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# Hypoxia- and radiation-inducible, breast cell-specific targeting of retroviral vectors

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**Abstract**

To facilitate a more efficient radiation and chemotherapy of mammary tumours, synthetic enhancer elements responsive to hypoxia and ionizing radiation were coupled to the mammary-specific minimal promoter of the murine whey acidic protein (WAP) encoding gene. The modified WAP promoter was introduced into a retroviral promoter conversion (ProCon) vector. Expression of a transduced reporter gene in response to hypoxia and radiation was analysed in stably infected mammary cancer cell lines and an up to 9-fold increase in gene expression demonstrated in comparison to the respective basic vector. Expression analyses in vitro, moreover, demonstrated a widely preserved mammary cell-specific promoter activity. For in vivo analyses, xenograft tumours consisting of infected human mammary adenocarcinoma cells were established in SCID/beige mice. Immunohistochemical analyses demonstrated a hypoxia-specific, markedly increased WAP promoter-driven expression in these tumours. Thus, this retroviral vector will facilitate a targeted gene therapeutic approach exploiting the unique environmental condition in solid tumours.


*Keywords:* CArG element; Hypoxia responsive element; Promoter conversion; Retroviral vector; Breast cell-specific expression targeting; Breast cancer xenografts; Mouse model

## Introduction

Hypoxia is one of the critical factors which prevent successful cancer therapy. The presence of hypoxic regions in tumours adversely affects the locoregional control and/or disease-free survival after primary radiotherapy, chemotherapy and surgery (De Schutter et al., 2005; Fyles et al., 1998, 2002; Hockel et al., 1996; Nordsmark et al., 2001; Zhou et al., 2005). Hypoxia is a special feature of solid tumours which is mainly due to their insufficient blood supply caused by a highly irregular, tortuous, and elongated vasculature with blind ends, incomplete endothelial linings and irregular blood flow (Kimura et al., 1996; Vaupel et al., 1991). Furthermore, cancer patients often suffer from anaemia which in addition leads to a generally reduced oxygen supply (Blohmer et al., 2005; Vaupel and Mayer, 2005). These factors contribute to the emergence of variable areas of transient and chronic hypoxia in distinct tumour regions. Low oxygenation of tumour cells, however, can accelerate malignant progression and metastasis and as a consequence contribute to a poor prognosis (Brizel et al., 1996; Dales et al., 2005; To et al., 2005). In this respect, it was shown that hypoxia leads to an upregulation of expression of anti-apoptotic factors such as Bcl-2 (Shimizu et al., 1995), whereas expression of cell adhesion molecules such as integrins is downregulated (Hasan

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et al., 1998). Furthermore, it was reported that expression of the urokinase-type plasminogen activator receptor (Rofstad et al., 2002) or the autocrine motility factor (Niizeki et al., 2002) is upregulated under hypoxic conditions. All these events facilitate tumour cell detachment, migration and invasion. With respect to radio- and chemotherapy of tumours, it has been shown that low oxygenated cells are three times more resistant to radiation-induced cell death than normoxic cells (Harrison and Blackwell, 2004). On the basis of this information, different strategies are currently developed to specifically target hypoxic tumour regions.

In order to achieve hypoxia-specific expression, hypoxia responsive elements (HREs) located within enhancer regions of various hypoxia-regulated genes such as those encoding erythropoietin (Epo), phosphogycerate kinase 1 (PGK1), vascular endothelial growth factor (VEGF), or lactate dehydrogenase A (LDH A) have been used (Greco et al., 2003). These HREs are *cis* acting sequences containing the core motif 5′-(A/G)CGT(G/C)(G/C)-3′ and several transcription factor binding sites, one of which is essential for hypoxic activation by binding the hypoxia-inducible factor (HIF) 1 (Semenza and Wang, 1992). Linking HREs to the constitutively active minimal CMV promoter has been shown to confer hypoxia responsiveness to gene expression (Greco et al., 2002; Marples et al., 2000). Similarly, the promoter of the early growth response (Egr)-1 gene has been used successfully to facilitate expression of a transgene following radiation treatment (Hallahan et al., 1995). Based on this, synthetic promoters, bearing the consensus sequence CC(A/T)6GG which is known as CArG element (Datta et al., 1992), were shown to specifically mediate activation of gene expression in response to radiotherapy in vitro (Greco et al., 2002). The successful combination of both elements, five copies of HREs coupled to nine copies of CArG elements (Epo/E9), in an approach addressing the problem of hypoxia in radiotherapy of solid tumours was published previously (Greco et al., 2002).

In order to further exploit this system for cancer gene therapy, a retroviral vector-based gene delivery system was adapted to enable a mammary tissue-targeted, hypoxia- and radiation-inducible expression of transferred genes. We have previously reported a highly effective vector system derived from murine leukaemia virus (MLV) which facilitates targeted expression of transduced genes by promoter conversion (Hlavaty et al., 2004). In this system, transgene expression in virus packaging cells is driven from the MLV promoter within the 5′ long terminal repeat (LTR). After infection and reverse transcription, the MLV promoter is replaced by the heterologous tissue-specific promoter which is copied from the 3′ LTR to the 5′ LTR (Mrochen et al., 1997; Saller et al., 1998). To obtain tissue-specific expression in this study, a 470-bp fragment of the murine whey acidic protein (WAP) promoter was used, which had been shown recently in a transgenic mouse model to target the expression of transferred genes exclusively to the mammary gland (Lipnik et al., 2005). Since retroviral vectors stably integrate into the genome of infected cells, first of all, the functionality of the combined Epo/E9 enhancer element in stably transfected mammary cancer cells was tested. Transient and chronic hypoxia as well as a fractionated X-ray exposure resulted in a 5- to 50-fold increase of EGFP expression dependent on the respective cell line. Based on these results, a promoter conversion vector was generated carrying an EGFP gene under the control of a hybrid promoter consisting of the Epo/E9 enhancer element directly linked to the WAP promoter sequence. By means of in vitro infection experiments, we could demonstrate not only inducibility of EGFP expression by hypoxia and radiation but also a preserved cell-type specificity of the modified WAP promoter. Finally, in vivo function analyses with stably infected human breast cancer cell xenografts clearly demonstrated enhanced EGFP expression in hypoxic tumour regions.

## Results

### *The Epo/E9 element facilitates long term expression of EGFP in response to hypoxia and radiation*

Primary experiments were designed to determine whether the combined synthetic hypoxia and radiation responsive enhancer element is functional in well differentiated human breast cancer cell lines, i.e., MCF7 and T47D, as well as in a cell line derived from a breast cancer metastasis, i.e., MDA-MB435S. In addition, the long-term expression response in these cells after stimulation was investigated. For this purpose, MCF7, T47D cells and MDA-MB435S cells were stably transfected with the expression vector pEpoE9GFP (Greco et al., 2002), and EGFP expression was determined by fluorescence-activated cell sorting (FACS).

For induction of expression by transient hypoxia, cells were incubated with $CoCl_2$ for 4 h, which mimics hypoxia, including the production of reactive oxygen species (Minchenko et al., 2005; Vengellur et al., 2005). A concentration of 2–4 μM was already sufficient to provoke hypoxic conditions as demonstrated by immunocytochemical staining of exposed T47D cells (data not shown). To investigate the duration of enhanced expression activity after 4 h of $CoCl_2$ treatment, cells were further maintained in normal medium at normoxic conditions for 24 h to 144 h. Each of the investigated cell lines showed an individual reaction with expression maxima at different time points varying between 72 h and 120 h (Fig. 1A). T47D cells showed a steady increase of EGFP expression within 144 h of re-oxygenation. In comparison to non-treated cells, EGFP expression levels in $CoCl_2$-treated human breast cancer cells increased 22- to 57-fold.

Two different methods were chosen to simulate chronic hypoxia in stably transfected breast cancer cells. Firstly, cells were incubated with individual amounts of $CoCl_2$ (0.1, 0.3 or 0.5 μM; see Materials and methods) for 12 h–96 h (Fig. 1B). Thereby, about a 6-fold increase in EGFP expression levels was achieved with T47D and MCF7 cells, whereas transfected MDA-MB435S cells revealed a slight increase (2-fold) after 96 h. Secondly, cells were incubated in a hypoxic chamber in the presence of an oxygen reduced atmosphere for a maximum of 96 h. EGFP expression was determined by FACS analysis at

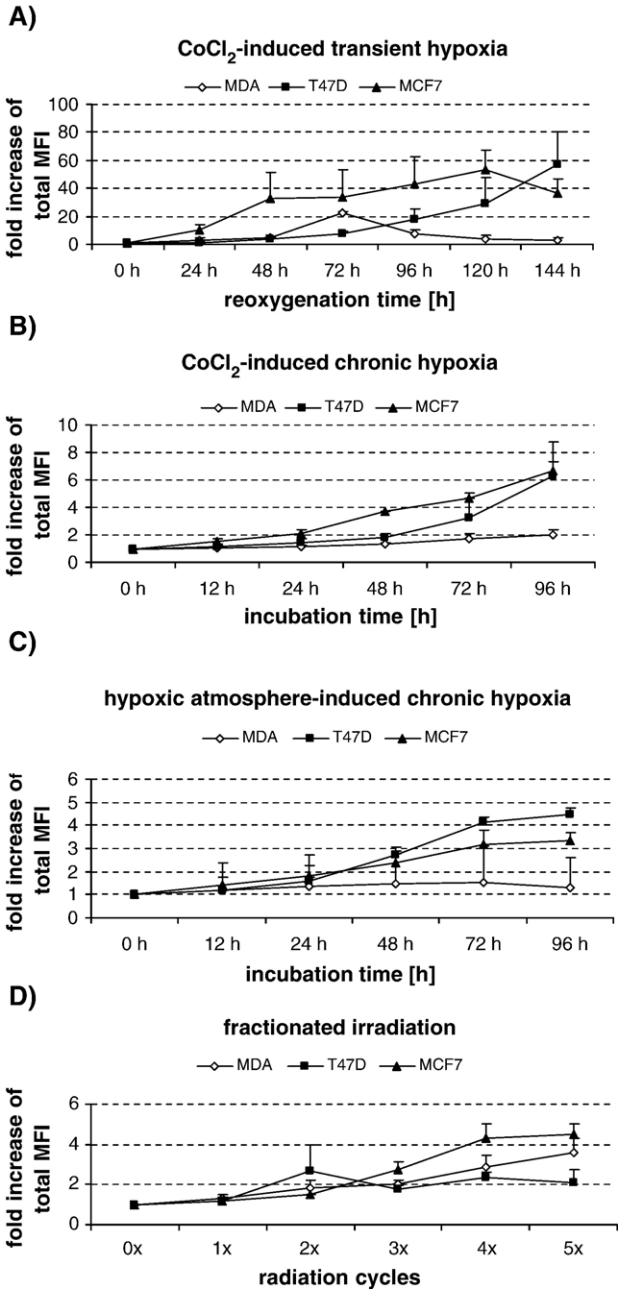


Fig. 1. (A–D): Effects of hypoxia and X-ray exposure on EGFP expression levels in human breast cancer cells stably transfected with pEpoE9GFP. EGFP expression levels were determined as the total mean fluorescence intensity (MFI) of EGFP expressing and non-expressing cells and are depicted as fold increase in relation to non-treated cells. Indicated are the mean values including standard deviations of three independent experiments. (A) Transient hypoxia induced by $CoCl_2$. Stably transfected T47D, MCF7 and MDA-MB435S cells were incubated with 2–4 μM $CoCl_2$ for 4 h and thereafter cultivated in the respective cell-specific medium under normoxic conditions for the indicated time spans. After re-oxygenation EGFP expression was determined by FACS. (B) Chronic hypoxia induced by $CoCl_2$. Cells were incubated for the indicated time spans with 0.1 to 0.5 μM $CoCl_2$ and subsequently analysed by FACS. (C) Cells were incubated for the indicated time spans in a hypoxic chamber and subsequently analysed by FACS. (D) Stably transfected T47D, MCF7 and MDA-MB435S cells were exposed up to five times to X-ray radiation (2.6 Gy each) and subsequently analysed by FACS.

different time points (Fig. 1C). Under these conditions, a 3- to 5-fold increase in EGFP expression levels was achieved depending on the respective cell line. Again, MDA-MB435S cells were barely inducible as demonstrated by an only 1.3-fold increase in expression levels after 96 h.

Since the Epo/E9 enhancer, besides the HRE, also contains 9 copies of radiation response elements, the stably transfected cell lines were tested for their reaction to X-ray exposure with respect to EGFP expression. In order to simulate a clinical radiation scheme (Truong et al., 2004; Whelan et al., 2002) cells were irradiated up to five times every 24 h with a dose of 2.6 Gy. 48 h after the last irradiation cells were harvested and analysed by FACS (Fig. 1D). Expression activity increased with repeated radiation doses. MCF7 cells showed a 4.5-fold increase in EGFP expression after five consecutive irradiations. MDA-MB435S cells revealed a 3.6-fold higher total MFI compared to non-irradiated cells. T47D cells showed a 2.4-fold increase in EGFP expression levels after four cycles of X-ray exposure.

### *Generation of a radiation- and hypoxia-inducible breast cell-specific retroviral vector*

Since the hypoxia- and radiation-inducible enhancer elements had been shown to be active in stably transfected human breast cancer cells, they subsequently were inserted into a retroviral promoter conversion (ProCon) vector carrying the mammary tissue-specific murine WAP promoter (Fig. 2B). To achieve this, the Epo/E9 enhancer element was amplified by PCR from pEpoE9GFP (Fig. 2A) and inserted into the plasmid pPCEW (Fig. 2B) 5′ of the WAP promoter within the 3′ LTR. The generated ProCon vector pPCE-EpoE9W (Fig. 2C) as well as the basic vector pPCEW (Fig. 2B) were stably transfected into 2GP19Talf amphotropic retroviral packaging cells (Pambalk et al., 2002). Stably transfected cell populations as well as single cell clones were isolated and virus titres were determined by real-time reverse transcriptase (RT)-PCR of viral RNA extracted from cell culture supernatant. Virus titres of $5 \times 10^6$/ml for cell populations and $1 \times 10^8$/ml for isolated virus producing cell clones were obtained. No significant differences in virus titres were observed when pPCE-EpoE9W transfected packaging cells were compared to those transfected with the basic vector pPCEW. Virus containing supernatant from high titre producing cell clones was then used to infect T47D, MCF7, and MDA-MB435S cells. Stably transduced cell populations and single cell clones were selected and further investigated for their response to hypoxia and radiation.

### *Stably pPCE-EpoE9W-transduced breast cancer cells respond to hypoxia and radiation*

In order to characterise the obtained stably transduced cell clones and populations they were analysed for hypoxia and radiation responsiveness by exposure to hypoxic conditions and X-ray irradiation as established before. As a control, cell populations and clones stably transduced with the basic vector PCEW lacking the Epo/E9 element were stimulated in the same

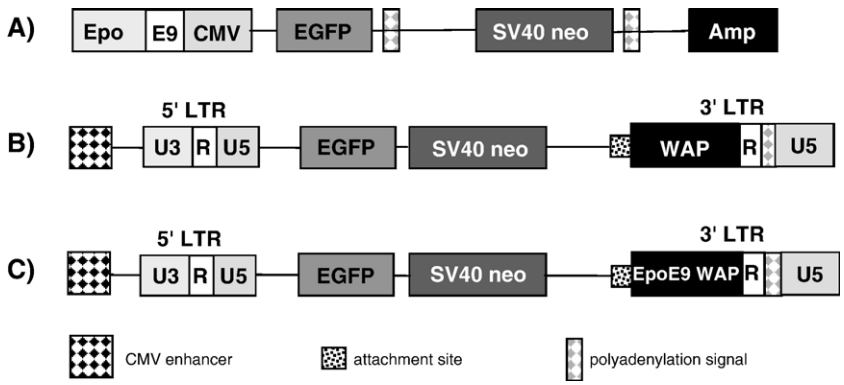


Fig. 2. Expression constructs and retroviral vectors. (A) The expression plasmid pEpoE9GFP carries five copies of a HRE derived from the erythropoietin gene (Epo) and nine copies of a CArG element of the Egr I gene (E9) which are coupled to a minimal CMV promoter (CMV). This promoter drives the expression of the marker gene EGFP which is followed by a SV40 polyadenylation signal. It contains a SV40 promoter/neomycin resistance gene (SV40 neo) selection cassette and an ampicillin resistance gene (Amp) as a bacterial selection marker. (B) The plasmid pPCEW contains a retroviral ProCon vector which is based on MLV. A CMV enhancer was inserted upstream of the 5′ long terminal repeat (LTR) within the plasmid backbone. The MLV promoter within the U3 region of the 3′ LTR was replaced by a 470-bp fragment of the murine WAP promoter. The vector contains an elongated attachment site and an additional polyadenylation signal downstream of the WAP promoter. EGFP was included as a reporter gene followed by a SV40-neo selection cassette. (C) The plasmid pPCE-EpoE9W contains in addition to the WAP promoter in the 3′ LTR the Epo/E9 enhancer element inserted directly upstream of the respective promoter sequence.

way. As summarised in Table 1, on average a 2-fold to 4.5-fold increase in EGFP expression levels was observed when PCE-EpoE9W-transduced breast cancer cells were exposed either to transient or chronic hypoxia. Interestingly, cells stably infected with PCEW in comparison to non-treated cells in some cases showed a decrease in EGFP expression levels when exposed to hypoxic conditions (Table 1). PCEW-transduced MCF7 cells exposed to transient hypoxia, however, revealed an about 2-fold increase in EGFP expression.

The radiation responsiveness of the newly generated vector was investigated by fractionated irradiation of stably transduced cells. Cells were irradiated five times with 2.6 Gy and, subsequently analysed by FACS. On average, dependent on the respective cell line tested, a 2-fold to 5-fold increase in expression levels was obtained (Table 1). In contrast to the results obtained with hypoxic conditions, X-ray exposure caused a marked increase of EGFP expression levels especially in PCEW-transduced T47D cells when compared to non-treated cells.

*The hypoxia responsiveness of the EpoE9-WAP promoter is mainly restricted to breast cancer cells*

In order to determine if the modified WAP promoter is still able to confer breast cell-specific gene expression in transduced cells, four different human mammary cancer cell lines (Hs578T, MDA-MB435S, T47D, MCF7) as well as five human non-mammary cancer cell lines including microvascular endothelial cells (HMEC), embryonic kidney cells (HEK293), primary normal human fibroblasts (huFi), a pancreatic cancer cell line (Panc1) and a cervix carcinoma cell line (HeLa) were infected with the respective retroviral vectors PCE-EpoE9W and PCEW (Fig. 3). Subsequently, EGFP expression in response to hypoxia was analysed and results obtained in non-mammary cancer cells compared to those obtained in mammary cancer cells. Non-selected populations of mammary cancer cells transiently infected with PCE-EpoE9W (Fig. 3A) show a clear increase of EGFP expression levels after cultivation in hypoxic conditions whereas the response to hypoxia in non-breast cancer cells besides endothelial cells (HMEC) is only modest (Fig. 3A). Cell populations which were transiently infected with the pPCEW vector, on average did not show a significant response with respect to expression activity (Fig. 3B). However, transduced HMEC again revealed an enhanced EGFP expression when treated with 0.5 μM $CoCl_2$.

In order to exclude an influence of cell line infectability or different viral titres, stably infected cell populations were generated. Stably transduced cells were cultured in a hypoxic chamber for 96 h and subsequently analysed by FACS. Comparable to the transiently infected cells mainly PCE-EpoE9W stably transduced breast cancer cell lines such as Hs578T, T47D and MDA-MB435S showed an increase in EGFP expression levels in response to hypoxia (Fig. 4A). Only a minimal effect was achieved with MCF7 cells which also did not react to hypoxic chamber-mediated hypoxia in the previous experiment (Fig. 3A, black bar). In comparison, EGFP expression levels in stably PCEW-infected cells on average rather decreased than increased (Fig. 4B). In summary, normalised to the basic WAP promoter ProCon vector PCEW the modified PCE-EpoE9W vector facilitated a 4.6-fold to 9.3-fold enhancement of EGFP expression in hypoxic breast cancer cell lines compared to a 1.6-fold to 4.3-fold stimulation of expression in hypoxic non-breast cancer cells (Fig. 4C).

*Stably transduced mammary cancer cell xenografts show enhanced marker gene expression in hypoxic tumour regions*

Following the in vitro analyses, inducibility of the modified WAP-promoter by hypoxia was also tested in vivo, since it is known that the maximum activity of the WAP promoter is achieved in a three-dimensional system (Lin et al., 1995). For this purpose, cell clones and populations of MCF7, T47D and

Table 1
Fold increase of EGFP expression in MDA-MB435S, T47D and MCF7 cells stably infected with PCEW or PCE-EpoE9W in response to hypoxia or X-ray exposure [a]

| | Transient hypoxia $CoCl_2$ | Chronic hypoxia $CoCl_2$ | Chronic hypoxia hypoxic chamber | Fractionated irradiation |
|---|---|---|---|---|
| MDA-PCE-EpoE9W pop | 2.6 ± 0.1 | 2.7 ± 1.7 | 2.1 ± 0.2 | 1.7 ± 0.08 |
| MDA-PCE-EpoE9W cl 5 | 3.0 ± 1.1 | 1.6 ± 0.4 | 1.9 ± 0.2 | 2.6 ± 1.0 |
| MDA-PCEW pop | 1.0 ± 0.1 | 0.8 ± 0.08* | 0.6 ± 0.08* | 0.8 ± 0.02 |
| MDA-PCEW cl 2 | 1.4 ± 0.8 | 0.7 ± 0.01* | 0.7 ± 0.05 | 1.3 ± 0.6 |
| T47D-PCE-EpoE9W pop | 3.2 ± 0.01 | 4.1 ± 0.07 | 3.1 ± 0.3 | 5.0 ± 0.1 |
| T47D-PCE-EpoE9W cl 3 | 3.9 ± 2.4 | 4.5 ± 1.5 | 2.3 ± 0.3 | 5.4 ± 0.6 |
| T47D-PCEW pop | 0.7 ± 0.1* | 1.1 ± 0.09 | 0.7 ± 0.1* | 6.4 ± 0.2 |
| T47D-PCEW cl 4 | 0.7 ± 0.3 | 1.4 ± 0.4 | 0.8 ± 0.07 | 3.1 ± 0.5 |
| MCF7-PCE-EpoE9W pop | 2.3 ± 0.4 | 2.7 ± 0.4 | 1.6 ± 0.2 | 2.2 ± 0.2 |
| MCF7-PCE-EpoE9W cl 11 | 2.0 ± 0.5 | 3.0 ± 0.1 | 1.1 ± 0.09 | 2.3 ± 0.3 |
| MCF7-PCEW pop | 2.2 ± 1.0 | 0.9 ± 0.1 | 0.6 ± 0.07* | 1.8 ± 0.07 |
| MCF7-PCEW cl 13 | 1.7 ± 0.5 | 1.0 ± 0.08 | 0.6 ± 0.1 | 1.9 ± 0.3 |

[a] The numbers represent mean values of three independent experiments including standard deviations.
* Significant decrease as determined by an unpaired *t* test.

MDA-MB435S cells stably transduced with PCE-EpoE9W or PCEW were implanted into the mammary fat pad of immune incompetent SCID/beige mice to allow formation of xenograft tumours. Five weeks after implantation of the tumour cells, mice were injected intraperitoneally with a single dose of pimonidazole hydrochloride to facilitate detection of hypoxic tumour regions (Cowen et al., 2004). Thereafter mice were sacrificed, tumours excised and prepared for immunohistochemical analyses. Consecutive tissue sections were stained either for detection of EGFP or hypoxic cells indicated by the presence of pimonidazole-protein adducts (Azuma et al., 1997). A high level EGFP expression was detected in xenografts derived from PCE-EpoE9W-transduced tumour cells mainly in hypoxic regions in the periphery of blood vessels (Figs. 5E, F) and in close vicinity to necrotic areas (Figs. 5G, H). In contrast, PCEW-transduced tumour cells showed maximal EGFP expression when located next to vessels and expression activity decreased adjacent to necrotic areas (Figs. 5A–D). Co-localisation of hypoxic and EGFP-expressing cells by means of fluorescence immunohistochemistry clearly demonstrated an enhanced expression activity of PCE-EpoE9W in hypoxic tumour cells (Figs. 6B, D). In tumour xenografts derived from cells infected with the basic vector PCEW, however, EGFP expression in hypoxic cells was hardly detected (Figs. 6A, C).

In order to quantify the effect of the Epo/E9 enhancer elements on WAP promoter-driven EGFP expression in vivo, FACS analysis of single cell suspensions obtained from six different tumour xenografts originating from a PCEW-, or respectively PCE-EpoE9W-infected T47D cell clone were performed. Both cell clones revealed comparable EGFP expression levels in vitro with more than 90% EGFP expressing cells and similar MFIs. FACS analysis of tumour tissue derived from PCEW-infected cells in contrast demonstrated that on average only 3.6% of the analysed cells expressed EGFP (Fig. 7, upper left panel). In comparison, tumours derived from PCE-EpoE9W-transduced cells on average revealed 53.1% green cells (Fig. 7, lower left panel). These results were confirmed by immunohistochemistry (Fig. 7, right panels). EGFP expression in tumour sections derived from PCEW-transduced T47D cells

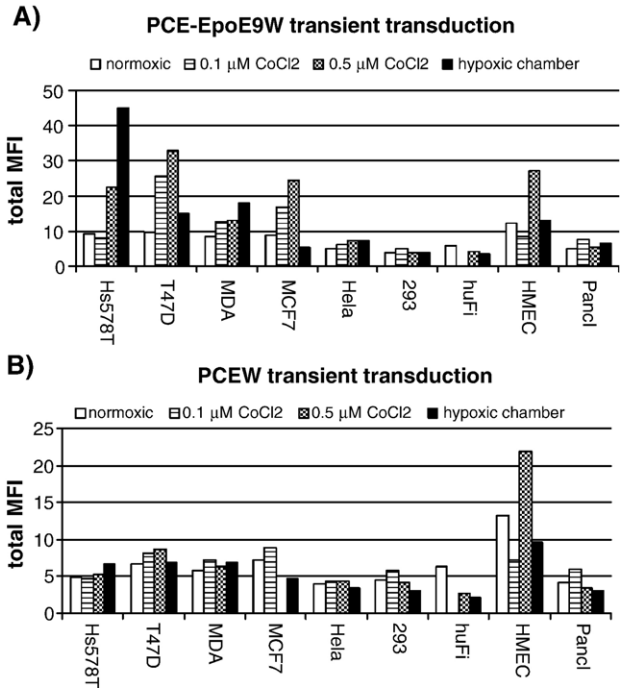


Fig. 3. Breast cell-specific, hypoxia-mediated induction of EGFP expression in transiently transduced human cell lines. Four different breast cancer cell lines (Hs578T, T47D, MDA, MCF7) and various non-breast cancer cell lines (HeLa, 293, PancI) as well as normal human fibroblasts (huFi) and a microvascular endothelial cell line (HMEC) were infected with PCE-EpoE9W (A) or with the basic vector PCEW (B). 72 h after infection cells were treated either with 0.1 or 0.5 µM $CoCl_2$ or cultured in an oxygen-reduced atmosphere to induce hypoxic conditions. After 96 h of treatment cells were analysed by FACS and compared to cells cultured under normoxic conditions.

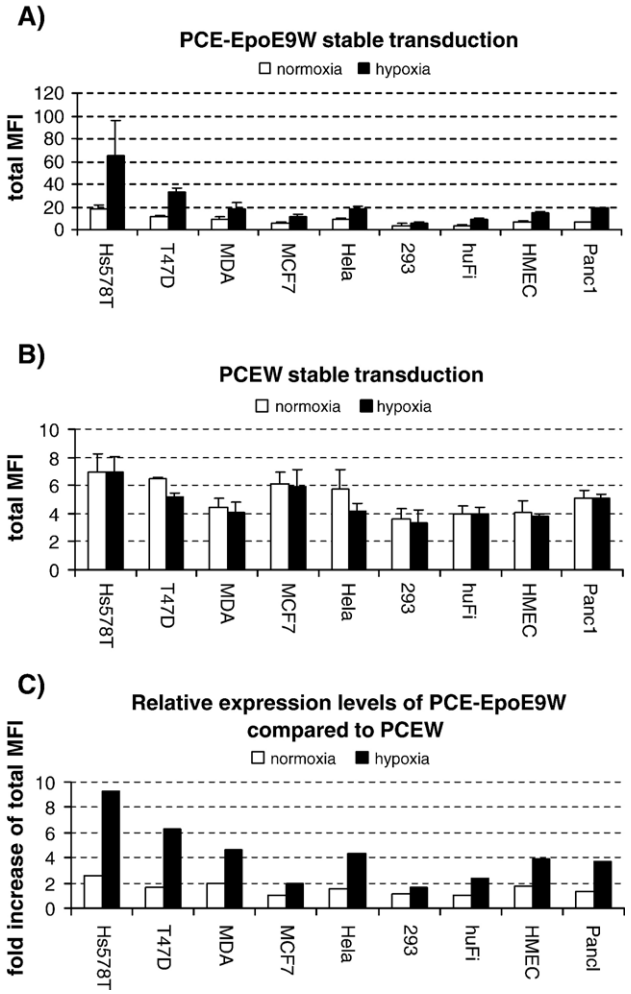


Fig. 4. Breast cell-specific, hypoxia-mediated induction of EGFP expression in stably transduced human cell lines. Selected, stably transduced populations of four different breast cancer and five non-breast cancer cell lines (see legend Fig. 3) were cultured for 96 h in an oxygen reduced atmosphere to induce hypoxic conditions. Subsequently the cells were analysed by FACS and compared to cells cultured under normoxic conditions. (A) Cells stably infected with PCE-EpoE9W; (B) cells stably infected with PCEW. The relative increase of expression levels in PCE-EpoE9W-infected cells compared to PCEW-infected cells under normoxic and hypoxic conditions is illustrated in (C).

was hardly detectable whereas tumours originating from PCE-EpoE9W-infected cells showed a marked expression of the transduced gene (Fig. 7, lower right panel).

## Discussion

Current approaches in treatment of solid tumours usually combine chemotherapy and radiotherapy. Hypoxia, a special feature of solid tumours, adversely affects therapeutic efficacy and may increase the risk for development of metastases and tumour recurrence (Brizel et al., 1996; Dales et al., 2005; Fyles et al., 2002; Hockel et al., 1996; To et al., 2005)). In the present study, we established a retroviral vector-based gene delivery system which exploits this specific tumour microenvironment thereby facilitating a targeted and efficient transgene expression in mammary tumour cells in vitro and in vivo.

HREs derived from the erythropoietin encoding gene have been previously shown to confer hypoxia inducibility to ubiquitously active viral core promoters such as CMV and SV40 (Greco et al., 2002; Wang et al., 2005). In addition, CArG elements (Datta et al., 1992) fused to a minimal CMV promoter also allowed induction of gene expression in response to gamma irradiation (Scott and Marples, 2004; Scott et al., 2000). These hybrid promoters have been used to drive expression of suicide genes for application in cancer gene therapy approaches (Brown and Wilson, 2004; Greco et al., 2002; Scott and Greco, 2004). A major prerequisite for gene therapy with viral vectors, however, is a targeted delivery of the therapeutic gene. Therefore, in the present approach synthetic hypoxia- and radiation-inducible elements were coupled to a minimal fragment of the mammary-specific murine WAP promoter (Lipnik et al., 2005; Öztürk-Winder et al., 2002). The hybrid WAP promoter was then introduced into a MLV-based retroviral promoter conversion vector, a vector system which allows tissue-specific expression of transferred genes in infected cells (Mrochen et al., 1997; Saller et al., 1998). Detailed analysis of the generated vector in vitro using three different stably transduced human breast cancer cell lines revealed cell type-specific gradually different responses to hypoxia with respect to reporter gene expression levels (Table 1). This was already evident when the respective elements fused to a minimal CMV promoter were analysed in stably transfected cells (Fig. 1). Especially $CoCl_2$-induced transient hypoxia provoked an individual time-dependent massive increase of EGFP expression levels ranging from 20-fold to 60-fold, which might be due to the production of reactive oxygen species during re-oxygenation followed by activation of HIF-1 (Denko et al., 2003; Neubauer, 2001). Although overall induction levels in stably infected cells were at least ten times lower, a clear benefit of the hybrid promoter (PCE-EpoE9W) in comparison to the basic WAP promoter (PCEW) was observed under hypoxic conditions (Table 1). Interestingly, expression levels of PCEW-infected cells chronically exposed to an oxygen reduced atmosphere in all three cell lines decreased in comparison to normoxic conditions, however, significant changes were only obtained with cell populations. This effect was not observed when cells were treated with fractionated irradiation. In these experiments, also PCEW-infected cells showed an increase of EGFP expression levels although to a lesser extent compared to PCE-EpoE9W-infected cells.

Having shown that the modified WAP promoter can be stimulated by hypoxia and radiation, the respective recombinant vector virus was tested for tissue specificity by infection of a number of breast cancer and non-breast cancer cell lines. In transiently as well as in stably transduced cell lines (Figs. 3, 4) cultivation under hypoxic conditions stimulated expression of PCE-EpoE9W predominantly in infected breast cancer cells. An increase above average was also obtained with microvascular endothelial cells in both PCEW- and PCE-EpoE9W-infected cells, when they were treated with 0.5 μM $CoCl_2$ (Fig. 3A, B hatched bars). This result therefore might indicate an overall enhanced response of these cells to these distinct hypoxic conditions rather than a specific effect on the introduced enhancer elements (Roland et al., 2000).

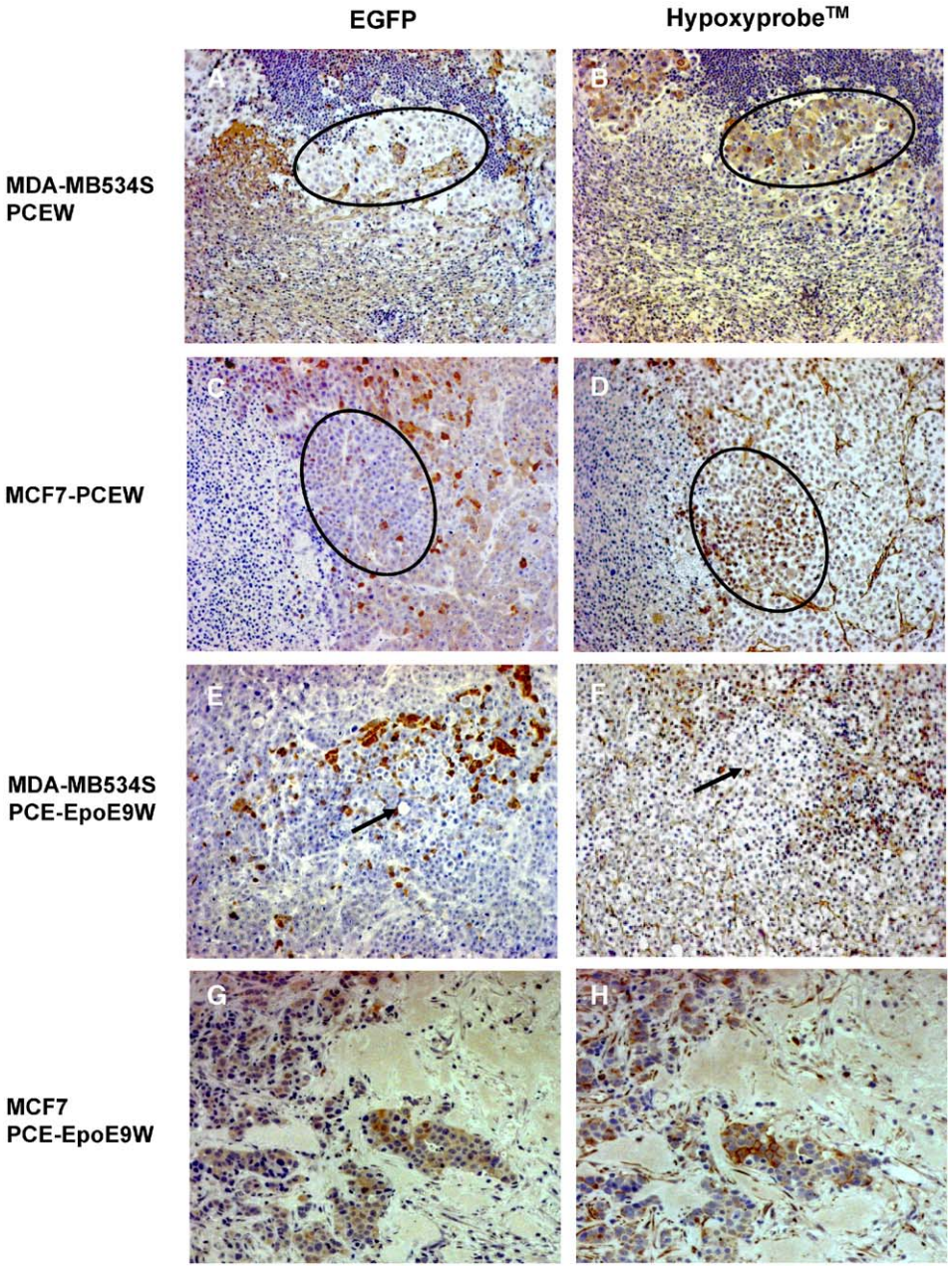


Fig. 5. Immunohistochemical analysis of EGFP expression in mammary cancer cell xenografts. Consecutive sections of xenograft tumours derived from MCF7 (A, B, G, H) or MDA-MB534S cells (C–F) stably infected either with PCEW (A–D) or PCE-EpoE9W (E–H) were stained for detection of EGFP (A, C, E, G) or pimonidazole-protein adducts (B, D, F, H) indicating hypoxic tumour areas. Circles indicate corresponding regions on consecutive sections. A blood vessel in sections E and F is marked by an arrow. Photomicrographs were taken at a magnification of 200×.

A comparable hypoxia-mediated expression targeting system was recently described with an adenovirus-associated virus vector system where a hypoxia-inducible, cardiac-specific expression of VEGF was achieved by linking an HRE to the myosin light chain promoter (Su et al., 2004). A retroviral vector carrying an internal hypoxia-inducible hybrid promoter consisting of the liver-specific α-fetoprotein (AFP) promoter coupled to a VEGF promoter-derived HRE was shown to enable a hepatoma cell-specific expression of therapeutically active thymidine kinase (Ido et al., 2001). Using this approach, Ido and co-workers managed to achieve a therapeutic effect even on xenograft tumours which were derived from low AFP-expressing hepatoma cells.

A clear increase of promoter activity was demonstrated by quantitative FACS analyses also in tumour tissue derived from PCE-EpoE9W-infected T47D mammary cancer cells compared to those infected with the vector PCEW bearing the basic WAP promoter (Fig. 7). Detailed immunohistochemical and immunofluorescence analyses strongly suggest that this increase in expression levels is due to the enhancer elements linked to the WAP promoter mediating expression within hypoxic and next to necrotic tumour areas (Figs. 5 and 6). Recently, it was

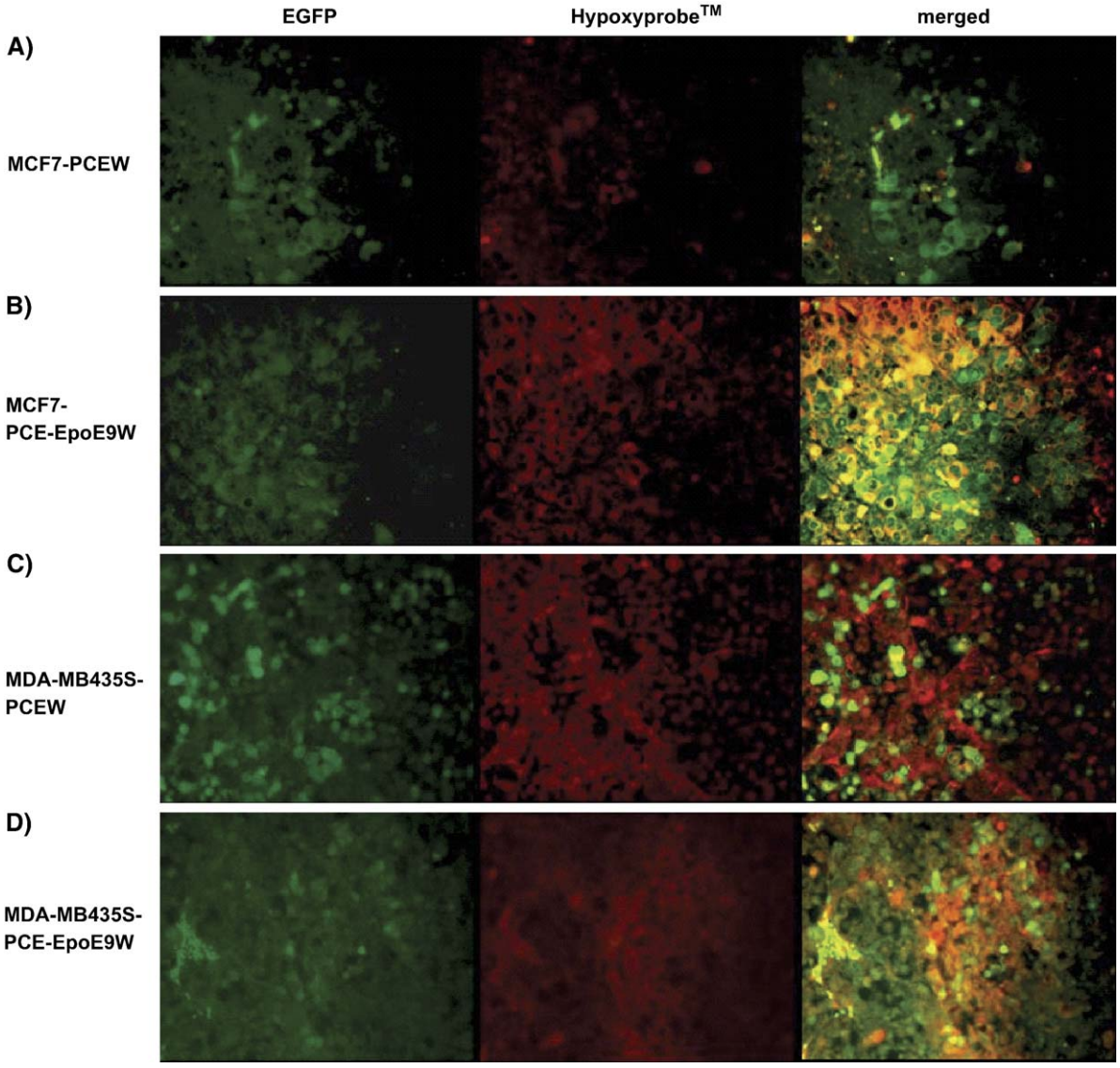


Fig. 6. Immunofluorescence analysis of EGFP expression in mammary cancer cell xenografts. Sections of tumours derived from MCF7 (A, B) or MDA-MB435 (C, D) breast cancer cells stably transduced with PCEW (A, C) or PCE-EpoE9W (B, D) were simultaneously stained for detection of EGFP (green fluorescence) or pimonidazole-protein adducts (Hypoxyprobe, red fluorescence). Colocalisation is indicated by an orange/yellow colour in merged pictures. Photomicrographs were taken at a magnification of 400×.

described that radiation (Rofstad et al., 2004) and various chemotherapeutic agents such as cisplatin (Park et al., 2002) and cyclophosphamide (Dorie and Kallman, 1992) can enforce tumour hypoxia. On the other hand, Greco et al. have shown that besides irradiation also cisplatin and doxorubicin activate promoters containing CArG elements (Greco et al., 2005). Thus, in conclusion, data strongly recommend the respective hypoxia and radiation responsive elements for an application in expression targeted gene therapy delivery systems.

## Materials and methods

### *Cell lines*

Human mammary adenocarcinoma cells (MCF7; ATCC No: HTB-22), were cultivated in Minimum Essential Medium with Earls Salts (MEM, Invitrogen Life Technologies) supplemented with 10% foetal bovine serum (FBS; Invitrogen LT), 0.1 mM non-essential amino acids (Invitrogen LT), 0.1 mM sodium pyruvate (Invitrogen LT) and 1 mM bovine insulin (Sigma-Aldrich). The human mammary carcinoma cell line T47D (ATCC no: HTB-133) was grown in RPMI 1640 medium (Invitrogen LT) supplemented with 10% FBS, 0.2 U/ml bovine insulin, 2 mM L-glutamine (Invitrogen LT) and 1 mM sodium pyruvate. The MDA-MB435S cell line (ATCC No: HTB-129), derived from a pleural effusion metastasis of a human mammary gland ductal carcinoma, was grown in Dulbecco's Minimum Essential Medium (DMEM, Invitrogen LT) supplemented with 15% FBS. The human breast carcinoma cell line Hs578T (ATCC No: HTB-126) was cultivated in DMEM supplemented with 10% FBS and 0.01 mg/ml bovine insulin. The pancreatic carcinoma cell line Panc1 (ATCC No: CRL-1469), the human

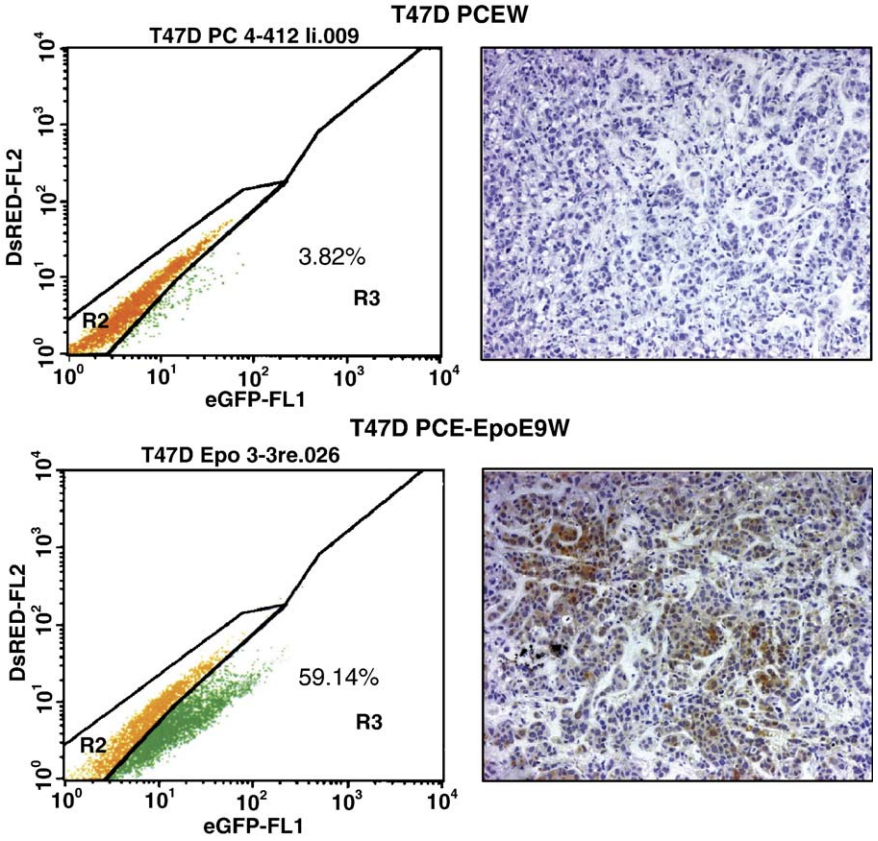


Fig. 7. Quantitative and qualitative analysis of EGFP expression in tumour xenografts derived from PCEW- or PCE-EpoE9W-transduced T47D cells. Parts of excised tumours were either digested with collagenase to obtain single cell suspensions and analysed by FACS (left panels) or embedded in paraffin for immunohistochemical detection of EGFP (right panels). The percentage of green fluorescent cells (area R3) contained in the amount of analysed cells is indicated. Representative data of one tumour out of six are shown. Photomicrographs were taken at a magnification of 200×.

embryonic kidney cell line HEK293 (ATCC No: CRL-1573) and the cervix carcinoma cell line HeLa (ATCC No: CCL-2) were cultured in DMEM supplemented with 10% FBS. The human microvascular endothelial cell line HMEC (Cambrex, Verviers, Belgium) was maintained in EGM medium (Cambrex) and primary normal human dermal fibroblasts (huFi; NHDF-c; PromoCell, Vienna, Austria;) were cultured in Amniomax C100 BASIC medium (Invitrogen, LT) supplemented with Amniomax C100 SUP (Invitrogen, LT). HEK293 derived amphotropic retroviral packaging cells 2GP19Talf (Pambalk et al., 2002), which stably provide the murine leukaemia virus (MLV) Env, Gag and Pol proteins, were grown in DMEM supplemented with 10% FBS. All cells were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$.

*Plasmids and retroviral vectors*

The expression plasmid pEpoE9GFP (Fig. 2A) is described elsewhere (Greco et al., 2002). The MLV-based retroviral ProCon vector pPCEW (Fig. 2B) is a modification of the vector pPCEMa published previously (Hlavaty et al., 2004). The U3 region of mouse mammary tumour virus (MMTV) located in the 3′ LTR of pPCEMa was replaced by a 470-bp fragment of the murine WAP promoter (Lipnik et al., 2005) giving rise to pPCEW. For generation of pPCE-EpoE9-W (Fig. 2C) the Epo/E9 enhancer elements from pEpoE9GFP were amplified by PCR using the oligonucleotide primers pEpoE9-for (5′-GATACACCGCGGGATCTGGCCCTACGTGCT-3′) and pEpoE9-rev (5′-GATACA CCGCGGATCGCCCAAA-TAAGGCCAA-3′). Both primers contain a *Sac*II restriction endonuclease binding site (underlined) to facilitate cloning. PCR was performed with 30 ng of pEpoE9GFP using *Taq* DNA Polymerase (Promega) for 30 cycles (1 min 94 °C, 30 s 70 °C, 1 min 74 °C). A 270 bp Epo/E9-specific fragment was isolated and introduced into a pCR-XL-TOPO PCR cloning vector (Invitrogen, LT) followed by DNA sequencing. The Epo/E9 element was then isolated from pCR-XL-TOPO by restriction digest with *Sac*II and inserted into the *Sac*II linearized vector pPCEW 5′ of the WAP promoter. The resulting vector pPCE-EpoE9W was finally sequenced with a primer binding within the WAP promoter sequence (WAP rev: 5′-AAGAGGCTG GCACAGCTCTA-3′).

*Transfection and flow cytometric analysis*

MCF7, T47D, MDA-MB435 and 2GP19Talf cells were transfected following a calcium phosphate co-precipitation protocol (Invitrogen LT). 5 μg of plasmid DNA were used per well of a 6-well-plate. Stable populations and single cell clones of transfected cells were selected by supplementing the

respective culture medium with 400 μg/ml Geneticin (G418 sulfate, Invitrogen LT). For fluorescence-activated cell sorting (FACS), cells were harvested and washed twice with phosphate buffered saline (PBS, pH 7.4, AppliChem, Bio-Chemica, Austria). Xenografts were digested to a single cell suspension by incubation in collagenase dissolved in PBS (Sigma; 5 mg/ml PBS) for 2 h at 37 °C with continuous mixing and shaking. After 2 h digested tissue was supplemented with DMEM/10% FCS and cells were harvested and washed as described above. Finally cell pellets were resuspended in 1 ml PBS and the cell suspension was filtered through gauze (Bückmann, Germany). Fluorescence analysis for detection of EGFP expression was performed using a FACScalibur (Becton Dickinson) at an excitation wave length of 488 nm (Becton Dickinson BP 530/30) and the CellQuest analysis software (Becton Dickinson).

*Retroviral infection*

Retrovirus producing cells were obtained by G418 selection of 2GP19Talf packaging cells transfected with the respective retroviral vector plasmids. Target cells were seeded the day before infection at a density of 4 to 6 × $10^5$ cells per well of a 6-well-plate. Virus producing cells were seeded at 4 × $10^6$ cells per well of a 6-well plate 2 days before the infection experiment. The next day, their culture medium was replaced by 2 ml of fresh DMEM/10% FBS. Twenty-four hours later, virus containing cell culture supernatant was filtered through a 0.45-μm filter (Sarstedt) and 1 ml, 100 μl or 10 μl thereof were used for infection of target cells in a total volume of 1 ml DMEM/10% FBS. The cells were incubated with recombinant virus for 6 h in the presence of 8 μg/ml polybrene (Sigma-Aldrich). Thereafter, medium was replaced with the respective cell type-specific culture medium. Cells were harvested 72 h after infection and analysed by FACS for determination of the number of infected cells. The viral titre (green fluorescent units (gfu)/ml) was estimated according to the percentage of EGFP positive cells. For generation of stably transduced cell clones and populations, infected cells were cultured in the presence of G418 (400 μg/ml) as described above.

*Generation of hypoxic conditions*

For generation of hypoxic conditions two different methods were applied. Cells were either seeded in duplicates in 6-well-plates and kept in a hypoxic chamber which was inflated for 10 min with 10% $CO_2$ compensated with nitrogen (Linde Gas, $H_2O$ <5 ppm, $O_2$ <3 ppm), or seeded in T25 flasks (Greiner) and cultured in the presence of cobalt (II) chloride (99.9% metal basis, Alfa Aesar, Johnson Matthey, Merck). For simulation of transient hypoxia 4 μM (MDA-MB435S) and 2 μM (T47D, MCF7) $CoCl_2$ were applied. Chronic hypoxia was simulated by incubating the cells in the presence of 0.1 μM (T47D), 0.3 μM (MCF7) and 0.5 μM (MDA-MB435S) $CoCl_2$ for up to 96 h. All experiments were performed three times and the mean values including standard deviations are depicted in the respective figures and tables. Significance of the obtained values ($P < 0.05$) was calculated applying an unpaired $t$ test (Graph PadPrism 3.0).

*X-ray exposure*

For irradiation of cells, 8 × $10^5$ MCF7 and T47D, and 4 × $10^5$ MDA-MB435S cells respectively were seeded in 6 cm Ø dishes. The day after seeding the culture medium was reduced to 1 ml, the cover was removed and the cells were irradiated with different doses of X-rays. The X-ray generator used (Dermaopan, Siemens, Austria) contained a 0.3-mm aluminium filter and was set to level 2 (29 kV) and 25 mA. The focus skin distance was 10 cm. The irradiation time varied according to the demanded dose which was determined using a gamma dosimeter (X-ray-gamma-dosimeter 27060; Kuhm Analytik, Klosterneuburg, Austria). Irradiation was applied for 24 s to achieve a dose of 2.6 Gy. After irradiation, 50 μg/ml gentamycin (Invitrogen, LT) was added to the culture medium. Cells were harvested and analysed 48 h after irradiation. All experiments were performed three times and the mean values including standard deviations are depicted in the respective figures and tables.

*Viral RNA extraction and real time RT-PCR*

For isolation of viral genomic RNA from particles, cell culture supernatant of virus producing cells was digested with 1.9 U benzonase (Merck) at 37 °C for 1 h to remove interfering cellular nucleic acids. Thereafter viral RNA was extracted from 140 μl supernatant using the QIAamp viral RNA mini kit (Qiagen). The amount of viral RNA was determined by EGFP-specific Taqman real-time RT-PCR. The sequences for oligonucleotide primers and the fluorogenic probe as well as the respective PCR conditions have been published previously (Klein et al., 2000). The detected fluorescence signals were analysed by using sequence detection software (SDS version 1.9.1; Applied Biosystems). The amount of viral RNA per 5 μl sample was calculated by using a serial dilution of either in vitro transcribed RNA or viral RNA as a standard. The reaction efficiency of each assay was calculated by the method described previously (Klein et al., 2001) and revealed only minimal differences (<5%).

*Establishment of tumour cell xenografts*

MCF7, T47D and MDA-MB435S single cell clones and populations, stably infected with PCE-EpoE9W or PCEW, respectively, were cultured to 90% confluency in T175 flasks. 5 × $10^6$ (MCF7, MDA-MB435) or 1 × $10^7$ (T47D) were mixed 1:1 (vol/vol) with matrigel (Becton Dickinson) and injected subcutaneously into the pectoral mammary fat pad of female SCID/beige mice (C.B-17/IcrHsd-Prkcd$^{scid}$ Lyst$^{bg}$; Harlan-Winkelmann, Bochen, Germany). Additionally, an estrogen pellet (1.7 mg, 21-day release, Innovative Research of America, Sarasota) was implanted subcutaneously in the flank of those mice which were injected with

MCF7 and T47D cells. Mice were kept in scantainers (Scanbur, Karlslunde, Denmark) under specific pathogen-free conditions. They had free access to standard diets (Altromin-R+M-H-1324, autoclaved; Altromin, Lage, Germany) and autoclaved tap water. Tumour sizes were measured twice a week in two dimensions with a calliper and the tumour volume was calculated according to the formula [l × w × w/2]. Five weeks post-implantation the mice were injected intraperitoneally with a single dose of HypoxyprobeTM-1 (60 mg/kg, pimonidazole hydrochloride, Chemicon) in order to visualize hypoxic tumour regions. 15 min later mice were sacrificed, xenografts were excised, fixed in formalin and analysed for EGFP expression and the presence of hypoxia by immunohistochemistry. These animal experiments were performed according to Austrian laws governing animal experimentation (GZ) BMBWK-68.205/0011-BrGT/2005.

*Immunohistochemistry*

Tumours were excised from the mammary fat pad, fixed in 4% buffered formaldehyde (pH 7.0) for 4–6 h at room temperature, and embedded in Histo-Comp (Vogel, Giessen, Germany) using an automatic embedding equipment (Tissue Tek VIP 2000; Miles Scientific Inc., Mishawaka, IN, USA). 5 μm-thick sections were prepared and mounted on glass slides pre-treated with poly-L-lysine (Sigma). Sections were deparaffinized in xylene and re-hydrated in a descending series of ethanol (100%, 96%, and 70%). Deparaffinized sections were pre-treated with 3% hydrogen peroxide in methanol for 15 min to inactivate endogenous peroxidase and rinsed in tap water. After that, slides were put into an immunostaining center (Sequenza, Shandon Scientific, Schwerte, Germany). For detection of EGFP, slides were incubated with 1.5% normal goat serum (DakoCytomation) for 30 min at room temperature followed by an overnight incubation with the primary antibody at 4 °C (rabbit anti-*Aequorea* victoria green fluorescent protein, Molecular Probes, Invitrogen, LT; 1:4000 in PBS). Slides were then washed in PBS and incubated with the peroxidase conjugated secondary antibody (anti-rabbit EnVision+ TM, DakoCytomation) for 30 min at room temperature. Subsequently, the sections were washed in PBS and slides developed for 10 min at room temperature in 0.1% 3,3′-diaminobenzidine (DAB) hydrochloride containing 0.03% hydrogen peroxide. The reaction was stopped by incubation in distilled water for 5 min. Finally, sections were counterstained with Mayer's hemalumn, dehydrated and mounted in DPX mounting medium (Fluka). For detection of pimonidazole-protein adducts, antigen retrieval was performed by incubating the slides with 0.1% protease (Sigma) at room temperature for 10 min. Sections were then washed with PBS for 10 min and incubated with 1% BSA in PBS (Sigma) for 5 min at room temperature to minimize unspecific background staining. The primary antibody incubation (mouse-anti pimonidazole, Hypoxyprobe-1 MAb1, Chemicon, Hampshire, UK; 1:100 in PBS) was performed at room temperature for 40 min. After washing in PBS the secondary antibody (anti-mouse LabVision+ TM, Labvision, Vienna, Austria) was applied for 30 min at room temperature. Subsequent steps were performed as described for EGFP staining. For the double fluorescence staining slides were incubated with 1.5% normal goat serum (DakoCytomation) for 30 min at room temperature followed by an overnight incubation with the primary antibody at 4 °C (rabbit anti-*Aequorea* victoria green fluorescent protein, Molecular Probes, Invitrogen, LT; 1:2000 in PBS). After a washing step with PBS for 5 min, a second protein block was performed as described above. Then the slides were incubated with the mouse-anti pimonidazole antibody (1:25 in PBS) for 2.5 h at room temperature and washed with PBS for 5 min. Slides were then incubated with the secondary antibodies Alexa Fluor goat anti-rabbit 488 (Vector laboratories, Vienna, Austria; 1:100 in PBS) and Alexa Fluor goat anti-mouse 568 (Vector laboratories; 1:100 in PBS) for 1 h. Finally the slides were washed with PBS for 5 min, with Aqua dest. for 2 min and mounted with Mowiol (Fluka).

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