum and propagated three times a week. HEK293-pEpProm-control and HEK293-pEpProm-TK, HEK293-EBNA1-pEpProm-control and HEK293-EBNA1-pEpProm-TK, SkBr3-pEpProm-control and SkBr3-pEpProm-TK cell lines were established by stable transfection using Metafectene (Biontex, Munich, Germany) according to the manufacturer's protocol. Stable transfectants were grown in 10% FCS DMEM standard medium containing 200μg/ml hygromycin (Calbiochem Merck KGaA, Darmstadt, Germany).

Transient transfections. pEpProm-control or pEpProm-GFP plasmids were transiently transfected in HEK293, HEK293-EBNA1 and SkBr3 cell lines using Metafectene (Biontex) as directed by the manufacturer.

Flow cytometry analysis. Cells ($1x10^5$ per sample) were incubated for 30 min with the EpCAM-specific antibody C215 (kind gift of Dr. H. Lindhofer, TrionPharma, Munich, Germany), washed once in PBS supplemented with 2% FCS and thereafter incubated for 15 min with FITC-conjugated secondary antibody. The cells were washed twice in PBS/FCS and cell surface expression of EpCAM analysed in a FACScalibur cytometer (Becton Dickinson, Heidelberg, Germany). GFP fluorescence of transiently transfected cells was analysed directly in a FACScalibur cytometer (FL-1 chanel).

Immunoblot analysis. $1x10^6$ cells were lysed in 100 μl lysis buffer (TBS/1% Triton and protease inhibitors, Roche complete™). Proteins were separated by SDS-PAGE and detected by specific antibodies (anti-GFP sc-9996 and anti-actin sc-1616, Santa Cruz, Heidelberg, Germany) in combination with HRP-conjugated secondary antibodies (Dako, Heidelberg, Germany) and the ECL reagent (Amersham Biosciences, Freiburg, Germany).

Reverse-transcription polymerase chain reaction. To analyze the mRNA expression level of HSV-TK in stable transfectants harbouring pEpProm-control or pEpProm-TK plasmids, $1x10^6$ cells were grown overnight in 10% FCS DMEM standard medium. Thereafter, total mRNA was isolated using the High Pure RNA Isolation Kit (Roche, Manheim, Germany) according to the manufacturer's protocol. RNA (1μg) was reverse transcribed into cDNA and PCR amplification of a fragment of the HSV-TK cDNA was performed with the following primers: HSV-TK-up: 5'-GAAAACCACCACCACGCAAC-3'; HSV-TK-down: 5'-ACCGCACCGTATTGGCAAGTAG-3', GAPDH-up 5'-TGTCGCTGTTGAAGTCAGAGGAGA-3', GAPDH-down 5'-AGAACATCATCCCTGCCTCTACTG-3'. PCR conditions: 95˚C 10 minutes, (95˚C 30sec, 58˚C 2min, 72˚C 1min) 30 cycles, 72˚C 10min.

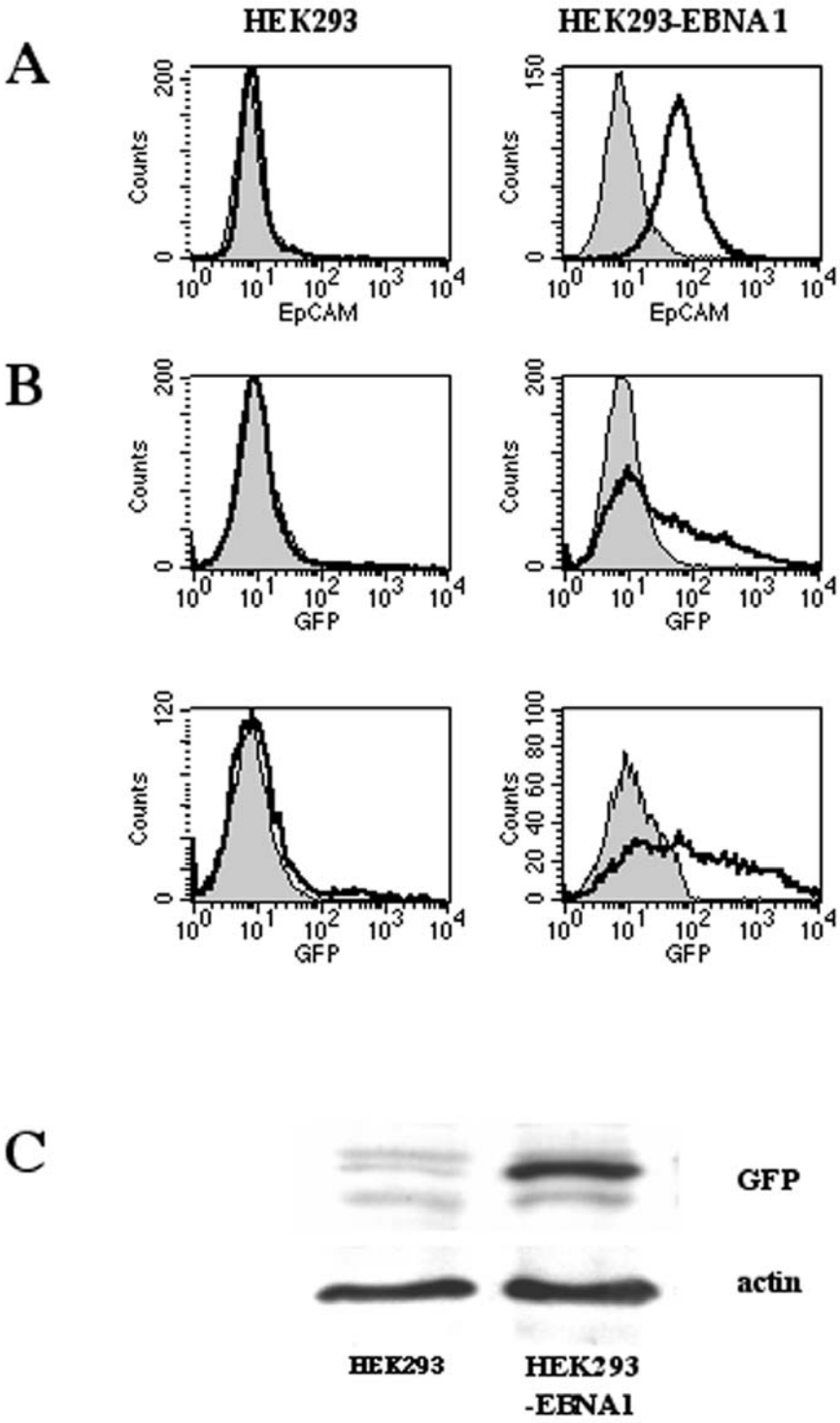

Figure 2. *GFP expression from pEpProm-GFP in HEK293 and HEK293-EBNA1 cells. (A) The expression of EpCAM on the cell surface of HEK293 and HEK293-EBNA1 cells was assessed by flow cytometry. Gray: control, black line: EpCAM. (B) Following the transient transfection of pEpProm-GFP (1 μg) in HEK293 and HEK293-EBNA1 cells (5x10^5), GFP fluorescence was assessed by flow cytometry after 24h (upper panels) and 48h (lower panels). (C) The expression of GFP protein was detected 48h after transient transfection in HEK293 and HEK293-EBNA1 cells by immunoblotting. Actin served as a control for equal protein loading in each lane. Shown are the representative results of three independent experiments.*

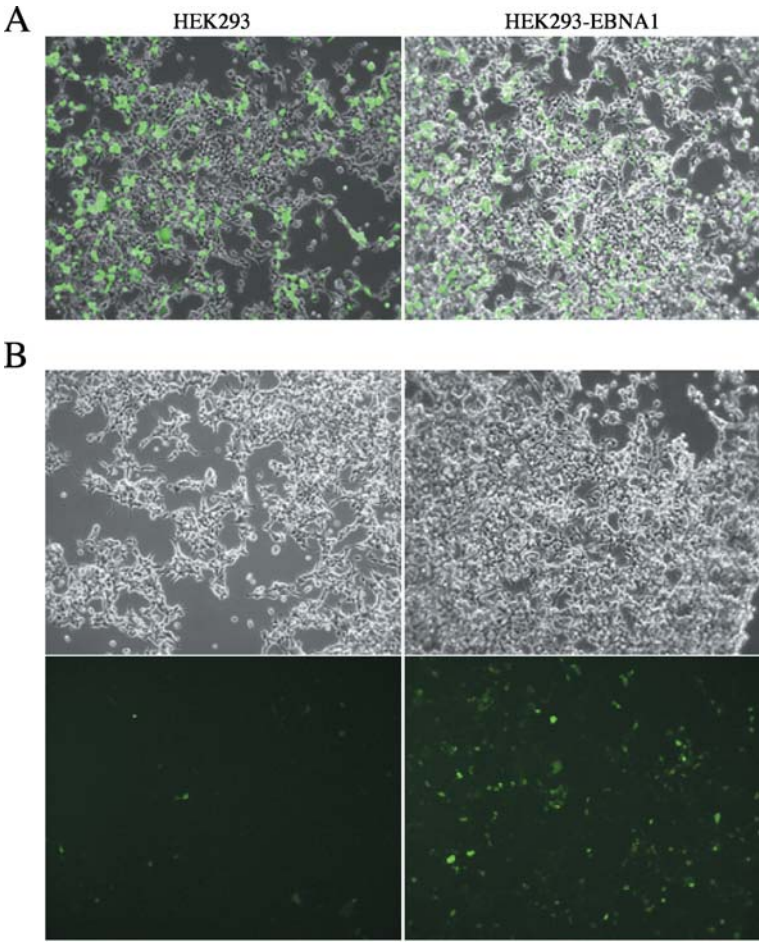

Figure 3. *Fluorescence microscopy detection of GFP in HEK293 and HEK293-EBNA1 cells. (A) For a transfection control, HEK293 and HEK293-EBNA1 cells ($5x10^5$) were transiently transfected with pEGFP-C1 (1 μg) and GFP fluorescence assessed after 24h. Shown are representative overlays of GFP fluorescence and bright light images from three independent experiments. (B) pEpProm-GFP (1 μg) was transiently transfected into HEK293 and HEK293-EBNA1 cells ($5x10^5$). GFP fluorescence was monitored 24h after transfection. Representative results from three independent experiments are shown (Bright light: upper panels; fluorescence: lower panels).*

Table I. *EpCAM cell surface expression and GFP fluorescence in HEK293 AND HEK293-EBNA1 cells. Levels are expressed as mean fluorescence intensity ratios resulting from flow cytometric measurements (MFI-R: EpCAM/isotype-control; pEp-Prom-GFP/pEp-Prom-control). Given are the mean MFI-R with standard deviations from two independent experiments at the indicated time points.*

	EpCAM	GFP (24h)	GFP (48h)
HEK293	1.05±0	1.1±0.2	1.78±0.03
HEK293-EBNA1	6.4±1.7	9.8±5	20±5.4

MTT-assay. The cellular metabolism was assessed in a standard MTT conversion assay as described elsewhere (13). Briefly, $3x10^3$ cells/well were plated in 96-well plates and analysed at the time points indicated.

Results

EpCAM promoter-mediated GFP gene expression in EpCAM-positive cells. The expression vectors pEpProm-control and pEpProm-GFP (Figure 1) were analysed in a cellular system composed of subclones of the human embryonic kidney (HEK) cell line, which differ in their EpCAM expression. HEK293 cells are EpCAM-negative, whereas HEK293-EBNA1 cells, which harbour an expression plasmid for the Epstein-Barr virus nuclear antigen 1 (EBNA1), express endogenous EpCAM at the cell surface as demonstrated by flow cytometry (Figure 2A). In order to analyse the EpCAM promoter-dependent GFP expression, pEpProm-control and pEpProm-GFP plasmids were transiently transfected into HEK293 and HEK293-EBNA1 cells. Thereafter, GFP expression and fluorescence was monitored by flow cytometry (Figure 2B), fluorescence microscopy (Figure 3B), and immunoblotting (Figure 2C). The GFP expression in the HEK293 cell line was sporadic and restricted to just a few cells. In contrast, GFP was readily expressed from the pEpProm-GFP vector in HEK293-EBNA1 cells. The respective levels of EpCAM and GFP in HEK293 and HEK293-EBNA1 cells were calculated as mean fluorescence intensity ratios (MFI-R) between control and GFP fluorescence (Table I). In HEK293-EBNA1 cells, GFP fluorescence was already strong 24h following transfection

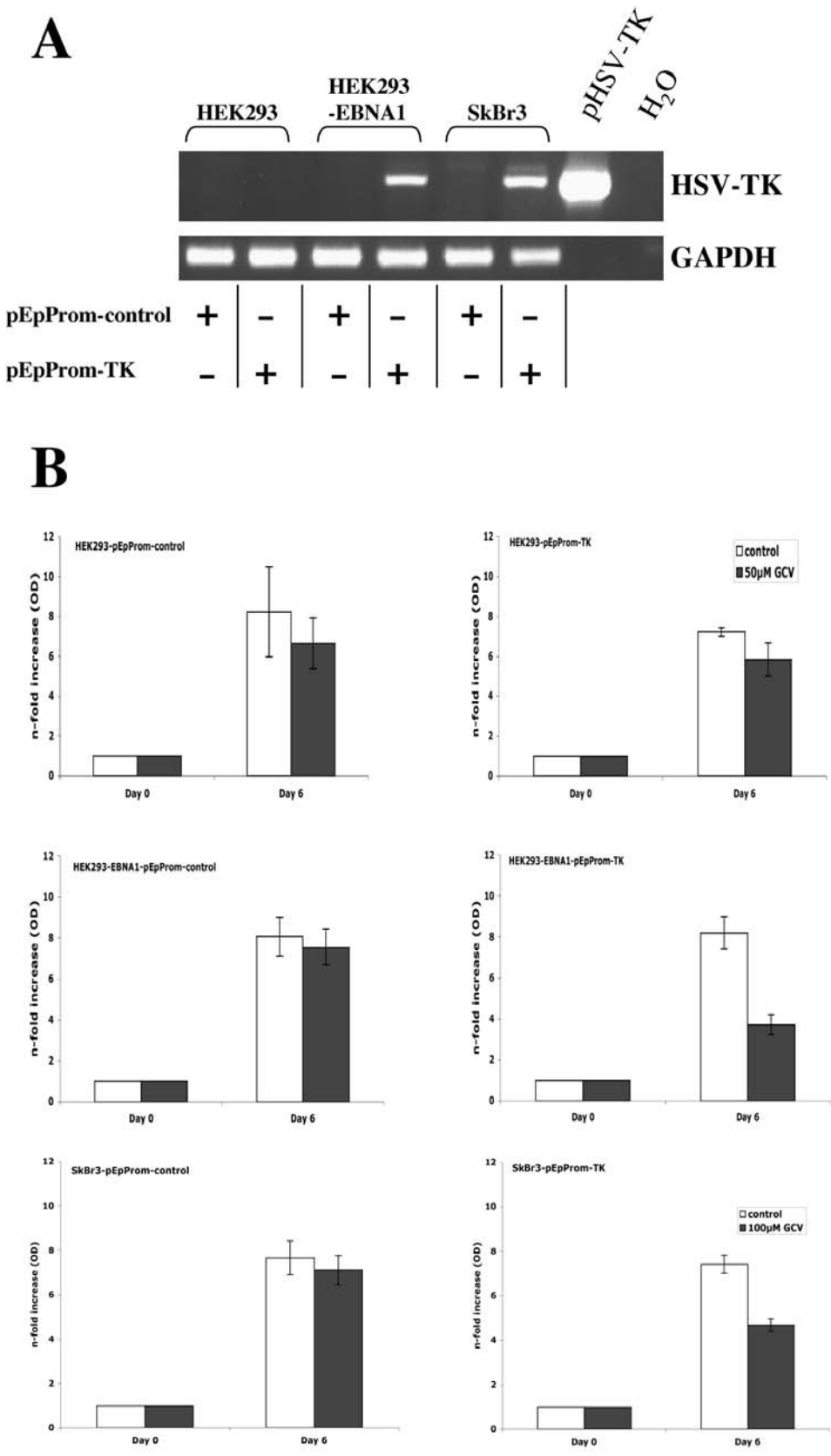

Figure 4. *HSV1-TK expression from pEpProm-HSV1-TK and ganciclovir sensitivity is dependent on EpCAM expression. (A) Stable HEK293, HEK293-EBNA1, and SkBr3 transfectants of pEpProm-HSV1-TK were tested for the expression of HSV1-TK mRNA by RT-PCR. GAPDH served as a control for equal cDNA levels, a plasmid for HSV1-TK as a positive control (pHSV-TK) and H_2O as a negative control. Shown are the representative results from three independent experiments. (B) Stable transfectants harbouring pEpProm-control or pEpProm-HSV1-TK in HEK293 cells (upper panels), HEK293-EBNA1 cells (middle panels) and SkBr3 cells (lower panel) were grown in the presence of the indicated concentrations of ganciclovir. At the indicated time points, the MTT conversion was assessed and given as the n-fold increase compared to day 0. Mean inductions with standard deviations from two independent experiments performed in triplicates are shown.*

and further increased at 48h after transfection (MFI-R 9.8±5 and 20±5.4, respectively). Furthermore, GFP expression in HEK293-EBNA1 cells was in a similar range compared with EpCAM (MFI-R 6.4±1.7). A slight increase was detectable in HEK293 cells 48h following the transient transfection of pEpProm-GFP (MFI-R 1.78±0.03), but not 24h after transfection (MFI-R 1.1±0.2). As expected, the transfection of pEpProm-control into either cell line did not result in the expression of GFP (data not shown). In addition, the transfection efficiency of HEK293 and HEK293-EBNA1 was comparable, as shown by transient transfection of a vector encoding GFP under the control of the cytomegalovirus early promoter (Figure 3A).

pEpProm-HSV1-TK transfection results in ganciclorvir sensitivity in EpCAM-positive cells. The EpCAM promoter is selectively re-activated in squamous cell carcinomas, but silent in healthy squamous epithelium, as deduced from the expression pattern of the EpCAM protein (7). Therefore, we tested the possibility of expressing the suicide gene HSV1-TK specifically in EpCAM-positive cells using the 1.1kb promoter fragment. Stable transfection of the pEpProm-HSV1-TK vector in HEK293, HEK293-EBNA1 and in the EpCAM-positive breast carcinoma cell line SkBr3 resulted in HSV1-TK mRNA transcription specifically in EpCAM-positive cells, *i.e.* HEK-293-EBNA1 and SkBr3 (Figure 4A). EpCAM-negative HEK293 cells displayed only minute amounts of HSV1-TK mRNA. To test the functionality of the introduced suicide gene, the sensitivity of HEK293, HEK293-EBNA1 and SkBr3 cell clones to ganciclovir treatment was assessed in a standard MTT assay. Ganciclovir sensitivity correlated positively with the expression of EpCAM protein and HSV1-TK mRNA: the metabolic activity of HEK293-EBNA1 and SkBr3 clones expressing HSV1-TK was markedly reduced upon ganciclovir treatment to levels representing 45% and 62% of untreated cells, respectively (Figure 4B). However, HEK293-pEpProm-HSV1-TK clones did not react to the addition of 50 μM ganciclovir into the culture medium over a time period of up to six days, as compared with control transfectants (Figure 4B).

Discussion

The tumour-associated antigen EpCAM is, in many ways, of great interest in cancer research. On the one hand, EpCAM has gained attention from the clinical aspect, reflected in its use as a target in several therapeutic approaches (5). On the other hand, basic research has focussed on the elucidation of the role of EpCAM at the molecular level, to understand its function in more detail. Besides effects exerted by this tumour antigen, such as the direct up-regulation of the proto-oncogenε c-myc (8), it is noteworthy that the presence and levels of EpCAM directly correlate with carcinogenesis and the grade of neoplasia (6). Molecular mechanisms involved in the re-activation of the promoter of the *epcam* gene, leading to the observed *de novo* expression, are not yet understood. The analysis of DNA sequences located upstream of the *epcam* gene revealed the presence of regulatory elements in a stretch encompassing 1.1 kilobases. The transcriptional activity of this 1.1kb DNA stretch closely correlated with the levels of EpCAM (11). So far, no inducer of this promoter sequence has been identified, however, NF-Î B signalling negatively influenced the transcriptional activity present in the 1.1kb fragment (10, 11).

On the basis of the compiled knowledge discussed above, we tested the potential of the 1.1kb EpCAM promoter fragment to specifically transcribe genes of interest in EpCAM-positive epithelial cells, including carcinoma cells. Utilising GFP as a marker protein allowed the simplified and quantifiable assessment of the transcriptional activity of the 1.1kb DNA fragment in cell lines depending upon the levels of EpCAM expression. As demonstrated in our HEK293 cellular model system, GFP expression from the pEpProm-GFP construct depended on the presence of nuclear factors regulating the endogeneous *epcam* promoter. Twenty-four hours after transfection, the fluorescence intensity of GFP was in the same range compared with EpCAM protein levels (MFI-R 9.8 ± 5 and 6.4 ±1.7, respectively). Notably, GFP protein accumulated in transiently transfected cells as demonstrated by the increased MFI-R at 48h post transfection (20 ± 5.4).

In a second line of experiments, we transcribed HSV1 thymidine kinase as a model therapeutic suicide gene from the 1.1kb promoter fragment. Despite a faint transcriptional activity of this fragment being measurable in EpCAM-negative HEK293 cells, the level was insufficient to confer ganciclovir sensitivity to these cells. In contrast, EpCAM-positive cells (HEK293-EBNA1 and SkBr3) displayed a strong transcriptional activity of the introduced promoter, resulting in the expression of HSV1-TK and ganciclovir sensitivity, as demonstrated by MTT experiments. However, these experiments also revealed an additional issue: although the level of EpCAM expression in SkBr3 is distinctly higher than in HEK293-EBNA1 (11), ganciclovir sensitivity in SkBr3 cells was not proportionally higher. This may be due to other factors, which contribute to the effect of the suicide gene, such as the susceptibility for apoptosis, chemo- and multiple drug resistance and metabolites enhancing ganciclovir effects (14-16). Thus, it is worth testing several gene candidates for a given tumour entity in order to define the most therapeutically potent suicide gene.

In summary, we demonstrated that the targeted gene expression of the model suicide gene HSV1-TK under the control of the 1.1kb EpCAM promoter fragment leads to ganciclovir sensitivity in EpCAM-positive HEK293-EBNA1

as well as in SkBr3 carcinoma cells. Thus, the 1.1kb EpCAM promoter fragment is a promising tool to deliver therapeutic suicide genes for the elimination of EpCAM-positive carcinoma cells.

References

1 Khare PD, Shao-Xi L, Kuroki M, Hirose Y, Arakawa F, Nakamura K and Tomita Y: Specifically targeted killing of carcinoembryonic antigen (CEA)-expressing cells by a retroviral vector displaying single-chain variable fragmented antibody to CEA and carrying the gene for inducible nitric oxide synthase. Cancer Res *61*: 370-5, 2001.

2 Di Paolo C, Willuda J, Kubetzko S, Lauffer I, Tschudi D, Waibel R, Pluckthun A, Stahel RA and Zangemeister-Wittke U: A recombinant immunotoxin derived from a humanized epithelial cell adhesion molecule-specific single-chain antibody fragment has potent and selective antitumor activity. Clin Cancer Res *9*: 2837-48, 2003.

3 Barker SD, Dmitriev IP, Nettelbeck DM, Liu B, Rivera AA, Alvarez RD, Curiel DT and Hemminki A: Combined transcriptional and transductional targeting improves the specificity and efficacy of adenoviral gene delivery to ovarian carcinoma. Gene Ther *10*: 1198-204, 2003.

4 Greenberger S, Shaish A, Varda-Bloom N, Levanon K, Breitbart E, Goldberg I, Barshack I, Hodish I, Yaacov N, Bangio L, Goncharov T, Wallach D and Harats D: Transcription-controlled gene therapy against tumor angiogenesis. J Clin Invest *113*: 1017-24, 2004.

5 Armstrong A and Eck SL: EpCAM: A new therapeutic target for an old cancer antigen. Cancer Biol Ther *2*: 320-6, 2003.

6 Litvinov SV, van Driel W, van Rhijn CM, Bakker HA, van Krieken H, Fleuren GJ and Warnaar SO: Expression of Ep-CAM in cervical squamous epithelia correlates with an increased proliferation and the disappearance of markers for terminal differentiation. Am J Pathol *148*: 865-75, 1996.

7 Balzar M, Winter MJ, de Boer CJ and Litvinov SV: The biology of the 17-1A antigen (Ep-CAM). J Mol Med *77*: 699-712, 1999.

8 Munz M, Kieu C, Mack B, Schmitt B, Zeidler R and Gires O: The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. Oncogene *23 (34)*: 5748-58, 2004.

9 Munz M, Hofmann T, Scheibe B, Gänge M, Junghanns K, Zeidler R and Gires O: The carcinoma-associated antigen EpCAM induces glyoxalase 1 resulting in enhanced methylglyoxal turnover. Cancer Genomics & Proteomics *1 (3)*: 241-8, 2004.

10 Gires O, Kieu C, Fix P, Schmitt B, Munz M, Wollenberg B and Zeidler R: Tumor necrosis factor alpha negatively regulates the expression of the carcinoma-associated antigen epithelial cell adhesion molecule. Cancer *92*: 620-8, 2001.

11 Gires O, Eskofier S, Lang S, Zeidler R and Munz M: Cloning and characterisation of a 1.1 kb fragment of the carcinoma-associated epithelial cell adhesion molecule promoter. Anticancer Res *23*: 3255-61, 2003.

12 Graham FL, Smiley J, Russell WC and Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol *36*: 59-74, 1977.

13 Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods *65*: 55-63, 1983.

14 Pasanen T, Karppinen A, Alhonen L, Janne J and Wahlfors J: Polyamine biosynthesis inhibition enhances HSV-1 thymidine kinase/ganciclovir-mediated cytotoxicity in tumor cells. Int J Cancer *104*: 380-8, 2003).

15 McNeish IA, Tenev T, Bell S, Marani M, Vassaux G and Lemoine N: Herpes simplex virus thymidine kinase/ganciclovir-induced cell death is enhanced by co-expression of caspase-3 in ovarian carcinoma cells. Cancer Gene Ther *8*: 308-19, 2001.

16 Degreve B, De Clercq E and Balzarini J: Selection of HSV-1 TK gene-transfected murine mammary carcinoma cells resistant to (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) and ganciclovir (GCV). Gene Ther *7*: 1543-52, 2000.

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