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Research Paper

Development of a Flexible and Potent Hypoxia-Inducible Promoter for Tumor-Targeted Gene Expression in Attenuated *Salmonella*

Asferd Mengesha[1]
Ludwig Dubois[1]
Philippe Lambin[1]
Willy Landuyt[2]
Roland K. Chiu[1]
Bradly G. Wouters[1]
Jan Theys[1,*]

[1]Department of Radiation Oncology (Maastro Lab); GROW Research Institute; University of Maastricht; Maastricht, the Netherlands

[2]Laboratory of Experimental Oncology/Radiobiology; University Hospital Gasthuisberg; K.U. Leuven; Belgium

*Correspondence to: Jan Theys; University of Maastricht; Maastricht Radiation Oncology (Maastro); Universiteitssingel 50/23; P.O. Box 616; 6200 MD Maastricht; The Netherlands; Tel.: +31.0.43.3882975; Fax: +31.43.3884540; Email. jan.theys@maastro.unimaas.nl

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KEY WORDS

Salmonella typhimurium, hypoxia, gene therapy, induction

ABBREVIATIONS

HIP	hypoxia-inducible promoter
GFP	green fluorescent protein
RFP	red fluorescent protein
FNR	fumarate and nitrate reduction regulator
RLU	relative light units
OD600nm	optical density
HRE	hypoxia response element
HIF-1	hypoxia-inducible factor

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ABSTRACT

To increase the potential of attenuated *Salmonella* as gene delivery vectors for cancer treatment, we developed a hypoxia-inducible promoter system to limit gene expression specifically to the tumor. This approach is envisaged to not only increase tumor specificity, but also to target those cells that are most resistant to conventional therapies. We demonstrate that the exponential growth of the attenuated bacteria is identical under normoxia and hypoxia. A hypoxia-inducible promoter (HIP-1) was created from a portion of the endogenous *Salmonella pepT* promoter and was shown to drive reporter gene expression under both acute and chronic hypoxia, but not under normoxia. Genetic engineering of the TATA- and FNR-box within HIP-1 allowed fine-tuning of gene induction, resulting in hypoxic induction factors of up to 200-fold. Finally, we demonstrate that HIP-1 can drive hypoxia-mediated gene expression in bacteria which have colonized human tumor xenografts in mouse models. Expression of both GFP and RFP under control of HIP-1 demonstrated an ~15-fold increase relative to a constitutive promoter when tumors were made hypoxic. Moreover, the use of a constitutive promoter resulted in reporter gene expression in both tumors and normal tissues, whereas reporter gene expressing using HIP-1 was confined to the tumor.

INTRODUCTION

Genetically modified, nonpathogenic bacteria that preferentially localize and proliferate in solid tumors have been studied as alternative gene delivery vectors in cancer treatment.[1,2] In one approach, strictly anaerobic nonpathogenic *Clostridium* spores that are specifically targeted towards the hypoxic/necrotic regions of the tumor, are being explored.[3] Another strategy involves the use of genetically engineered auxotrophic *Salmonella typhimurium* strains with strongly reduced virulence as potential gene delivery system.[4,5] *Salmonella* are facultative anaerobes that are able to grow under both aerobic and anaerobic conditions. To develop a safe gene delivery vector, wild type *Salmonella typhimurium* was attenuated by chromosomal deletion of the *pur*I and *msb*B genes, thereby increasing safety and reducing toxicity.[6] Both bacterial and tumor-related factors have been implicated for its preferential accumulation in tumors. *Salmonella* are motile, and following their adherence to the tumor vasculature, they can invade in and spread throughout the tumor area.[7] Within the tumor, areas of necrosis and apoptosis may provide additional nutrients such as purines required by the organism. In addition, the tumor may provide an immunosuppressive environment that inhibits the clearance of *Salmonella*.[8,9] However, following the administration of attenuated *Salmonella* to tumor-bearing animals, it has been observed that not only the tumor but also normal tissues are colonized, albeit transiently and to a lesser extent. Obviously and since the recombinant strains used so far all employed a strong constitutive promoter to drive the therapeutic gene of interest, this biodistribution pattern can cause undesired side effects and negatively influence the specificity of the *Salmonella* mediated gene transfer system.[10,11] One way to address this issue and to obtain controlled gene expression when using attenuated *Salmonella* is to exploit the unique hypoxic tumor microenvironment.

Hypoxia is a common characteristic of most malignant tumors and is considered to be an independent prognostic factor of poor outcome in a variety of tumor types. Although hypoxia leads to resistance to radiotherapy and most chemotherapeutic drugs, and to increased malignancy, it also represents a potential opportunity for obtaining tumor-specific gene expression.[12,13] One promising way to achieve this goal, is to place a therapeutic gene under the transcriptional control of a hypoxia regulatory element.

In *E. coli* and *Salmonella*, the Fumarate and Nitrate Reduction regulator (FNR) regulates the global response to the transition between aerobic and anaerobic growth. FNR is a bifunctional protein that acts both as a hypoxic sensor and a hypoxia-responsive transcription factor.[14-16] The sensory domain contains four cysteine residues that act as ligands for an oxygen-labile [4Fe-4S] cluster. Acquisition of a [4Fe-4S] cluster initiates the formation of FNR homodimers that bind DNA site-specifically and regulate transcription from target promoters. Under aerobic conditions the [4Fe-4S] clusters are disassembled and the FNR dimers dissociated to form nonDNA-binding monomers.[17,18] All FNR-regulated promoters are characterized by the presence of one or more FNR-binding sites. We thus reasoned that a FNR responsive promoter would be an attractive candidate to exploit for restricting therapeutic gene expression specifically to the tumor, thereby increasing the specificity of the *Salmonella* mediated gene delivery system. Moreover, targeting *Salmonella* to hypoxic areas represents another major advantage as these regions are most resistant to effective conventional cancer treatment.

We therefore tested the potential hypoxic induction of a promoter (HIP) derived from the FNR-dependent pepT promoter in attenuated *Salmonella typhimurium*.[19] Here, we demonstrate that *Salmonella* grow as efficiently in both normoxic and hypoxic conditions and show that hypoxia induced bacterial promoters have the potential to increase the specificity of the *Salmonella* mediated gene delivery system by establishing spatial control of gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions. Attenuated *Salmonella typhimurium* VNP20009 [*msbB*$^-$, *purI*$^-$, *xyl*$^-$ and *EGTA*^{r}] was obtained from Vion Pharmaceuticals (New Haven, CT, USA). *Escherichia coli* TG1 obtained from Gibco BRL (Invitrogen, Carlsbad, CA, USA) was used as host for all recombinant DNA manipulations. Detailed descriptions of the plasmids used in this work are listed in Table 2. The pZEP08 and pZEP16 carrying GFP$^+$ were a kind gift from Dr. Jay Hinton (IFR, Norwich Research Institute, UK). The dsRed.T4 was obtained from Dr. Bumann (Max Planck Institute, Germany). For strain manipulations and maintenance, cells were grown at 37°C in liquid Luria-Bertani (LB) medium containing 0.5% Bacto-yeast extract, 1% Bacto-tryptone, and 1% NaCl (Gibco BRL) and on LB agar plates using standard procedures. When required, ampicillin and chloramphenicol were added to the medium to a final concentration of 50 and 12 μg/ml respectively.

Bacterial strains harboring the recombinant plasmids were grown overnight in liquid LB, with shaking at 37°C. The following day cultures were diluted twice (1:100) when an optical density (OD_{600nm}) of 0.3 was reached. Cultures were again diluted 100 fold and experiments were started. In order to determine the bacterial count, 100 μl of culture was serially diluted and plated on LB agar. Bacterial colonies were counted after overnight incubation at 37°C. Aerobic conditions were obtained by shaking the culture vigorously (250 rpm/min). For anaerobic induction experiments, cultures were incubated in an hypoxic chamber (MACS Anaerobic Workstation; Don Whitley Scientific, Shipley, UK (5% CO_2 -95% N_2) at 37°C with shaking conditions as in the aerobic cultures. All the media and buffers used for anaerobic experiments were made hypoxic and kept in the anaerobic chamber for days before onset of the experiments.

For acute stress hypoxia experiments, cultures were transferred to the hypoxic workstation following an aerobic dilution cycle as described. Samples were taken at different time points, OD_{600nm} defined and the bacteria stored on ice prior to a luciferase assay. For chronic hypoxia experiments, cultures were maintained in the anaerobic chamber for days and the luciferase assay was performed following two cycles of mid log growth with in the anaerobic chamber.

Table 1 **Oligonucleotide primers used in this study**

Oligonucleotide	Sequence[a]
FFmod_F1	5'-TGCAGGGATAAAATTGATCTGAATCAATATTTG-TCTT-3'
FFmod_R1	5'-AGACAAATATTGATTCAGATCAATTTTATCCCTG-CAA-3'
PepTm_F1	5'-CAGGGATAAAAGGATCCGACGCAATATTTGT-CTTT-3'
PepTm_R1	5'-AAAGACAAATATTGCGTCGGATCCTTTTATCC-CTG-3'
pFNRg_F1	5'-CATCTAGAGAGTGGTTATTGCGCCATG-3'
pFNRg_R1	5'-CACTCGAGGCGGAAAAATCAGGCAACG-3'
pPepT_F1	5'- CAGGTACCCAGAAAGCGTGTCAAA-3'
pPepT_R1	5'- CAAGATCTCTTTTCGTGACAACATT-3'
pPepT GFP_F1	5'-CACCCGGGCAGAAAGCGTGTCAAA-3'
pPepT GFP_R1	5'-CATCTAGACTTTTCGTGACAACATT-3'

[a]Sequences underlined indicate locations of restriction sites used for cloning.

DNA manipulations and vector construction. All general DNA manipulations in *E. coli* were carried out as described by Sambrook et al.[20] Restriction endonucleases and DNA-modifying enzymes were purchased from New England Biolabs (NEB Ltd, Ipswich, UK) and used as indicated by the suppliers. Chromosomal DNA was extracted from *E. coli* and *Salmonella* using the Gene Elute Bacterial Genomic DNA kit mini (Sigma, St. Louis, MO, USA). PCR was performed on the extracted chromosomal DNA as a template. Primers used in this study are described in Table 1 and were designed using the published sequence of *pepT* gene [EMBL: STPEPT, AC: M62725] and FNR gene [EMBL: STPEPT, AC: AE008893]. PCR products were synthesized with Hot Star DNA polymerase (Qiagen GmbH, Westburg, Hilden, Germany) to generate the 3' deoxyadenosine necessary for direct cloning into pGEM-T Easy vector (Promega Corp, Madison, WI) and sequenced using standard T3/T7 forward and reverse primers (ABI prism 310 Genetic Analyzer, Applied Biosystems). Fragments for subcloning were isolated from low melting agarose using the QiaQuick gel extraction kit (Westburg, Hilden, Germany) and restriction sites incorporated into the primers were then used to subclone fragments into the pSP-Luc+NF expression vector (Promega Corp, Madison, WI) and pZEP-08.[21] Transformation into *E. coli* TG1 (*supE hsdΔ5 thi Δ* [*lac-proAB*]) was performed using chemocompetent cells (Gibco BRL) obtained with the RbCl method. Introduction of recombinant plasmids into attenuated *Salmonella* was done by electroporation [25 μF, 400 and 2.5 kV,] using 0.2 mm cuvettes. Plasmid DNA isolations were performed using the Gene Elute plasmid miniprep kit (Sigma, St. Louis, MO, USA).

Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions using pGEM-HIP-1 construct as template. Complimentary oligonucleotides were designed to contain the desired mutation (Table 1), flanked by the unmodified sequence to anneal to the same sequence on opposite

Table 2 **Strains and plasmids used in this study**

Strains or Plasmids	Genotype	Reference
E. coli TG1 cells	(*supE hsdΔ5 thi Δ(lac-proAB) F′ [traD36 pro AB*$^+$ lacI^q *lacZΔM15]*	Gibco BRL
Salmonella Typhimurium [VNP2009]	(*msbB*$^-$, *purI*$^-$, *xyl*$^-$ and EGTA^r)	Vion Pharm.
Plasmids		
pGEM-T Easy vector	MCS, Ap^r, LacZα	Promega
pSp-Luc+NF fusion vector	MCS, Ap^r, LUC+NF	Promega
pSp-HIP-1-Luc+NF	pSp-Luc+NF, HIP-1promoter	This study
pSp-HIP-1$_{MUT}$-Luc+NF	HIP-1promoter, Δ FNR box	This study
pSp-HIP-1$_{TATA}$-Luc+NF	HIP-1promoter, TATA box modified	This study
pSp-HIP-1$_{FNR}$-Luc+NF	HIP-1promoter, FNR box optimized	This study
pSp-HIP-1-Luc+NF/FNR	HIP-1promoter, over expressing FNR gene	This study
pSp-HIP-1$_{MUT}$-Luc+NF/FNR	HIP-1 promoter, Δ FNR box over expressing FNR gene]	This study
pZEP-pepT-GFP$^+$	ΦHIP-1-*gfp*$^+$, pZEP08 derivative (Cm^r Km^s)	This study
HIP-1-DsRed.T4	DsRed.T4, HIP-1 promoter	This study
prpsM-DsRed.T4	DsRed.T4, rpsM promoter	This study
pZEP-08	pBR322 derivative (*mob*$^+$/*bla*$^+$ Φ*gfp*$^+$ Cm^r Km^r)	21
pZEP-16	Φ*rpsM′-gfp*$^+$, pZEP08 derivative (Cm^r Km^s)	21
pDsRed.T4	MCS, Ap^R, DsRed	25

Ap^r, ampicillin resistant; Km^r, kanamycin resistantant; Km^s, kanamycin sensitive; Cm^r, chloramphenicol resistant; MCS, multiple cloning site. Indicates a transcriptional gene fusion.

strands of the template plasmid. Sample reactions for PCR based site directed mutagenesis are prepared as follows: 5 µl of 10X reaction buffer, 5 µl (25 ng) each oligonucleotide primers, 1 µl of dNTP (5 mM) and 25–50 ng of template DNA, mixed to a final volume of 50 µl. PCR reactions were carried out using 1 µl Pfu DNA polymerase (2.5 U/µl). The thermal cycling parameters were: 1 cycle (30 s, 95°C),[18] cycles (30 s, 95°C; 1 min 48–55°C) and 1 cycle (10 min, 68°C). All mutations were verified by sequencing.

Determination of in vitro promoter activities. Promoter activities were determined by monitoring luciferase activity in vitro using a luciferase assay protocol (Promega, Corp, Madison, WI). Ninety microliters aliquots were taken from the 1:100 diluted overnight cultures at different time intervals, mixed with 10 µl of 1 M K_2HPO_4 (pH 7.8), 20 mM EDTA and quick-frozen on dry ice. Following collection of all samples, they were equilibrated to room temperature and incubated for 10 min with 300 µl freshly prepared lysis mix (1X cell culture lysis reagent (CCLR), 1.25 mg/ml lysozyme and 2.5 mg/ml BSA). Twenty microliter aliquots of sample and 100 µl of luciferase assay reagent were then mixed into a luminometer tube and luciferase activity measured (10 s, delay 2 s) in a LUMAC type luminometer (Perstorp Analytical Company) at room temperature. Activities are given as relative light units (RLU) after subtraction of the instrumental background. Data are normalized by dividing the RLU by OD_{600nm} at each time point. Induction factors are calculated by dividing the RLU/OD_{600nm} of the induced sample displayed at the times indicated by that of the matched, noninduced samples. The induction factor of 1.0 represents no induction.

Western blot analysis. Tumor and normal tissue samples were homogenized in 1 ml of PBS. Five-hundred microliters of homogenate was subsequently mixed with 500 µl of TrisHCl (pH = 7.5, 100 mM) and samples were lysed by 4 x 30 s sonications. Samples were kept on ice for at least 2 min between the sonications. Protein concentrations were determined by a Bradford assay with BSA as a standard. Lysed samples were separated by sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE). Transfer of proteins to nitrocellulose membranes was performed at 100 V during 1 hour at 4°C (BioRad PowerPac 300). Nitrocellulose membranes were blocked with 0.5% Blotting Grade Blocker non fat dry milk (BioRad) in PBS/Tween 0.2% overnight at 4°C and subsequently incubated with a 1:1000 dilution of anti-GFP monoclonal antibody (Living Colors A.v. mAb JL-8, Clontech) during 2 hours at room temperature. Membranes were washed in PBS/Tween 0.2% and incubated for 1 hour with horseradish peroxidase-conjugated goat anti-mouse IgG and autoradiographed using the ECL Western Blotting detection system as described by the supplier (Amersham Biosciences).

In vivo experiments. Female adult NMRI nu/nu mice were used to assess the in vivo responsiveness of the recombinant attenuated *Salmonella* to hypoxia. 1.5 x 10^6 human colorectal carcinoma (HCT116) cells were injected subcutaneously in the left and right abdominal flank under minor anesthetics. When tumors reached a predefined volume (300 mm^3), recombinant bacteria (2 x 10^6 colony forming units) carrying the pZEP-HIP-1-GFP$^+$ or HIP-1-DsRed.T4 plasmid were systemically administered via a lateral tail vein. Recombinant *Salmonella* harboring the pZEP-rpsM-GFP$^+$ or rpsM-DsRed.T4 (resulting in constitutive activation of the reporter genes), or empty bacteria (VNP20009) injected in mice, were used as control. Tumor colonization was allowed for seven days, while selection antibiotics were added to the drinking water (chloramphenicol for the GFP$^+$ constructs at 500 mg/l and ampicillin for the DsRed.T4 constructs at 20 mg/l). In the first set of experiments animals were analyzed on the BonSAI imager for noninvasive evaluation of reporter gene expression. Tumors and normal tissues were then excised and processed for Western blot analysis. In another set of experiments, one tumor was removed immediately for colonization testing, further histological processing as described previously.[5,22] In the contra lateral tumor, production of fluorescent protein was monitored for several hours using the bonSAI optical imager (Siemens AG, München, Germany). All animal experiments were conducted in accordance with local institutional guidelines, approved by the Animal Ethics Committee of the University.

RESULTS

Effect of oxygen on the growth of *Salmonella.* *Salmonella* are gram-negative facultative anaerobes and grow readily in simple culture media, under both aerobic and anaerobic conditions. Since the effects of hypoxia on the growth pattern of *Salmonella* are only poorly described, we compared the growth characteristics of *Salmonella* under both hypoxic and normoxic conditions by measuring the optical

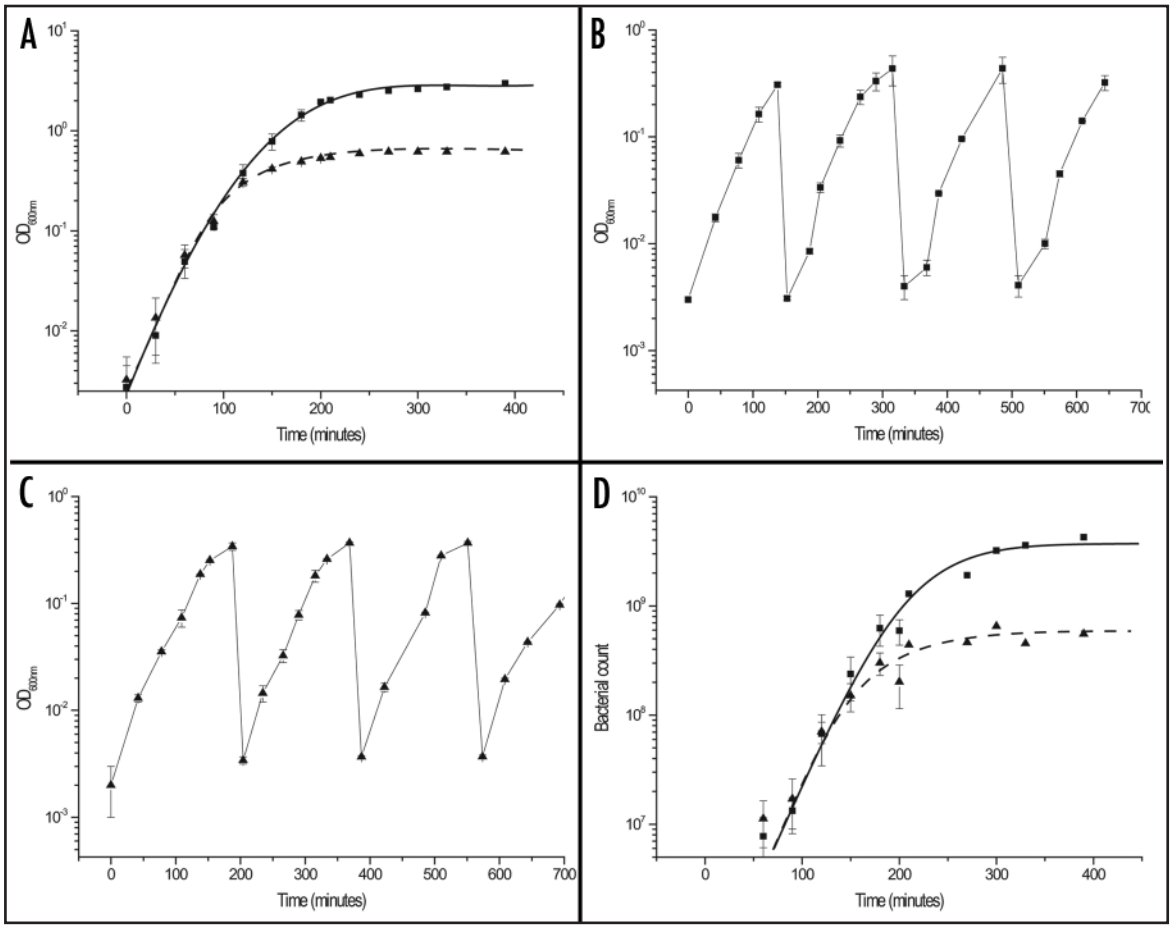

Figure 1. Growth characteristics of attenuated *Salmonella typhimurium* under both hypoxic and normoxic conditions. (A) Bacterial growth defined by optical density ($OD600_{nm}$) as a function of time under aerobic (■) and anaerobic (▲) conditions. Cultures are maintained under aerobic (B) or anaerobic (C) growth conditions for 4 cycles of mid exponential growth by serial 10-fold dilutions with fresh media each time when the bacteria reach an OD_{600nm} of ~0.3. (D) Bacterial growth defined by cell count as a function of time under aerobic (■) and anaerobic (▲) conditions. Each data point represents the mean of 3-4 independent experiments.

density (OD_{600nm}) as a function of time. We observed that the growth rate of the bacteria during the exponential phase was identical under both conditions, but that the cells plateau at a lower density in an anaerobic environment (Fig. 1A). To test whether this effect was directly due to hypoxia itself or whether this was the consequence of hypoxia-mediated depletion of nutrients, their growth was evaluated following long term hypoxic exposure. Cultures were maintained under hypoxic conditions for several generations by repeated serial 100-fold dilutions with fresh anaerobic media each time when bacterial growth reached mid-exponential phase. Interestingly, as observed for bacteria growing in normoxic conditions, the bacteria continued to grow under prolonged hypoxia without any evidence for growth inhibition, provided the nutrient supply in the growth media was replenished (Fig. 1B and C). We subsequently investigated whether hypoxic exposure influences long term bacterial viability by plating the bacteria at different time points after hypoxic growth and assaying for colony survival (Fig. 1D). Similar to our observations with cell density measurements, no difference in survival was observed in normoxic versus hypoxic conditions. Importantly, these growth kinetic studies allowed us to evaluate gene expression in aerobic and hypoxic cells over long periods of time without the confounding effects of early induction of stationary plateau phase, thereby enabling precise and quantitative comparisons of reporter gene expression profiles at the cellular level in the subsequent experiments.

Use of HIP-1 promoter to drive hypoxia specific gene expression. Having demonstrated that the hypoxic and normoxic growth rate of attenuated *Salmonella* are identical, we investigated whether we could develop the wild-type strain into a prokaryotic hypoxia-regulated vector with spatially controlled gene expression. Analogous to the eukaryotic context in which the HRE is the recognition site for the hypoxia-inducible factor-1 (HIF-1), an oxygen-responsive global transcription factor (FNR) induces the expression of a number of bacterial genes during anaerobic growth following binding to a consensus FNR recognition sequence. Based on available literature data, the FNR-regulated *pepT* gene promoter was chosen as a starting point for our investigations.[16] *pepT* is transcribed from two promoters. The P1 promoter is activated by anaerobiosis and requires the activator FNR, while the other promoter, located upstream of P1, is constitutive and unaffected by anaerobiosis.[23] We isolated the P1 fragment, designated it the Hypoxia Inducible Promoter-1 (HIP-1), cloned it upstream of the firefly luciferase coding sequence in the expression vector and used it to transform the attenuated *Salmonella* strain. To test the FNR dependent activation of the HIP-1 promoter in the recombinant bacteria, an in vitro transcription assay was performed under hypoxic conditions.

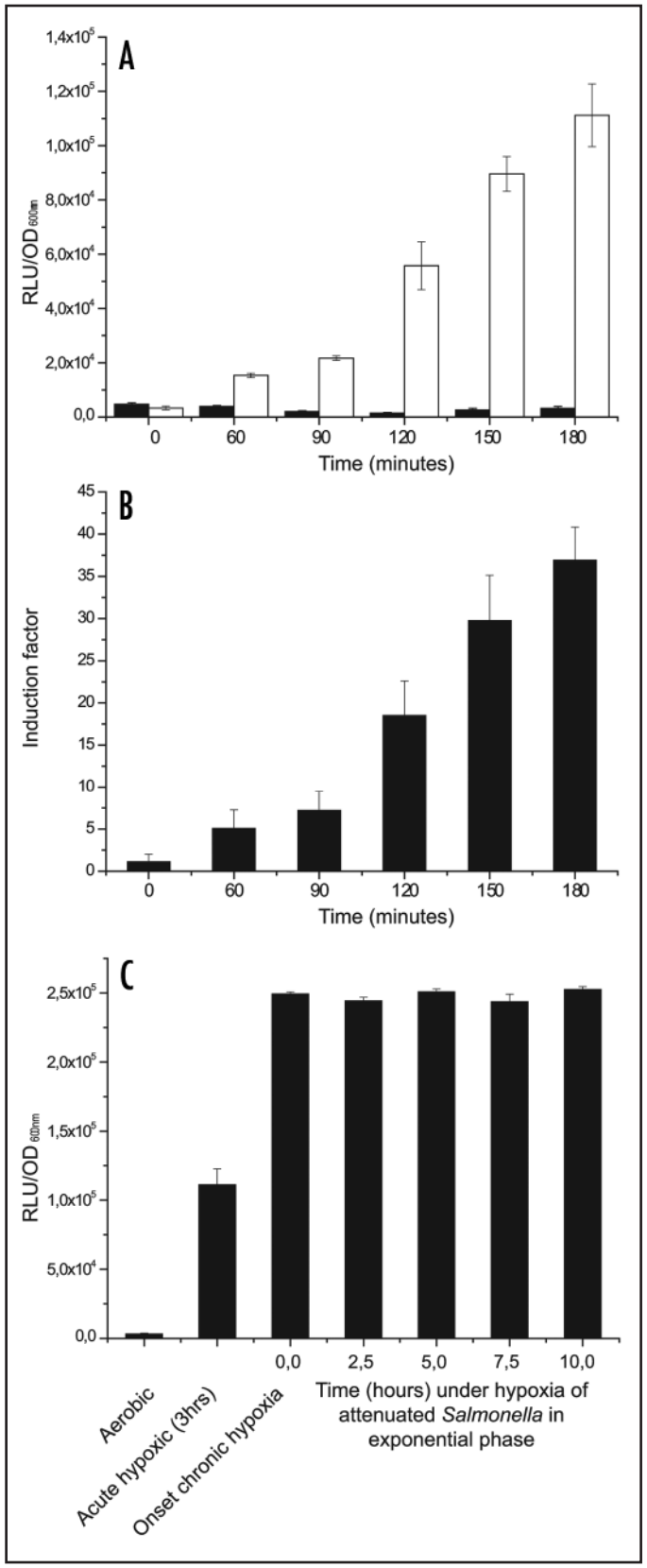

Figure 2. HIP-1 promoter drives hypoxia specific gene expression. (A) Normalized luciferase activity of attenuated *Salmonella* carrying the HIP-1-luciferase construct under aerobic (black bars) and hypoxic (white bars) conditions. (B) Induction factor of wild type (wt) HIP-1 promoter activity as a function of time. (C) HIP-1 promoter activity under chronic state hypoxia. Attenuated *Salmonella typhimurium* were allowed to grow in the hypoxic chamber for two to three days prior to the start of the luciferase assay. After two cycles of mid-exponential growth in strictly hypoxic conditions, enzyme activity was determined every two to three hours while maintaining the exponential growth. Samples were taken when the bacteria reached OD_{600nm} ~0.3 at each growth cycle. Data from the acute hypoxic experiment are included for comparison. Time 0 hours indicates the first time point for the chronic hypoxia experiment. The data represent from three to four independent experiments. Error bars represent the standard errors of the mean (SEM).

In a first set of experiments, we evaluated the inducibility upon acute transition from aerobic to anaerobic growth. Following two cycles of mid exponential growth under aerobic growth conditions, cultures were transferred to the hypoxic chamber and kept under hypoxic conditions in exponential growth phase for up to three hours to monitor at the cellular level whether hypoxia-induction of luciferase takes place. Samples were taken at different time points and luciferase activity assessed. The wild type HIP-1 promoter was induced immediately following hypoxic exposure and reporter protein activity further increased during the follow-up period, whereas the basal activity under aerobic conditions remained low (Fig. 2A). In these experiments, we ensured cells were continuously kept in exponential phase during the induction period and performed normalization towards measured OD_{600nm} values, which we have shown to be equivalent to bacterial number. This allowed us to precisely calculate induction factors following hypoxic exposure, without influence of other growth parameters (Fig. 2B). A 37-fold increase in reporter gene activity was observed within three hours of hypoxic treatment, establishing the hypoxia-inducibility of the HIP-1 promoter during an acute change from aerobic to hypoxic conditions. To evaluate the induction following long term chronic hypoxia exposure and to ensure that the induction observed following acute hypoxia is not transient, we tested the expression profile of the reporter gene during several generations of prolonged chronic hypoxia. Cells were grown for several days under continuous hypoxic exposure. HIP-1 driven gene expression was then subsequently evaluated over a period of ten hours, during which cells were kept in exponential phase. The observed reporter gene expression was not only maintained during the entire follow-up period, but was also found to be 2–3 fold higher compared to the induction observed in the acute hypoxic exposure. This indicates a constant hypoxia-induced gene expression in all individual *Salmonella* bacteria (Fig. 2C). Gene expression levels rose from 37-fold at three hours following acute transition to hypoxia to ~85-fold under chronic hypoxia.

HIP-1 driven expression is mediated by FNR. The putative FNR binding site (GTGA-N_4-CGCAA) in the HIP-1 promoter is centered at position -41.5, upstream of the transcription start site. To confirm the pivotal role of the FNR box in regulating the induction following hypoxia, mutations were introduced by site directed mutagenesis resulting in the destruction of the binding site (Table 3). The promoter containing the desired mutation was then cloned into the luciferase expression vector and tested for its activity under hypoxia. The mutation affecting the FNR binding region of the HIP-1 promoter completely abolished the hypoxia inducibility of the HIP-1 promoter (Fig. 3A). This is in line with a previous report where an A to G change at position -45 in the FNR binding site of

the narG promoter totally abolished the anaerobic expression profile.[24]

Since we use a high copy plasmid-based expression system, endogenous FNR protein may not be sufficient to activate all available FNR boxes. Therefore, we overexpressed FNR by cloning the FNR coding sequence on the same expression plasmid under the control of its own promoter to result in a maximum desired induction under hypoxic conditions. The combination of the wild type HIP-1 and the overexpressed FNR gene indeed resulted in a significant increase in expression compared to the wild type promoter alone, with an average 2.5-fold higher induction in response to hypoxia ($p < 0.05$, Student's t-test) (Fig. 3B). Overexpression of the FNR gene did not affect the activity of the HIP-1 with a deleted FNR-box (data not shown). Both sets of data clearly established that the FNR gene and the FNR box are responsible for the observed hypoxia response.

Table 3 **Nucleotide sequence of the wild type and modified HIP-1 promoter region**

Type of Promoter	Sequence[a]
HIP-1	GGATAAAAGTGACCTGACGCAATATTTGTCTTTTCT TGCTTAATAAT GTTGTCA
HIP-1 *TATA*	GGATAAAAGTGACCTGACGCAATATTTGTCTTTTCT TGCTTTATAAT GTTGTCA
HIP-1$_{FNR}$	GGATAAAATTGATCTGAATCAATATTTGTCTTTTCT TGCTTAATAAT GTTGTCA
HIP-1$_{mut}$	GGATAAAAGGATCCGACGCAATATT GTCTTTTCT TGCTTAATAAT GTTGTCA

[a]The highly conserved 5-base inverted repeat of the FNR consensus sequence is boxed. The putative -10 hexamer are underlined. The arrow indicates the transcription start site.

Optimization of HIP-1 promoter. Having shown that the HIP-1 promoter can drive hypoxia specific gene expression and that the FNR binding site is responsible for its inducibility, we further aimed at improving the specificity and expanding the applicability of the system by optimizing the -10 region and the FNR box. The 5'-AATAAT-3' hexamer (-12 to -7) matching the TATA box of the wild-type (wt) HIP-1 promoter has five of the six bases matched to those of the consensus sequence. We performed a single nucleotide substitution changing the -10 region of the putative FNR-dependent promoter to match the -10 consensus sequence for σ^{70} promoters (TATAAT) (Table 3) and predicted that the -10 consensus sequence would enhance the activity of this promoter, resulting in elevated gene expression under anaerobic conditions. (Fig. 4A) shows the expression profile of the modified promoter compared to the wild type. Correction of the TATA-box increased expression by a factor of 4. This increase was observed in both basal and induced conditions (Fig. 4A), resulting in overall similar induction factors as obtained when applying the wt promoter construct (Fig. 4C). We then investigated whether we could decrease the basal activity by mutating the FNR binding site to the consensus sequence. Interestingly, basal expression levels were now barely detectable under aerobic conditions (Fig. 4B). The absolute expression levels with the FNR-optimized promoter sequence following acute hypoxia were similar to these observed with the wt promoter. The acute inducibility of the system could thus be increased from 37-fold in the wt promoter to 83-fold in the promoter with an optimized FNR box (Fig. 4C). Taken into account that the maximal expression levels under chronic hypoxia are even 2–3 fold higher, this inherently means that use of this promoter can induce gene expression levels up to ~200-fold. Recombinant *Salmonella* harboring a construct in which the promoter had both the TATA- and FNR-box mutated to their respective consensus sequences, behaved similar like the promoter with the modified TATA-box alone (data not shown). This is likely due to saturation effects, as the higher basal levels resulting from optimizing the TATA-box might mask the effect of the optimized FNR sequence in our high copy plasmid-based system.

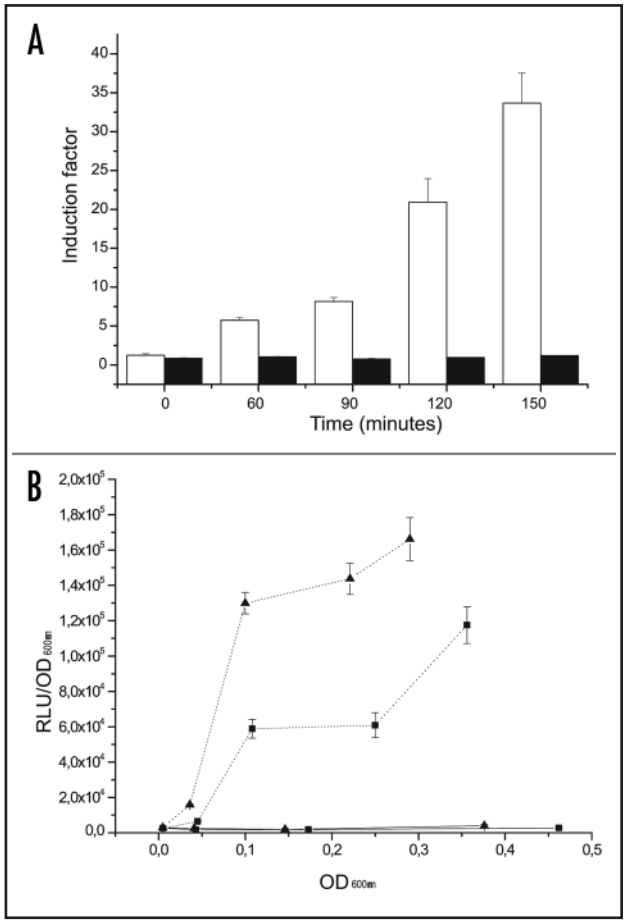

Figure 3. HIP-1 driven expression is FNR dependent. (A) Induction factors of the HIP-1 promoter wt construct (white bars) and the HIP-1 promoter with the deleted FNR-box (black bars) at the indicated time points following onset of hypoxic conditions. (B) Normalized luciferase activity in attenuated *Salmonella* carrying the wt HIP-1 promoter (■) and HIP-1 promoter with overexpressed FNR gene (▲) under hypoxic (dashed line) and normoxic (straight line) conditions as a function of optical density. Enzyme activity was determined as a function of optical density as the rate of bacterial growth differed between recombinant *Salmonella* harboring either constructs. The data represent three independent experiments. Vertical bars represent SEM.

In vivo validation of the HIP-1 promoter activity. As the in vitro data provide strong evidence of luciferase expression by attenuated *Salmonella* under hypoxic conditions and since the bacteria preferentially accumulate in tumors, we subsequently aimed to demonstrate that the HIP-1 promoter was capable of tumor-specific gene expression and importantly of exhibiting hypoxia-inducibility in an in vivo context. Animals harboring HCT116 human colorectal xenografts were therefore systemically injected with *Salmonella* that harbor HIP-1 driven GFP$^+$ or DsRedT4 reporter genes. Both genes are derivatives of the well known fluorescent reporters GFP and RFP, respectively, and are adapted for use in a prokaryotic background.[25]

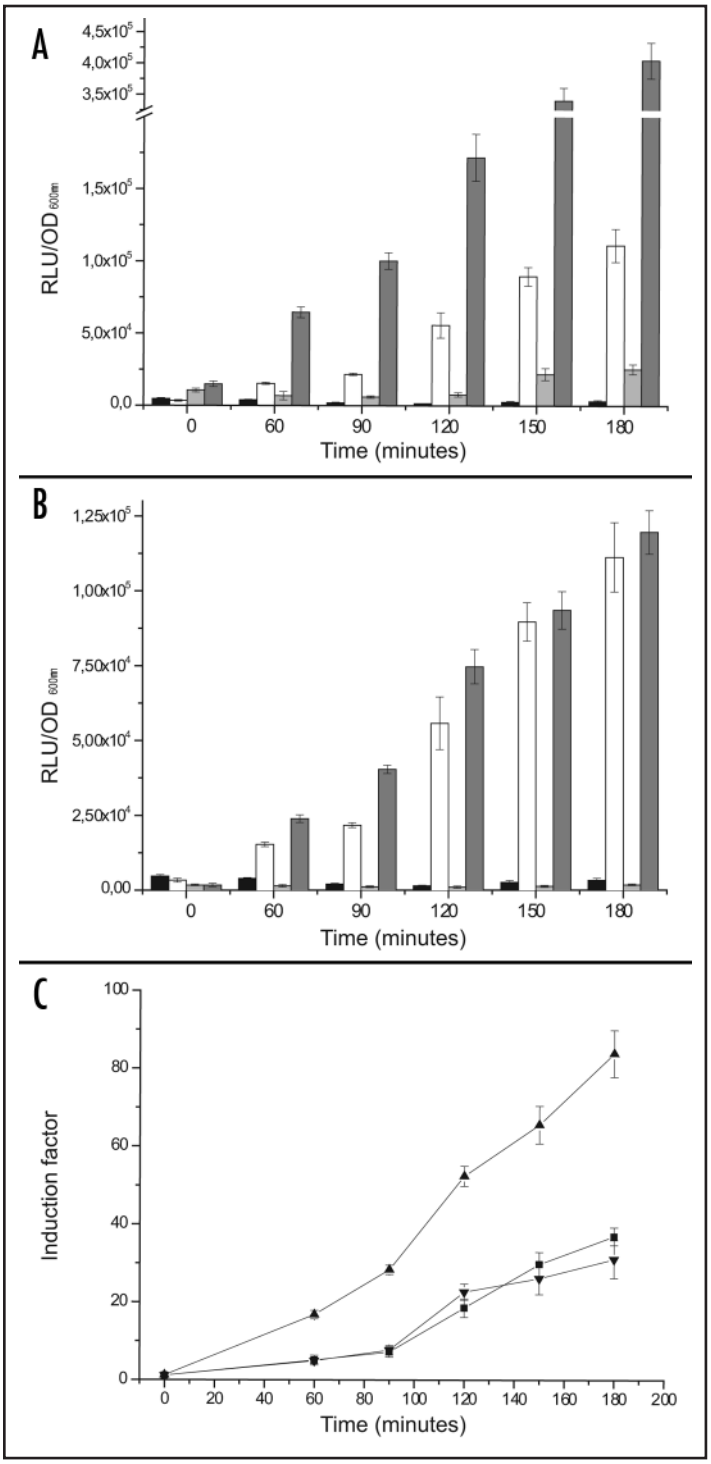

Figure 4. Effect of regulatory sequence modification on the HIP-1 promoter activity. (A) wt HIP-1 promoter activity under normoxic (black bars) and hypoxic (white bars) conditions compared to activity of HIP-1 promoter with optimized TATA-box under normoxic (light grey bars) and hypoxic (dark grey bars) conditions. (B) wt HIP-1 promoter activity under normoxic (black bars) and hypoxic (white bars) conditions compared to activity of HIP-1 promoter with optimized FNR-box under normoxic (light grey bars) and hypoxic (dark grey bars) conditions. optimized HIP-1 promoter under normoxic hypoxic conditions. (C) Induction factors of the wt HIP-1 (▼), the HIP-1 with optimized TATA-box (■) and the HIP-1 with optimized FNR-box (▲). Data represent results from 3 independent experiments. Vertical bars represent the SEM.

Control animals received empty bacteria or recombinant bacteria carrying the same plasmid but with reporter gene expression under control of the constitutive rpsM promoter.[21] In a first set of experiments, we aimed to evaluate the presence of reporter protein in different tissues. At 7 days following injection, tumor and normal tissues were excised and homogenates prepared to quantify colonization and expression of the GFP reporter. All tumors (up to 10^{10} cfu/g tumor tissue) and normal tissues (up to 10^7 cfu/g tissue in liver and spleen) were well colonized. GFP was strongly expressed in tumors colonized with recombinant *Salmonella* harboring constructs with GFP driven off the constitutive rpsM promoter and was also detected in tumors colonized with *Salmonella* in which GFP expression was controlled by HIP-1 (Fig. 5A). We also attempted to detect reporter signals in normal tissues. Although we could readily detect GFP in normal tissues (liver, spleen) when expression was driven by the rpsM promoter, no signal could be demonstrated in normal tissues of animals carrying the HIP-1 GFP$^+$ construct (data not shown).

Xenografts growing in mice are known to be heterogeneous and to contain substantial areas of hypoxia. We thus expected a priori that the colonized tumors would demonstrate HIP-1 promoter activity and this is likely responsible for the fact that we can detect in our Western blots GFP at different levels in tumor extracts containing HIP-1 GFP$^+$ recombinant bacteria. However, this does not show directly that the hypoxia per se is responsible for the observed reporter gene expression. Therefore, to definitively demonstrate the in vivo inducibility of HIP-1, we chose to evaluate changes in reporter activity in individual tumors in which the oxygenation state was dramatically altered. This was accomplished by sacrificing the animals containing the colonized tumors. The production of the fluorescent reporter protein (GFP$^+$ or DsRed.T4) was monitored prior to sacrifice and for several hours afterwards using non-invasive animal imaging technology (BonSAI, Siemens). Tumors were confirmed to contain high levels of *Salmonella* bacteria (up to 10^{10} cfu/g tumor tissue) (data not shown). Despite the fact that these tumors are known to contain already substantial hypoxia at the onset of the measurements, the level of GFP$^+$ under the control of HIP-1 promoter was induced and progressively increased during the entire follow up period in comparison to the level of GFP$^+$ driven by the constitutive rpsM promoter (Fig. 5B). We also evaluated the HIP-1 promoter driving the red Dsred.T4 gene, which exhibits substantially less background than the GFP$^+$ constructs.[10] Quantitative evaluation of fluorescence induction following induction of tumor hypoxia yielded highly similar results to GFP$^+$. A representative illustration of the tumor colonized with attenuated *Salmonella* carrying the pepT-DsRed.T4 recombinant plasmid is shown in Figure 5C.

DISCUSSION

Success of gene therapy as a treatment for cancer will ultimately depend on the targeting of therapeutic gene expression specifically to the tumor site.[26-28] One of the most promising ways to accomplish this is by exploiting the lower oxygen levels found in tumors compared to normal tissues. This strategy of using tumor hypoxia has a second potential advantage, because hypoxic areas of the tumor are the most refractory to traditional types of cancer treatment. Most experimental tumors and a wide variety of clinical tumors are characterized by the presence of very low pO_2 values.[29] Even in small clinical metastases, relatively large a-vascular regions, in general constituting 25–75% of the tumor mass, are often present.[30] In this study, we demonstrate that the prokaryotic attenuated *Salmonella* gene delivery vector can

be genetically engineered to spatially control gene expression using a hypoxia-inducible promoter. Results were obtained with a fragment of the FNR-responsive pepT promoter. Analogous eukaryotic hypoxia-inducible gene expression vectors have been developed for cancer gene therapy, based on the presence of hypoxia response elements (HREs), with varying success.[31-33]

In order to be able to use a prokaryotic hypoxia-driven spatial level of gene expression, we evaluated the growth pattern of attenuated *Salmonella* and demonstrated that the hypoxic exponential growth and survival of attenuated *Salmonella* is identical to that under normoxia. Moreover, in the presence of sufficient nutrients continued proliferation under hypoxic conditions was possible. The plateau observed at lower cell density under hypoxic compared to normoxic conditions can thus most likely be attributed to increased loss of nutrients and not directly to the presence of hypoxia per se. This finding is highly relevant since the irregular tumor vasculature in vivo often fails to supply growing tumors with sufficient oxygen, resulting in areas of diffusion-limited chronic hypoxia. Thus, stable transgene expression over an extended period, as shown here, may be necessary for therapeutic application of the hypoxia-regulated promoter in vivo. Consistent with the necessity of hypoxia for activation of HIP-1, our in vivo data confined the HIP-1 driven signal to the tumor. Indeed, no signals in normal tissues could be detected when the reporter was driven off the hypoxia-inducible promoter. It may therefore be well anticipated that the use of a hypoxia-inducible promoter will eventually result in increased therapeutic ratio.

We subsequently sought to further fine-tune the specificity and potency of the HIP-1 hypoxia-responsive promoter. The specificity (relative hypoxia/normoxia expression) could be increased by engineering the FNR box to its consensus sequence. Our first attempt to increase the potency (absolute expression levels), was to increase the amount of FNR activator protein in order to activate all available FNR boxes present in our high copy plasmid reporter. Interestingly, we demonstrated that this resulted in an additional 2.5-fold induction upon hypoxia. In other words, it can be anticipated that integration of the therapeutic gene as a single copy in the bacterial host's chromosome, where the FNR-box can consequently be more readily activated, may lead to even higher induction factors. Although it has yet to be shown in this system, the efficacy of single copy gene expression in attenuated *Salmonella* has already been demonstrated in in vivo anti-tumor experiments.[10] In that context, we also showed that absolute expression levels can be enhanced by a factor of 4 by mutating the -10 TATA-box to its consensus sequence. Overall, these data show that engineering of the HIP-1 promoter can be used to maximize the inducibility and/or vary the absolute gene expression levels. This may be necessary to achieve optimal expression levels of the therapeutic gene.

We also conducted in vivo experiments to assess the hypoxia-inducibility of HIP-1 in combination with non-invasive imaging. Our results indicated that reporter protein levels originating from tumors colonized with constitutive rpsM-driven GFP-expressing attenuated *Salmonella* were similar, whereas they were variable across tumors when GFP expression was driven off the HIP-1 promoter. This finding was not surprising, since hypoxic levels can vary significantly from one tumor to another. In addition, mouse and human tumors are characterized not only by reduced overall oxygenation, but also by a very heterogeneous spatial distribution of oxygen. Tumor hypoxia is often characterized as "chordal' indicating the presence of hypoxia at the diffusion limit (typically 100 um) around every blood vessel.[34] Consequently, hypoxia exists throughout the entire tumor, but only in localized regions. Moreover, tumor hypoxia does not correlate well with tumor size. Thus, rather than attempting to correlate reporter signal with tumor size, or overall tumor oxygenation, we chose to evaluate the ability of the reporter to respond to a change in tumor oxygenation. This can be accomplished by administration of oxygen modifying drugs or by applying techniques such as tumor clamping to reduce blood flow.[35,36] However, the most straightforward and easiest approach involves sacrifice of the animal.

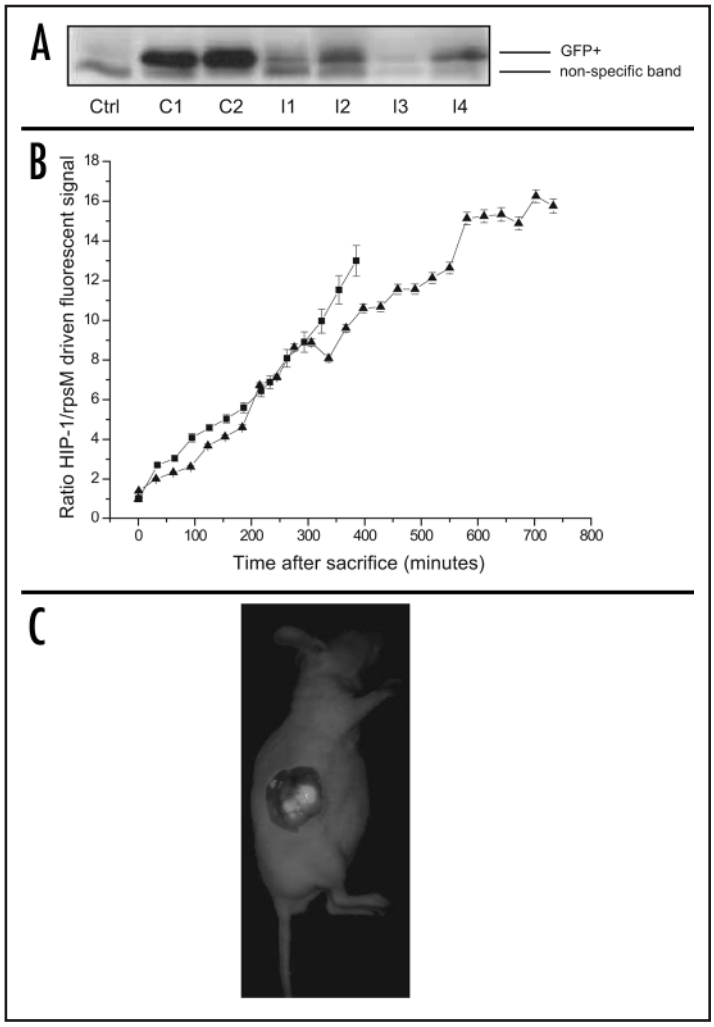

Figure 5. In vivo validation of HIP-1 promoter activity. (A) Western blot for GFP in cell extracts from HCT116 tumors from NMRI nu/nu mice injected i.v. 7 days before sacrifice with empty VNP20009 (Ctrl) or recombinant *Salmonella* expressing GFP driven by the constitutive rpsM promoter (indicated as C) or by the inducible HIP-1 (indicated as I) . 250µg of tumor cell extract was loaded on the gel. A tumor extract colonized with nonrecombinant VNP20009 was used as negative control. Different numbers represent extracts from independent tumors. (B) GFP+ (■) and DsRed.T4 (▲) reporter gene activity in *Salmonella*, carrying the HIP-1 inducible or the rpsM constitutive promoter, injected in colorectal tumor bearing NMRI-nu (nu/nu) mice. Normalized ratio of the HIP-1 to rpsM driven fluorescent reporter gene activity is shown as a function of time. Data represent results from two independent experiments for each reporter gene. Vertical bars represent the SEM. (C) Illustration of HIP-1 driven DsRed.T4 reporter gene expression 12 hours after sacrificing the animal. The fluorescent excitation light was provided by a 150 W halogen lamp, filtered for a wavelength of 500–555 nm. The fluorescent light emitted from the animal was detected by a charge-coupled device camera after filtering for wavelengths of 610 ± 23.5 nm. Depending on the fluorescence intensity, integration times ranged from 1.4 to 2.0 s.

The loss of blood flow results in the rapid development of anoxia throughout the tumor and ensures that all previous aerobic areas also are depleted of oxygen. Subsequent comparison of the induction pattern of reporter signals from the HIP-1 versus the constitutive rpsM promoter in time, validated the in vivo hypoxia-inducibility of the system. The lower in vivo induction levels as compared to the in vitro findings are most likely due to the fact that these tumors contain substantial areas of hypoxia prior to sacrifice. It is thus not possible to measure the basal (aerobic) gene expression levels. Despite this complication, however, the trend in the gene expression profile following hypoxic treatment was very similar to that observed in vitro.

In summary, our data show the potential for tumor-specific expression with supporting molecular biology with regards to FNR and engineered improvements of the promoter region in terms of specificity and potency. We provide a proof-of-principle that an inducible bacterial promoter system can be employed to target the hypoxic region of solid tumors. Since hypoxia has been shown to be present in most solid human tumors and to be a primary cause of treatment failure for conventional therapeutic agents, we believe that this approach will act in a complementary way to current radiotherapy and chemotherapy treatments, which preferentially kill well oxygenated cells. Therefore, the application of this hypoxia-driven targeting approach might be applicable to an extensive patient population.

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