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Construction and Characterization of Multiple Human Colon Cancer Cell Lines for Inducibly Regulated Gene Expression

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Abstract Validation of targets for cancer drug discovery requires robust experimental models. Systems based on inducible gene expression are well suited to this purpose but are difficult to establish in several epithelial cell types. Using the recently discovered transcriptional transactivator (rtTA2^S-M2), we developed a strategy for fast and efficient generation of Tet On cells. Multiple clones of HCT116, SW480, and HT29 human colon cancer cells for doxycycline-regulated gene expression were constructed that constitutively express green fluorescent protein (GFP) for selection/maintenance purposes. The cell lines displayed good fold inducibility (49–124× HCT116; 178–621× SW480; 261–787× HT29) and minimal leakiness after transient transfection with a luciferase reporter or with vectors driving inducible expression of red fluorescent protein (dsRed2), constitutively active c-Src or dominant negative K-Ras4B. The clones preserved their transformed phenotype as demonstrated by comparing their properties to respective wild type cells, in terms of growth in vitro and in vivo (as tumor xenografts), cell cycle traverse, and sensitivity to drugs used in chemotherapy. These engineered cell lines enabled tightly controlled inducible gene expression both in vitro and in vivo, and proved well suited for construction of double-stable cell lines inducibly expressing a protein of interest. As such they represent a useful research tool for example, to dissect oncogene function(s) in colon cancer. Supplementary material for this article be found at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/94/suppmat_welman.doc. J. Cell. Biochem. 94: 1148–1162, 2005.

Key words: colon cancer; Tet On; rtTA; doxycycline; rtTA2^S-M2; model system

Cancer is a complex genetic disease resulting from mutations leading to uncontrolled cell growth. It is believed that these mutations accumulate over time and cause abnormalities in function and interdependence of signaling cascades involved in control of normal cell behavior [Hanahan and Weinberg, 2000]. Transformed phenotypes have been associated with inhibition or activation of a number of signaling pathways. In the case of colorectal cancer, the best characterized changes involve mutations of APC, p53, K-Ras, and βcatenin, defects in mismatch repair genes, and up-regulation of the non-receptor tyrosine kinase c-Src [Kinzler and Vogelstein, 1996; Frame, 2002; Narayan and Roy, 2003]. The contribution of these and other changes to colon cancer progression is only partially understood. Elucidating the mechanisms essential for promotion and maintenance of transformed phenotype in colon cancer cells requires reliable, tightly controlled experimental models. Such models

Arkadiusz Welman and Christopher Cawthorne have contributed equally to this study.

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might help to define new drug targets for improved therapeutic intervention in colorectal cancer, the second highest cause of cancer related death in the west and where there is a pressing need for improved therapies [Gill et al., 2003].

Currently most of the studies on the mechanisms involved in colorectal carcinogenesis utilize either rodent models (e.g., the well established APC^{Min} mice model) or human colon cancer cells (grown in vitro or in vivo as xenografts) [Park and Gazdar, 1996; Heyer et al., 1999; Gayet et al., 2001]. Both approaches have advantages and disadvantages. Animal models provide the complexity of fully developed colonic environment, but rodent cells are known to differ from human cells with respect to the events required for transformation [Hahn and Weinberg, 2002; Rangarajan and Weinberg, 2003]. On the other hand, human colon cancer cells represent the true human genetic background but cannot be readily studied in proper colonic microenvironment. Surgical orthotopic implantation of human colon cancer cells into the colon of nude mice represents an attempt to combine the advantages of both approaches, however, it is technically difficult and at present its use is rather restricted [Hoffman, 1999].

In comparison to transgenic animals, the established colon cancer cell lines provide much more flexibility in functional studies on the roles of specific gene products in transformation and in response to chemotherapy. They are often well characterized and are amendable for genetic manipulations [Park and Gazdar, 1996; Gayet et al., 2001]. In many studies human colon cancer cell lines have been stably transfected with constructs driving constitutive expression of diverse genes of interest (e.g., [Windham et al., 2002; Sekharam et al., 2003; Wang et al., 2003]). These stably transfected cell lines have contributed significantly to our better understanding of colorectal cancer. Unfortunately, the results obtained using such cell lines might be compromised by spontaneously arising compensatory mechanisms to the effects of transgene product and/or by accumulation during the selection process of unspecific differences (genetic and epigenetic) between transgene-expressing cells and control cells.

The drawbacks of stable transfected cell lines constitutively expressing a given gene can be overcome by its inducible expression. Several inducible systems based on different principles have been described (reviewed in [Fussenegger, 2001]). Due to its properties, the Tet On version of the tetracycline-regulated system seems to be ideally suited for studies on gene function in human cancer cells [Berens and Hillen, 2003]. The Tet On system has been successfully used in a variety of cell lines, in diverse organs of transgenic animals and is currently validated for the purposes of human gene therapy (reviewed in [Corbel and Rossi, 2002; Zhu et al., 2002; Berens and Hillen, 2003]). It consists of two structural elements: (i) a cassette constitutively expressing a tetracycline-regulated reverse transcriptional transactivator (rtTA) and (ii) a responsive element encoding a gene of interest under the control of an inducible promoter activated by binding of the rtTA. Binding of the rtTA to the inducible promoter (and its activation) occurs only in the presence of tetracycline-analog doxycycline (Dox). Improvements and modifications to this system have been reported [Baron and Bujard, 2000; Corbel and Rossi, 2002; Zhu et al., 2002; Berens and Hillen, 2003], however, despite many potential benefits, establishing of doxycycline-regulated gene expression in human cancer cells often proves to be a difficult and time consuming task. Construction of such inducible models usually involves two consecutive steps: (i) in the first step a so called Tet On cell line stably transfected with rtTA expression cassette is generated (ii) in the second step, the Tet On cells are stably transfected with a responsive element containing the investigated gene under control of an inducible promoter [Gossen et al., 1995]. Alternative viral based single-step strategies do exist, however, they are not commonly used and posses several drawbacks (see "Discussion").

In order to facilitate studies on gene function in colon cancer, we concentrated on experimental approaches leading to doxycycline-regulated gene expression in a panel of human colon cancer cells with diverse genetic backgrounds. We developed a fast and 100% efficient strategy for construction of Tet On cell lines based on the chicken βactin promoter [Fregien and Davidson, 1986] and a recently discovered doxycycline-regulated transcriptional transactivator with improved characteristics ($rtTA2^{S}$-M2) [Urlinger et al., 2000]. Several human colon cancer Tet On cell lines have been created and characterized with respect to growth properties, cell cycle, and drug sensitivity. These cell lines

are suitable for inducible gene expression in vitro and in vivo (grown as xenografts) and for generation of double-stable cell lines inducibly expressing a gene of interest. Both, the methodology described and the established Tet On cell lines will facilitate the development of new inducible models for studies on gene function in human cancer cells.

MATERIALS AND METHODS

Plasmid Constructs and Adenoviruses

The pEGFP-N1 plasmid driving constitutive expression of EGFP under control of the cytomegalovirus (CMV) promoter was purchased from Clontech (Palo Alto, CA). The pN1pβactinEGFP plasmid that contains the βactin promoter instead of the CMV promoter was obtained by cloning the EGFP coding sequence into EcoRV-XbaI sites of the previously developed pN1pβactinEGFP(−) plasmid [Welman et al., 2000].

The mammalian expression vector pN1pβactin-IRES-EGFP was generated by recloning the IRES-EGFP module from the pI1G plasmid (gift of Dr. H. Albrecht, FMI, Basel) into the XbaI site of the pN1pβactinEGFP(−) vector [Welman et al., 2000]. The pN1pβactin-IRES-EGFPactin plasmid was constructed by replacing EGFP in the pN1pβactin-IRES-EGFP with the EGFP-actin fusion construct described by Hagmann et al. [1999]. The pN1pβactin-IRES-EGFP and pN1pβactin-IRES-EGFPactin plasmids were subsequently used to create the pN1pβactin-rtTA2^{S}-M2-IRES-EGFP and pN1pβactin-rtTA2^{S}-M2-IRES-EGFPactin vectors, which enable simultaneous expression of the rtTA2^{S}-M2 transcriptional transactivator and EGFP or EGFP-actin marker protein. This was achieved by cloning the rtTA2^{S}-M2 gene (PCR amplified from the pUHrT62-1 vector supplied by Prof. W. Hillen, University of Erlangen [Urlinger et al., 2000]) into the EcoRV cloning site located between the pβactin promoter and the IRES sequence.

The pTRE-Luc reporter vector encoding firefly luciferase under control of the Tet-dependent pTRE promoter and the pTKhygro plasmid used as the selection marker for generation of double stable cell lines were purchased from Clontech.

The pBILuc-Src527F and pBILuc-V12K-Ras4BΔt3aa plasmids expressing firefly luciferase and constitutively active mutant of c-Src (c-Src527F) or truncated (deletion of the C-terminal three amino acids), cytosolic version of the constitutively active mutant of K-Ras4B under control of the Tet-dependent bidirectional promoter were constructed using modified version of commercially available pBI vector (Clontech) and PCR amplified DNA fragments encoding c-Src527F, V12K-Ras4BΔ t3aa, and firefly luciferase. The pBIdsRed2 and pBIdsRed2-Src527F plasmids inducibly expressing red fluorescent protein alone (pBIdsRed2) or in combination with c-Src527F (pBIdsRed2-Src527F) were created in similar way.

The adenoviral vector pAd TRE β-gal encoding β-galactosidase under control of the Tet-inducible pTRE promoter was generated using pAd Easy system (Strategene, La Jolla, CA). The TRE β-galactosidase cassette from pTRE LacZ (Clontech) was inserted into pShuttle of the pAd Easy system. pShuttle TRE β-galactosidase and pAd Easy were cotransformed into BJ5183 competent cells (Strategene) to generate pAd TRE β-gal. The functional adenoviruses were produced by transfection of this plasmid into the HEK293 packaging cell line and purified using the BD Adeno-X Virus Purification kit (Clontech).

Cell Cultures and Transfections

HCT116, SW480, and HT29 cells were maintained in McCoys5A, DMEM and RPMI1640 media respectively (Gibco/Invitrogen, Paisley, UK). All the media were supplemented with 10% fetal calf serum, 100 u/ml penicillin, and 100 μg/ml streptomycin.

Transfections were performed by mixing 20 μg of plasmid DNA with $\sim 2 \times 10^7$ cells suspended in 400 μl of Optimem with Glutamax (Gibco; Cat. No. 51985-026) in a 4 mm electroporation cuvette (Equibio, Cat. No. ECU104, Ashford, UK) and subsequent electroporation (simple pulse, 260 V, 1,050 μF) using EasyjecT Plus electroporator (Equibio).

Selection of Stable Cell Lines and Microscopy

The stable Tet On cell populations were generated by selection of cells transfected with pN1pβactin-rtTA2^{S}-M2-IRES-EGFP or pN1pβactin-rtTA2^{S}-M2-IRES-EGFPactin in medium containing 600 μg/ml G418 (Gibco) for 20 days, followed by flow cytometric sorting of GFP-positive cells. Single cell clones were picked using sterile cotton buds after low density seeding in Petri dishes and expanded to establish independent Tet On cell lines.

Double stable cell lines expressing c-Src527F and/or luciferase under control of Dox responsive pBI promoter were constructed by cotransfection of Tet On clones with pTKhygro and pBILuc-Src527F or pBILuc (vector identical to pBILuc-Src527F but lacking the Src527F gene), and subsequent selection in the presence of 150–200 μg/ml of hygromycin (Roche Molecular Biochemicals, Mannheim, Germany) for 16 days. Single cell clones were picked using sterile cotton buds, expanded and screened by polyacrylamide gel electrophoresis and Western blotting or by luciferase assay.

For live cell imaging cells grown on glass coverslips were mounted in purpose-build observation chamber and images were captured using a cooled CCD camera and METAMORPH Imaging Software (Universal Imaging Media, PA), as described [Hagmann et al., 1998].

Doxycycline Treatment and Luciferase Assay

Cells were seeded in 6-well plates (Corning, Corning, NY) at densities leading to 40–60% confluency 24 h later, when the medium was exchanged for the fresh medium containing (induction) or not (control) 2 μg/ml doxycycline (Clontech). After the next 24 h cells were washed with PBS, harvested and analyzed by polyacrylamide gel electrophoresis and Western blotting or by luciferase assay.

Luciferase assays were performed using Luciferase Assay System (Promega, Cat. No. E1500, Madison, WI) according to the manufacturer's instructions.

Subcellular Fractionations

Cells transfected with pBILuc-V12K-Ras4BΔ t3aa were seeded in 10 cm Petri dishes at densities leading to 40%–60% confluency 24 h later. They were treated for 24 h with doxycycline (no dox in controls), washed with cold PBS, resuspended in 1 ml of ice-cold hypotonic buffer (10 mM HEPES pH 7.5; 10 mM NaCl; 1 mM $MgCl_2$; 1 mM $CaCl_2$; 1 mM KCl) containing protease inhibitors cocktail (Sigma, Poole, UK), incubated on ice for 15 min and homogenized by 25 passages through a $25G^5/_8$ (0.5×16) needle. After centrifugation for 5 min at 500*g*, 900 μl of the postnuclear supernatant was centrifuged for 30 min at 120,000*g*. The supernatant (soluble fraction) was quantitatively transferred to another tube and the pellet (membraneous fraction) resuspended in 200 μl of hypotonic buffer. Appropriate volumes of 5× SDS-sample buffer were added to each fraction. Equal proportions of each fraction were analyzed by polyacrylamide gel electrophoresis and Western blotting.

Antibodies, Polyacrylamide Gel Electrophoresis, and Western Blotting

SDS–PAGE was performed using 10% polyacrylamide gel. Proteins were blotted onto PVDF membranes (Perkin-Elmer, Boston, MA), incubated with the appropriate first antibody (monoclonal mouse anti v-Src; Oncogene Research Products, Cat. No. OP07 (Nottingham, UK), polyclonal rabbit anti Src[pY^{418}]; Biosource, Cat. No. 44-660 (Camarillo, CA) or monoclonal rat anti Ras; Oncogene Research Products, Cat. No. OP01) and a horseradish peroxidase-coupled anti-mouse, anti-rabbit, or anti-rat secondary IgG (Dako, Glostrop, Denmark). This was followed by detection with the Western Lightning Chemiluminescence Reagent Plus from Perkin-Elmer.

In Vitro Growth and Analysis of S-Phase by BrdU Incorporation

To determine growth curves, cells were seeded at the density 1×10^5/well in 6-well plates and counted daily for a period of 6 days using a hemocytometer.

For cell cycle analysis cells were seeded at 7.89×10^6 per T75 tissue culture flask and pulse-labeled for 20 min at 37°C with 1 μM 5-bromo-2′-deoxyuridine (5-BrdU, Sigma) on day 4 (logarithmic phase of growth). They were subsequently trypsinised, and prepared for flow cytometry as described by Terry and White [2001]. Flow cytometry was performed on a Becton Dickinson FACSVantage cytometer (Becton Dickinson, San Jose, CA) using the 488 nm line of the Enterprise laser (Coherent, Palo Alto, CA) set to excite at 250 mW. Thirty-thousand cells were analyzed per sample. Forward and orthogonal light scatter were collected together with green fluorescence (FITC conjugated antibody; 530 ± 30 nm, log scale) and red fluorescence (DNA-bound propidium iodide; 630 ± 22 nm, linear scale). Cell clumps were excluded by fluorescence pulse processing.

MTT Assay

To determine the effect of cytotoxic drugs on selected cell lines a semi-automated cytotoxicity assay was performed. We employed a method originally described by Mosmann [1983].

Briefly, 100 μl aliquots of 1×10^3cells/well were plated in 96-well microplates and incubated overnight. On the next day 100 μl of medium containing various concentrations of drugs were added to each well and the cells were incubated for another 72 h. Subsequently, 50 μl of 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (=MTT, Sigma) was added to each well and the plates were returned to the incubator for 3 h. This was followed by aspiration of the medium and excess MTT, and solubilization of formed formazan precipitate in 100 μl of DMSO. The plates were then read using a multiscan microplate reader (Titretech/Flow Laboratories, Irvine, UK) at 540 nm with subtraction at 620 nm to allow for turbidity. The percentage growth inhibition (relative to control wells) and IC50 values (concentration of compound required to inhibit 50% growth) were calculated. 5-Fluorouracil (5-FU) was obtained from Sigma and Oxaliplatin from Alexis Biochemicals, Nottingam, UK.

Tumor Xenografts

The HCT116, SW480, HT29 wild type cells and selected Tet On clones were grown as subcutaneous xenografts in 8-week-old female CD-1 nude mice following intradermal injection of 5×10^6 cells in 0.1 ml of media 1 cm from the tail base on the midline. Mice were housed in an individually ventilated caging system on a 12 h light/12 h dark environment maintained at constant temperature and humidity. They were fed a standard irradiated diet and allowed water ad libitum. At the size 600–800 mm^3 tumors were excised and inspected under dissecting microscope or analyzed after H&E staining. All procedures were carried out in accordance with the Scientific Procedures Act 1986 and in line with the UKCCCR guidelines 1999, by approved protocols (Home Office Project License numbers 40/1770 and 40/2328).

Adenoviral Infections and β-Galactosidase Assay

The Tet-responsive adenovirus vector (pAd TRE β-gal) was delivered by the intra-tumoral injection of 10^8 pfu in a 50 μl volume when tumors reached ~200 mm^3. The animals in the "induced" group were given doxycycline in drinking water (2 mg/ml in 1% sucrose solution) from 48 h prior adenovirus injection until the end of the experiment 48 h after injection (the "control" group received drinking water with 1% sucrose). The animals were subsequently sacrificed, tumors were excised and snap frozen in liquid nitrogen.

For β-galactosidase assay tumor pieces were homogenized in passive lysis buffer (Promega). After sonification and centrifugation for 30 min at 13,000*g*, 100 μl of the resulting supernatant was mixed with 245 μl of PBS. Subsequently, 100 μl of substrate (4 mg/ml *o*-nitrophenyl-β-D-galactopyranoside) and 45 μl of assay reagent (10 mM $MgCl_2$, 0.45M β-marcapthoethanol) were added and the samples were left on ice for 5 min. This was followed by incubation at 37°C for 1 h. The reaction was terminated by addition of 510 μl of 1M Na_2CO_3 and OD at 420 nm was measured. In controls 100 μl of lysis buffer was used instead of tumor lysate. The results were normalized for protein content determined using 10 μl of the starting lysate.

RESULTS

In human Colon Cancer Cells the βactin Promoter Is Better Suited for Long-Term Transgene Expression Than the CMV Promoter

Viral promoters are frequently used to establish cell lines stably transfected with a gene of interest. Recent evidence, however, indicates that in human cancer cells strong eukaryotic promoters might be better suited for long-term transgene expression than promoters of viral origin [Gopalkrishnan et al., 1999; Teschendorf et al., 2002]. We directly compared the ability of the chicken βactin promoter and the CMV promoter to drive constitutive expression of GFP in three commonly used human colorectal cancer cell lines. As shown in Figure 1, upon transfection with analogous vectors driving GFP transcription under control of either CMV or βactin promoter and 14 days of selection in the presence of the neomycin analog G418, the amount of GFP positive cells was approximately 2–3 fold higher in cell populations transfected using the vector containing the βactin promoter. This result indicates that employing βactin promoter increases the success rate of long-term transgene expression in human colon cancer cells compared to the more widely used CMV promoter.

An Efficient Method for the Generation of Human Colon Cancer Tet On Cells

We combined the advantages of the chicken βactin promoter [Fregien and Davidson, 1986],

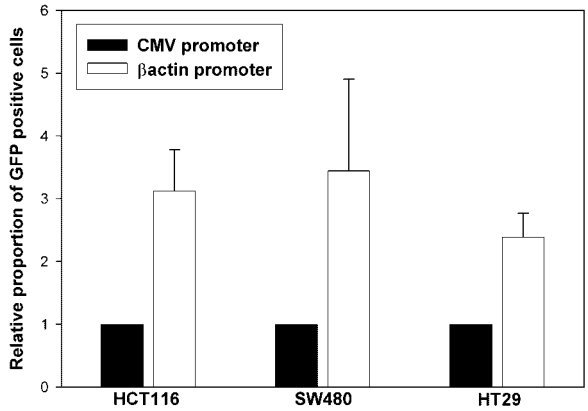

Fig. 1. Comparison of the efficiency of cytomegalovirus (CMV) and βactin promoters in supporting prolonged transgene expression in selected human colon cancer cell lines. Indicated cells were electroporated with analogously built plasmids driving constitutive expression of GFP under control of either CMV or βactin promoter (pEGFP-N1 plasmid or pN1pβactinEGFP plasmid, respectively). The cells were selected for 14 days in the presence of G418 and the proportions of GFP positive cells were estimated using fluorescent microscope. The results are presented relative to CMV promoter (arbitrary 1), for each vector they are based on four selections performed after two independent electroporations.

a recently developed improved version of doxycycline-regulated reverse transcriptional transactivator (rtTA2^S-M2) [Urlinger et al., 2000], the IRES sequence of the encephalomyocarditis virus [Jang and Wimmer, 1990], and the EGFP marker protein [Cubitt et al., 1995] to construct pN1pβactin-rtTA2^S-M2-IRES-EGFP, a vector constitutively expressing rtTA2^S-M2 along with EGFP (Fig. 2). In addition to the listed elements this plasmid contains a kanamycin/neomycin resistance cassette allowing its propagation in bacteria in the presence of kanamycin and selection of the transfected mammalian cells in the presence of G418. This vector was used to transfect a panel of human colon cancer cell lines: HCT116, SW480, and HT29. The transfected cells were first selected in the presence of G418 and subsequently sorted by flow cytometry to isolate GFP positive cell populations. Single cell clones have been established from those populations and (upon transfection with a reporter vector encoding luciferase under control of a tetracycline inducible promoter) tested for their ability to induce gene expression in the presence of Dox. All the clones screened were positive. The inducibility differed between the cell lines and between the clones within a given cell line, and was in the range from 4.95×10^1 to 7.87×10^2 (see supplementary Tables 1, 2, and 3). We subsequently

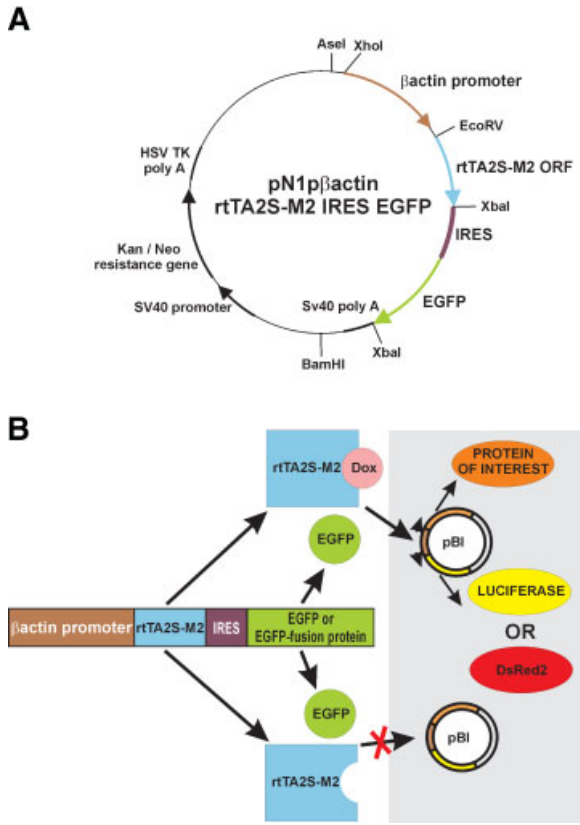

Fig. 2. Schematic of the pN1pβactin-rtTA2^S-M2-IRES-EGFP plasmid (**A**) and functional principles of the strategy used (**B**). Plasmid map shows individual component elements of the vector including the rtTA2^S-M2 ORF, βactin promoter, and the kanamycin/neomycin resistance gene. The shadowed box in (B) represents the constructs introduced into the cells after previous stable transfection with pN1pβactin-rtTA2^S-M2-IRES-EGFP or pN1pβactin-rtTA2^S-M2-IRES-EGFPactin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

decided to test if the EGFP within the pN1pβactin-rtTA2^S-M2-IRES-EGFP vector could be substituted with an EGFP-fusion protein without affecting vector potency for generation of Tet On cell lines. Use of GFP-fusion constructs enables real-time live-cell studies of many different proteins. The ability to combine inducible expression of one protein of interest with the use of another protein of interest constitutively expressed as EGFP-fusion chimera would be of scientific benefit. For that purpose pN1pβactin-rtTA2^S-M2-IRES-EGFPactin plasmid encoding EGFP-actin fusion protein instead of EGFP alone was constructed. This construct was used to generate a panel of stable transfected clones in the SW480 cell background. When electroporated with a luciferase reporter plasmid, all the clones displayed Dox

driven inducibility in the range similar to the SW480 cells transfected with pN1pβactin-rtTA2^{S}-M2-IRES-EGFP vector (supplementary table 4). The actin architecture of these clones was readily visible under fluorescent microscope providing the evidence that combination of Dox-regulated expression of one gene with simultaneous observation of another gene expressed as EGFP-fusion protein is possible (Fig. 3). Importantly the expression of rtTA2^{S}-M2 and EGFP/EGFP-actin in all the established Tet On cell lines lasted for months in the absence of selection and no decrease in Dox mediated inducibility was seen over time (data not shown).

In vitro Characterization of Generated Human Colon Cancer Tet On Cell Lines and Development of a Vector for dsRed2-Based Live Visualization of Inducible Gene Expression at a Single Cell Level

Three Tet On clones for each of the cell lines have been selected for further characterization on the basis of their superior inducibility and minimal residual leakiness (Fig. 3 and supplementary Tables 1–4). First, we checked if the inducibility detected in the enzymatic luciferase assays could be seen at the protein level with proteins other than firefly luciferase. For that purpose, two bicistronic vectors were generated: pBILuc-Src527F and pBILuc-V12K-Ras4BΔt3aa encoding luciferase and either a constitutively active mutant of c-Src (Src527F) or a dominant negative, cytosolic mutant of K-Ras4B (V12K-Ras4BΔt3aa) under control of a Dox-regulated bidirectional promoter (see "Materials and Methods"). As demonstrated in Figure 4A,B, these plasmids were able to trigger efficient production of respective proteins when transiently transfected into SW480AWE53 cells. Western blots with appropriate antibodies failed to detect any leakiness in the absence of Dox although the more sensitive luciferase assays indicated some (very low) degree of leakiness (Fig. 4A,B). Comparable results have been obtained with other Tet On clones tested (data not shown).

Luciferase activity linked to inducible expression of a gene of interest provides a well-defined and very sensitive read-out, however, in transiently transfected cells it does not enable functional studies to be carried out at the single cell level. To overcome this problem we constructed an additional bicistronic vector pBIdsRed2 in which Dox-regulated expression of a gene of interest can be directly coupled to expression of red fluorescent protein allowing easy microscopic identification of live cells in which the transgene has been successfully induced. We tested this vector using Src527F mutant. Figure 4C demonstrates

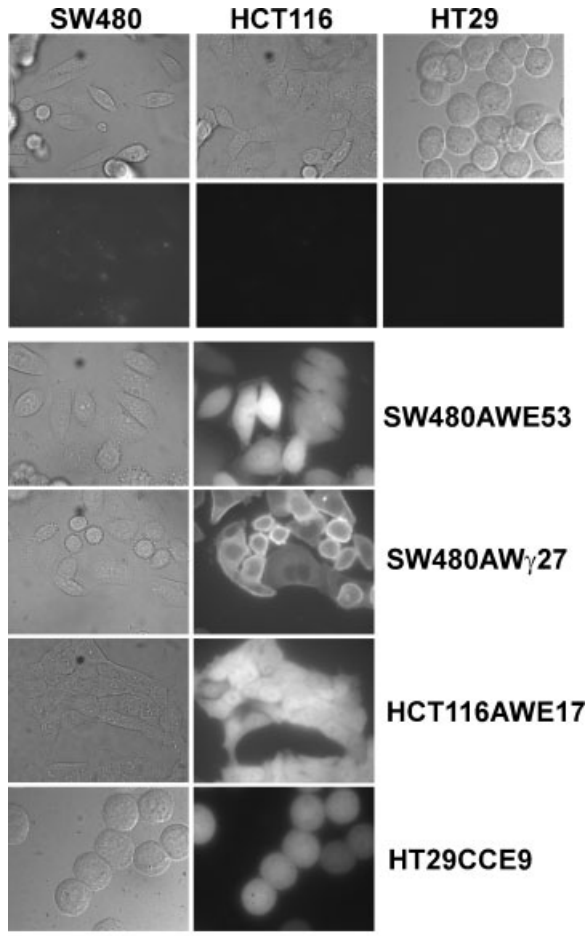

Fig. 3. Representative data showing the morphology, fluorescent properties, and inducibility of selected Tet On clones compared to respective wild type cells. Bright field and fluorescent images of SW480, HCT116, HT29, SW480AWE53, SW480AWγ27, HCT116AWE17 and HT29CCE9 cells (**top**) and inducibility of indicated clones determined after transient transfection with pTRE Luc reporter plasmid (**bottom**).

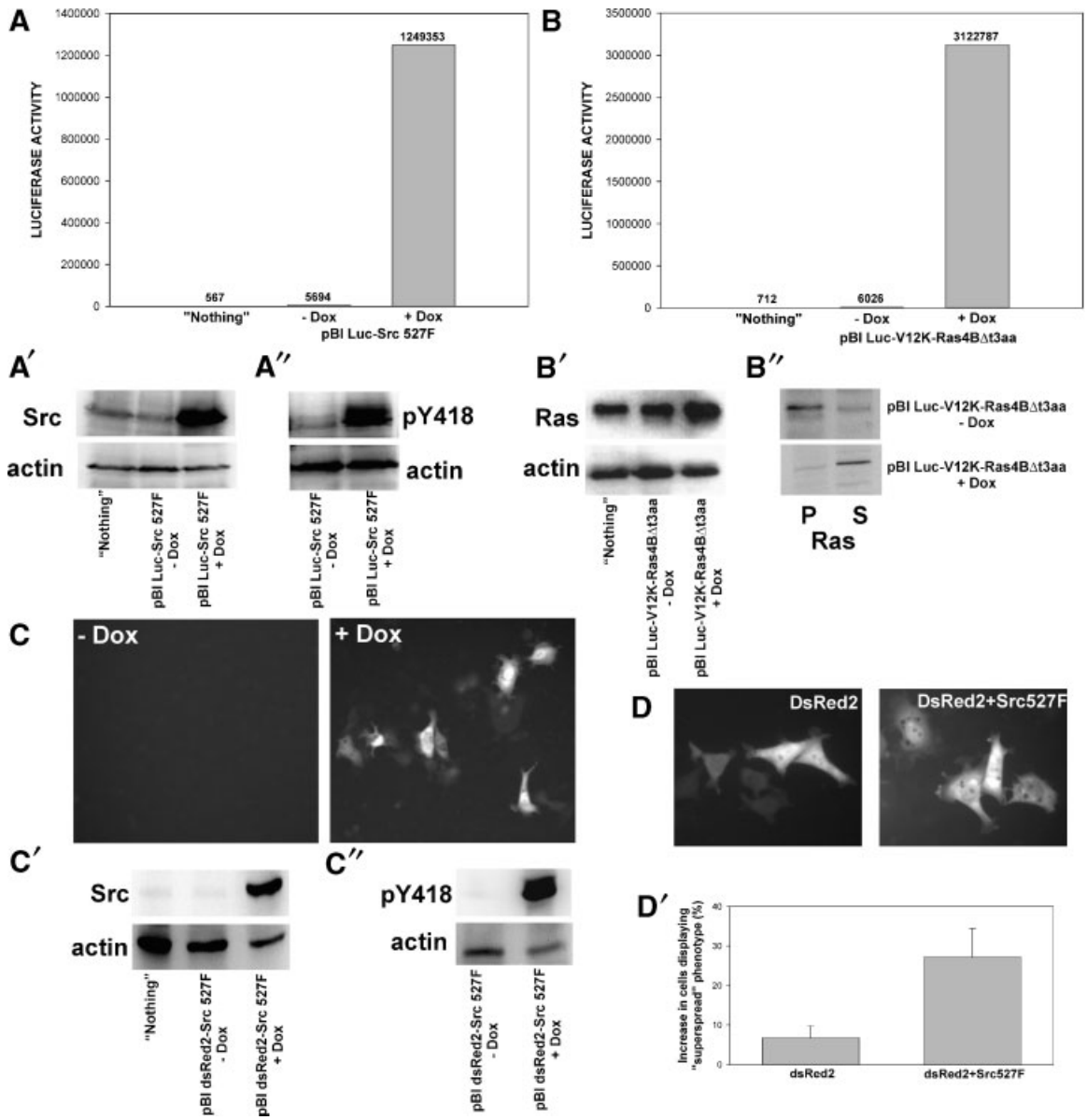

Fig. 4. Inducible co-expression of firefly luciferase or dsRed2 with constitutively active mutant of c-Src or dominant negative cytosolic mutant of K-Ras4B in SW480AWE53 cells. The cells were transfected with pBI Luc-Src527F plasmid, treated with Dox, and subjected to luciferase assay (**A**) or Western blotted for total Src (**A′**) and activated Src (pY418 antibody) (**A″**). Alternatively, the cells were transfected with pBILuc-V12K-Ras4BΔ t3aa plasmid, treated with Dox and subjected to luciferase assay (**B**) or Western blotted for Ras in total cell lysate (**B′**) or membraneous and soluble fractions (**B″**). The cells transfected with pBIdsRed2-Src527F plasmid were grown in the presence or absence of Dox and photographed under fluorescent microscope (**C**) or Western blotted for total Src (**C′**) and activated Src (**C″**). Actin blots represent loading controls. The data are representative of at least two independent experiments. The consequences of inducible expression of Src527F on morphology of SW480AWE53 cells were determined at the single cell level after transfection with pBIdsRed2-Src527F or pBIdsRed2, and doxycycline treatment. **Panel D** illustrates the morphology of typical dsRed2 positive cells transfected with pBIdsRed2 plasmid and ''superspread'' cells transfected with pBIdsRed2-Src527F plasmid. The increase in percentage of cells displaying ''superspread'' morphology after induction of dsRed2 or dsRed2 + Src527F is shown in (**D′**), the neighboring untransfected cells were used as a reference point. Data are based on cell counts after three independent electroporations.

that SW480AWE53 cells electroporated with pBIdsRed2-Src527F (a plasmid driving simultaneous inducible expression of dsRed2 and Src527F) displayed very good inducibility in the presence and undetectable background expression levels in the absence of Dox. The pBIdsRed2-Src527F vector was subsequently employed to study the consequences of inducible overexpression of Src527F in SW480AWE53 cells. As shown in Figure 4D, triggering

Src527F production by adding doxycycline to the cell culture medium resulted in an increased number of cells displaying a "superspread" phenotype. Many of these cells also possessed large, dsRed2 free vesicular structures (Fig. 4D and data not shown). These results suggest that in SW480 colorectal cancer cells c-Src signaling might be involved in regulation of cell adhesion and vesicle trafficking/formation.

We next characterized growth properties and drug sensitivity of selected Tet On cell lines. As shown in Figure 5A, their growth patterns were overall similar to the growth patterns of respective parental cell lines. Differences in growth curves were observed only between the wild type SW480 cells and the SW480AWE Tet On clones. The SW480 cells represent a very heterogeneous cell line consisting of cells of various sizes and morphologies with the potency to acquire mesenchymal-like or epithelial phenotypes [Barker and Clevers, 2001]. The noted diversity in the growth curves of SW480AWE clones and wild type SW480 cells most likely reflects this heterogeneity. This is supported by the fact that the characterized SW480AWE53, AWE21 and AWE74 clones all have similar "flat" phenotype and reach confluency faster than wild type SW480 cells (Fig. 3 and data not shown). The analysis of BrdU labeled cells demonstrated that the cell cycle distribution of the generated Tet On clones did not differ from their wild type counterparts (Fig. 5C). Similarly, the established Tet On clones and wild type cells showed comparable properties with respect to their sensitivity to drugs used in colon cancer chemotherapy: 5-FU (Fig. 5B) and oxaliplatin (data not shown).

Characterization of Constructed Tet On Cells In Vivo

Genetic manipulations combined with prolonged selection process might cause unspecific changes to cellular behavior. Although our experiments in vitro failed to detect any major differences between the generated Tet On clones and the parental cell lines, they did not exclude the possibility that these cells had lost their tumorigenic potential in vivo. To rule out that possibility, we injected cells from the clone of each cell line with the highest inducibility (HCT116AWE17, SW480AWE53, and HT29CCE9) subcutaneously into nude mice. The injected cells were able to grow and form tumors within similar time frame to the respective wild type cells (Fig. 6A–C). The cellular architecture of these tumors resembled that of tumors formed by wild type cells (data not shown). In contrast to wild type xenografts, the Tet On tumors displayed strong green fluorescence when placed under a dissecting microscope fitted with GFP optics (Fig. 6D). They were also able to support inducible gene expression in the presence of Dox when injected with adenovirus encoding β-galactosidase under control of a Dox-regulated promoter (Fig. 7). These results confirmed the in vivo functionality of the established Tet On clones.

Utility of Described Colon Cancer Tet On Cells for Generation of Double-Stable Cell Lines Inducibly Expressing a Gene of Interest

Construction of Tet On cells represents the first step in generation of double-stable cell lines for doxycycline-regulated expression of a gene of interest. In order to verify whether our Tet On clones are suitable for construction of such double-stable cell lines we transfected a few of them with vectors encoding constitutively active mutant of c-Src (c-Src527F) and/or luciferase under control of Dox responsive pBI promoter. As exemplified for SW480AWE53 clone in Figure 8, multiple stable cell lines expressing c-Src527F and/or luciferase in the Dox dependent manner could be established (their properties will be reported elsewhere). This result confirmed that the described human colon cancer Tet On clones represent a valuable tool for generation of cell lines inducibly expressing a gene of interest.

DISCUSSION

We have developed a high throughput approach for construction of stable human cancer Tet On cell lines for doxycycline-regulated gene expression. This approach overcomes many of the problems encountered during generation of such cells in the past (including elaborate screenings for positive clones, low frequency of truly positive clones after selection process, instability and low inducibility of established Tet On cell lines) and enables easy construction of unrestricted amounts of positive Tet On clones displaying a broad range of inducibility within a relatively short period of time. Importantly, cell lines established using this method preserve the transformed phenotype of parental wild type cells and are able to form tumors in vivo (Figs. 5 and 6).

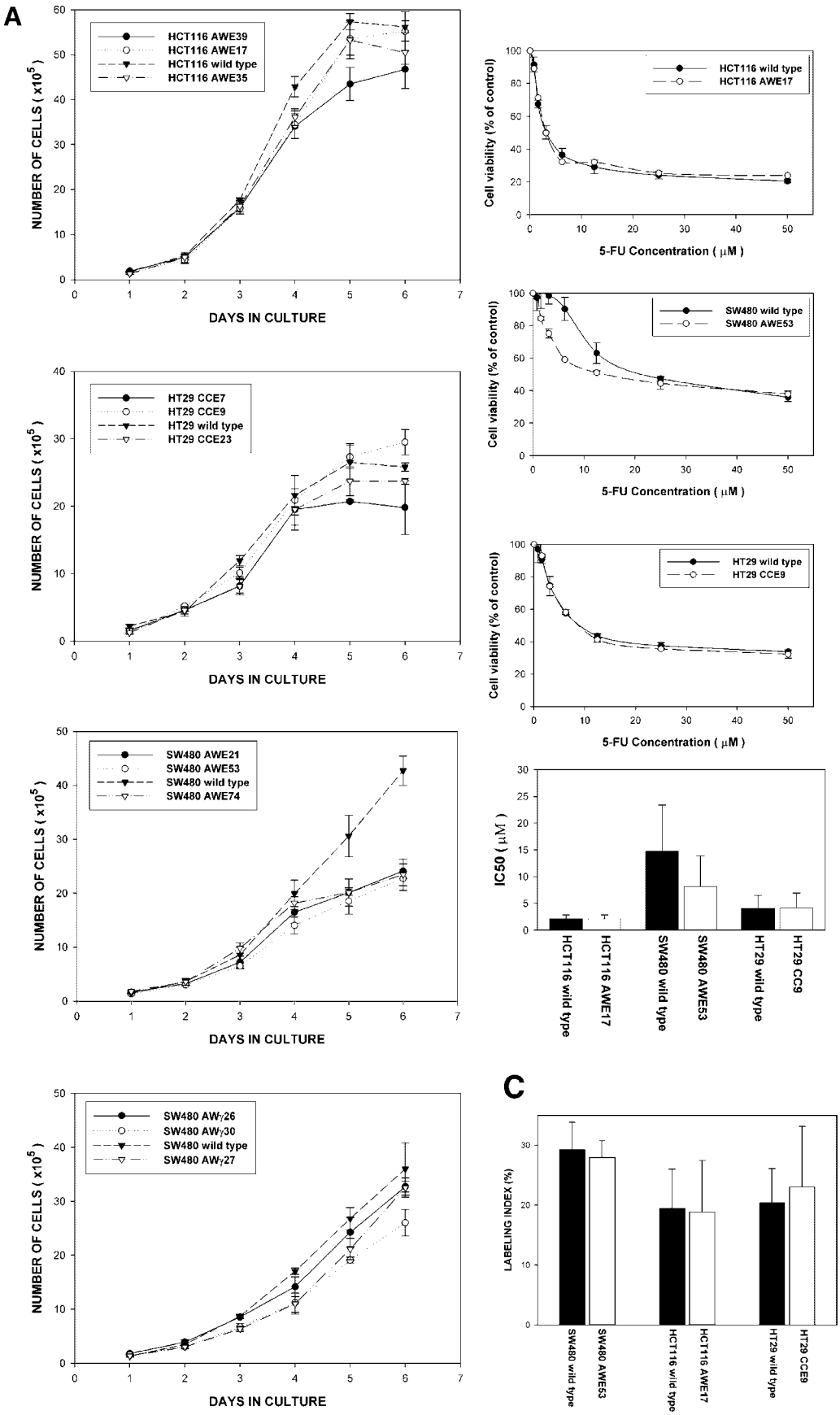
A
B
C
HCT116 AWE39
HCT116 AWE17
HCT116 wild type
HCT116 AWE35
NUMBER OF CELLS (x10^5)
DAYS IN CULTURE
HT29 CCE7
HT29 CCE9
HT29 wild type
HT29 CCE23
SW480 AWE21
SW480 AWE53
SW480 wild type
SW480 AWE74
SW480 AWγ26
SW480 AWγ30
SW480 wild type
SW480 AWγ27
Cell viability (% of control)
5-FU Concentration (μM)
SW480 wild type
SW480 AWE53
HT29 wild type
HT29 CCE9
IC50 (μM)
HCT116 wild type
HCT116 AWE17
SW480 wild type
SW480 AWE53
HT29 wild type
HT29 CC9
LABELING INDEX (%)
SW480 wild type
SW480 AWE53
HCT116 wild type
HCT116 AWE17
HT29 wild type
HT29 CCE9

Fig. 5.

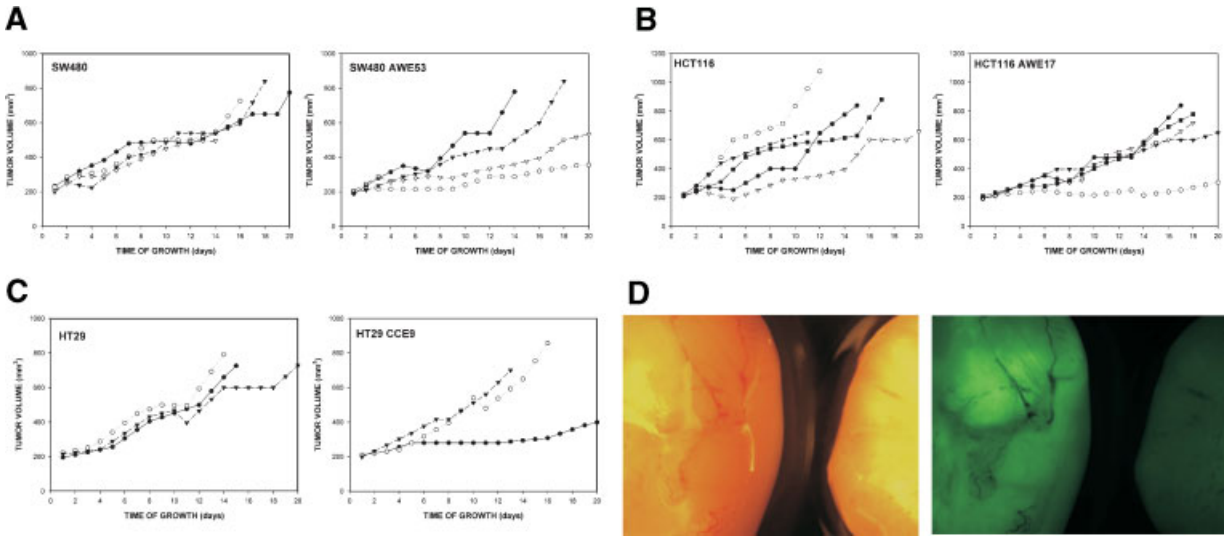

Fig. 6. In vivo properties of selected Tet On clones. Growth characteristics of xenografts derived from SW480AWE53 (**A**), HCT116AWE17 (**B**), HT29CCE9 (**C**) clones and respective wild type cells (A, B, C) (each line represents an independently grown tumor from the point it reached 200 mm^3). **D**: Bright field and fluorescent images of tumors formed by HCT116AWE17 (left) and wild type HCT116 cells (right).

The system is based on the chicken βactin promoter [Fregien and Davidson, 1986] and a recently discovered, improved version of Dox-dependent transcriptional transactivator ($rtTA2^S$-M2) [Urlinger et al., 2000] coupled to EGFP marker protein [Cubitt et al., 1995] via the IRES sequence of the encephalomyocarditis virus [Jang and Wimmer, 1990]. Combination of these elements seems to be a key factor for high efficiency of the strategy described. In the past generation of Tet On cells relied mainly on the use of strong viral promoters [Gossen et al., 1995]. Recently it has been realized that non-viral promoters might be better suited for this purpose due to lower cytotoxicity and lower levels of epigenetic silencing in transfected mammalian cells. For example Gopalkrishnan et al. [1999] directly compared the utility of the CMV promoter and human EF1α promoter for driving stable expression of rtTA in human melanoma cell line HO-1. Whereas no stable Tet On clones could be obtained after transfection with the CMV promoter based constructs, about 25% of cells selected after transfection with constructs utilizing EF1α promoter were positive [Gopalkrishnan et al., 1999]. Inability to support continual strong expression from promoters of viral origin has been documented in several other cell types including human colon cancer cells [Gorman et al., 1985; Sleigh, 1987; Hasegawa et al., 1990; Li et al., 1992; Miller and Rizzino, 1995; Teschendorf et al., 2002]. The βactin promoter represents one of the strongest non-viral promoters available and drives gene expression in a broad range of diverse cell types and tissues [Fregien and Davidson, 1986; Xu et al., 2001]. It is well tolerated by established cell lines, primary cell cultures, and in vivo in transgenic animals [Fregien and Davidson, 1986; Fischer et al., 1998; Kosuga et al., 2000; Ramezani et al., 2000; Xu et al., 2001; Chung et al., 2002; Roelandse et al., 2003]. It proved to be able to drive high and stable levels of expression of different transgenes over prolonged periods of time in diverse experimental systems [Fischer et al., 1998; Kosuga et al., 2000; Ramezani et al., 2000; Chung et al., 2002; Roelandse et al., 2003]. Our results comparing the efficiency of the CMV promoter and βactin promoter in supporting long-term GFP expres-

Fig. 5. In vitro properties of selected Tet On clones. **A**: Growth curves of indicated HCT116AWE, HT29CCE, SW480AWE, and SW480AWγ clones compared to respective wild type cells (representative of two independent experiments performed in quadruplicate). **B**: Sensitivity of wild type cells and indicated Tet On clones to 5-FU. Results of representative MTT assays performed in triplicates (**top**) and histogram comparing calculated IC50 values (based on three independent experiments performed in triplicates) (**bottom**). **C**: Comparison of the BrdU labeling index of parental cell lines and SW480AWE53, HCT116AWE17, and HT29CC9 clones (data based on three independent experiments).

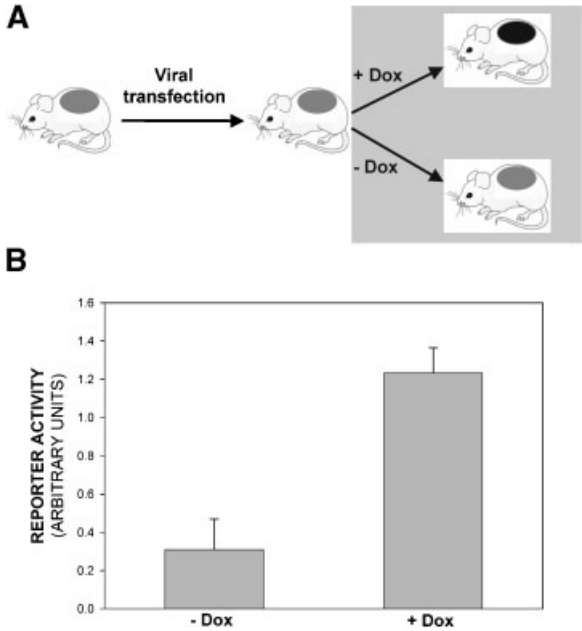

Fig. 7. Virus-mediated inducible expression of β-galactosidase by HT29CCE9 cells in vivo. **A**: Schematic of the experiment. **B**: β-galactosidase activity in xenografts derived from doxycycline treated animals (+Dox) and untreated control mice (−Dox). There were three animals per group.

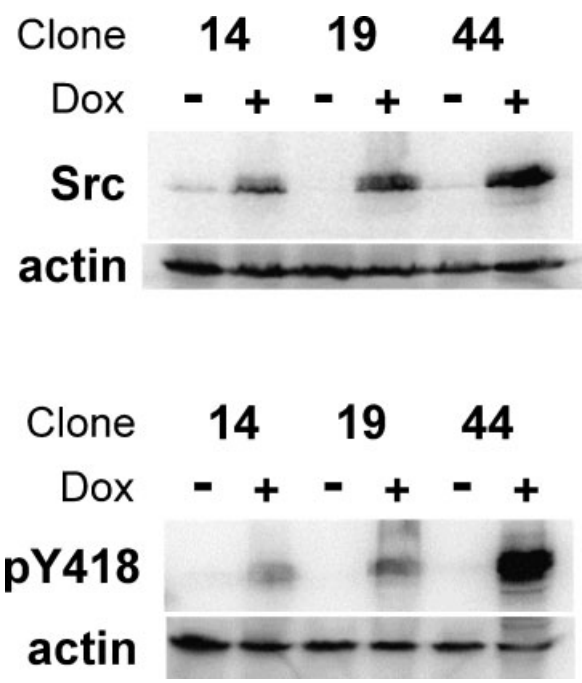

Fig. 8. Inducible expression of c-Src527F in SW480AWE53 cells stably transfected with pBI Luc-Src527F (double-stable SW480 cells). Indicated clones were grown for 24 h in the presence or absence of Dox, harvested, and Western blotted for total Src (**top**) or activated Src (pY418 antibody, **bottom**). Actin blots represent loading controls.

sion (Fig. 1) are certainly in agreement with the idea that eukaryotic promoters might be better suited than viral promoters for prolonged transgene expression in mammalian systems. The use of the improved mutant of the Dox-regulated reverse transcriptional transactivator (rtTA2^S-M2) constitutes another important aspect of the strategy described. This new mutant has significantly enhanced stability in human cells compared to the earlier developed and commonly used rtTA transactivator [Urlinger et al., 2000]. Additionally, rtTA2^S-M2 displays better sensitivity to doxycycline and shows more favorable kinetics of inducible gene expression (including lower leakiness) both in vitro and in vivo [Urlinger et al., 2000; Salucci et al., 2002; Koponen et al., 2003]. Coupling rtTA2^S-M2 to EGFP via the IRES sequence enables easy selection of positive clones and subsequent monitoring of the cells for maintenance purposes, a feature very useful in human cancer cells where genetic instability and chromosomal rearrangements are common [Gisselsson, 2003]. As demonstrated in the "Results" section, there is a 100% correlation between expression of EGFP and potential for Dox driven inducible gene expression (supplementary material, Tables 1–4 available at http://www.mrw.interscience.wiley.com/suppmat/0730-2312/suppmat/94/suppmat_welman.doc). This confirms previous observations showing a very high success rate of IRES based selection principles [Rossi et al., 1998; Izumi and Gilbert, 1999; Knott et al., 2002; Qu et al., 2004]. The green fluorescence of the stable cell lines enables their easy identification in vitro (for example in mixed cultures with other cell types) and non-invasive imaging of Tet On tumors in vivo (data not shown). Importantly, we have shown that in our system an EGFP-fusion protein can be used instead of EGFP alone. This opens the exciting possibility of combining live cell imaging of one protein of interest expressed as an EGFP-fusion construct with simultaneous inducible expression of another protein of interest. Considering the number of functional GFP-fusion constructs described in the literature, this approach might prove very attractive for future studies [van Roessel and Brand, 2002]. Although the work presented has concentrated on human colon cancer cells we have the evidence that the method can be successfully applied to other cell types (unpublished observations).

The developed Tet On clones represent a very useful tool for studies on gene function(s) in colon cancer. They are well suited for short term experiments based on transient transfection (Fig. 4) and for the construction of double-stable cell lines constitutively able to inducibly express a gene of interest (Fig. 8). Although single-step methods for generation of double-stable cell lines based on multi-cistronic virus-mediated gene delivery have been described [Hofmann et al., 1996; Chtarto et al., 2003; Koponen et al., 2003], those systems often utilize viral promoters prone to possible shutdown in human cancer cells as discussed above. In addition construction of a virus is usually time consuming and frequently requires increased safety levels. Moreover, viral vectors are often very large and contain virus specific genetic elements that might affect the basic characteristics of the cells established using those approaches as compared to their wild type progenitors. We demonstrated that the Tet On cell lines established using our plasmid-based strategy are stable in terms of rtTA2^{S}-M2 and EGFP expression. They are also similar to their parental cell lines in terms of growth properties in vitro and in vivo, cell cycle traverse and drug resistance—a prerequisite of a good inducible cancer model system. Few other human colon cancer cell lines for doxycycline-regulated gene expression have been described by others in the past, however, they represent single clones rather than series of independently generated clones [Kobayashi et al., 2000; Dietz et al., 2002; van de Wetering et al., 2002]. In addition, the majority of them have not been fully characterized with respect to tumorigenicity and functionality in vivo [Kobayashi et al., 2000; Dietz et al., 2002; van de Wetering et al., 2002]. Considering the fact that the HCT116, SW480, and HT29 cells represent human colon cancer cell lines with different morphological and molecular characteristics it might be expected that the panel of Tet On cell lines described here will contribute to better understanding of the molecular pathology of human colon cancer.

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REFERENCES

Barker N, Clevers H. 2001. Tumor environment: A potent driving force in colorectal cancer? Trends Mol Med 7:535–537.

Baron U, Bujard H. 2000. Tet repressor-based system for regulated gene expression in eukaryotic cells: Principles and advances. Methods Enzymol 327:401–421.

Berens C, Hillen W. 2003. Gene regulation by tetracyclines. Constraints of resistance regulation in bacteria shape TetR for application in eukaryotes. Eur J Biochem 270:3109–3121.

Chtarto A, Bender HU, Hanemann CO, Kemp T, Lehtonen E, Levivier M, Brotchi J, Velu T, Tenenbaum L. 2003. Tetracycline-inducible transgene expression mediated by a single AAV vector. Gene Ther 10:84–94.

Chung S, Andersson T, Sonntag KC, Bjorklund L, Isacson O, Kim KS. 2002. Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. Stem Cells 20:139–145.

Corbel SY, Rossi FM. 2002. Latest developments and in vivo use of the Tet system: Ex vivo and in vivo delivery of tetracycline-regulated genes. Curr Opin Biotechnol 13: 448–452.

Cubitt AB, Heim R, Adams SR, Boyd AE, Gross LA, Tsien RY. 1995. Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 20:448–455.

Dietz S, Rother K, Bamberger C, Schmale H, Mossner J, Engeland K. 2002. Differential regulation of transcription and induction of programmed cell death by human p53-family members p63 and p73. FEBS Lett 525: 93–99.

Fischer M, Kaech S, Knutti D, Matus A. 1998. Rapid actin-based plasticity in dendritic spines. Neuron 20:847–854.

Frame MC. 2002. Src in cancer: Deregulation and consequences for cell behaviour. Biochim Biophys Acta 1602: 114–130.

Fregien N, Davidson N. 1986. Activating elements in the promoter region of the chicken beta-actin gene. Gene 48:1–11.

Fussenegger M. 2001. The impact of mammalian gene regulation concepts on functional genomic research, metabolic engineering, and advanced gene therapies. Biotechnol Prog 17:1–51.

Gayet J, Zhou XP, Duval A, Rolland S, Hoang JM, Cottu P, Hamelin R. 2001. Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene 20:5025–5032.

Gill S, Thomas RR, Goldberg RM. 2003. Review article: Colorectal cancer chemotherapy. Aliment Pharmacol Ther 18:683–692.

Gisselsson D. 2003. Chromosome instability in cancer: How, when, and why? Adv Cancer Res 87:1–29.

Gopalkrishnan RV, Christiansen KA, Goldstein NI, DePinho RA, Fisher PB. 1999. Use of the human EF-1alpha promoter for expression can significantly increase success in establishing stable cell lines with consistent expression: A study using the tetracycline-inducible system in human cancer cells. Nucleic Acids Res 27: 4775–4782.

Gorman CM, Rigby PW, Lane DP. 1985. Negative regulation of viral enhancers in undifferentiated embryonic stem cells. Cell 42:519–526.

Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. 1995. Transcriptional activation by tetracyclines in mammalian cells. Science 268:1766–1769.

Hagmann J, Grob M, Welman A, van Willigen G, Burger MM. 1998. Recruitment of the LIM protein hic-5 to focal contacts of human platelets. J Cell Sci 111:2181–2188.

Hagmann J, Burger MM, Dagan D. 1999. Regulation of plasma membrane blebbing by the cytoskeleton. J Cell Biochem 73:488–499.

Hahn WC, Weinberg RA. 2002. Modelling the molecular circuitry of cancer. Nat Rev Cancer 2:331–341.

Hanahan D, Weinberg RA. 2000. The hallmarks of cancer. Cell 100:57–70.

Hasegawa T, Nakada S, Nakajima T, Oda K, Kawata M, Kimura H, Sekiya S. 1990. Expression of various viral and cellular enhancer-promoters during differentiation of human embryonal carcinoma cells. Differentiation 42:191–198.

Heyer J, Yang K, Lipkin M, Edelmann W, Kucherlapati R. 1999. Mouse models for colorectal cancer. Oncogene 18: 5325–5333.

Hoffman RM. 1999. Orthotopic metastatic mouse models for anticancer drug discovery and evaluation: A bridge to the clinic. Invest New Drugs 17:343–359.

Hofmann A, Nolan GP, Blau HM. 1996. Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette. Proc Natl Acad Sci USA 93: 5185–5190.

Izumi M, Gilbert DM. 1999. Homogeneous tetracycline-regulatable gene expression in mammalian fibroblasts. J Cell Biochem 76:280–289.

Jang SK, Wimmer E. 1990. Cap-independent translation of encephalomyocarditis virus RNA: Structural elements of the internal ribosomal entry site and involvement of a cellular 57-kD RNA-binding protein. Genes Dev 4:1560–1572.

Kinzler KW, Vogelstein B. 1996. Lessons from hereditary colorectal cancer. Cell 87:159–170.

Knott A, Garke K, Urlinger S, Guthmann J, Muller Y, Thellmann M, Hillen W. 2002. Tetracycline-dependent gene regulation: Combinations of transregulators yield a variety of expression windows. Biotechniques 32:796–806.

Kobayashi T, Sawa H, Morikawa J, Zhang W, Shiku H. 2000. Bax induction activates apoptotic cascade via mitochondrial cytochrome c release and Bax overexpression enhances apoptosis induced by chemotherapeutic agents in DLD-1 colon cancer cells. Jpn J Cancer Res 91: 1264–1268.

Koponen JK, Kankkonen H, Kannasto J, Wirth T, Hillen W, Bujard H, Yla-Herttuala S. 2003. Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression in vitro and in vivo. Gene Ther 10:459–466.

Kosuga M, Enosawa S, Li XK, Suzuki S, Matsuo N, Yamada M, Roy-Chowdhury J, Koiwai O, Okuyama T. 2000. Strong, long-term transgene expression in rat liver using chicken beta-actin promoter associated with cytomegalovirus immediate-early enhancer (CAG promoter). Cell Transplant 9:675–680.

Li M, Hantzopoulos PA, Banerjee D, Zhao SC, Schweitzer BI, Gilboa E, Bertino JR. 1992. Comparison of the expression of a mutant dihydrofolate reductase under control of different internal promoters in retroviral vectors. Hum Gene Ther 3:381–390.

Miller K, Rizzino A. 1995. The function of inducible promoter systems in F9 embryonal carcinoma cells. Exp Cell Res 218:144–150.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63.

Narayan S, Roy D. 2003. Role of APC and DNA mismatch repair genes in the development of colorectal cancers. Mol Cancer 2:41.

Park JG, Gazdar AF. 1996. Biology of colorectal and gastric cancer cell lines. J Cell Biochem Suppl 24:131–141.

Qu Z, Thottassery JV, Van Ginkel S, Manuvakhova M, Westbrook L, Roland-Lazenby C, Hays S, Kern FG. 2004. Homogeneity and long-term stability of tetracycline-regulated gene expression with low basal activity by using the rtTA2S-M2 transactivator and insulator-flanked reporter vectors. Gene 327:61–73.

Ramezani A, Hawley TS, Hawley RG. 2000. Lentiviral vectors for enhanced gene expression in human hematopoietic cells. Mol Ther 2:458–469.

Rangarajan A, Weinberg RA. 2003. Opinion: Comparative biology of mouse versus human cells: Modelling human cancer in mice. Nat Rev Cancer 3:952–959.

Roelandse M, Welman A, Wagner U, Hagmann J, Matus A. 2003. Focal motility determines the geometry of dendritic spines. Neuroscience 121:39–49.

Rossi FM, Guicherit OM, Spicher A, Kringstein AM, Fatyol K, Blakely BT, Blau HM. 1998. Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16. Nat Genet 20:389–393.

Salucci V, Scarito A, Aurisicchio L, Lamartina S, Nicolaus G, Giampaoli S, Gonzalez-Paz O, Toniatti C, Bujard H, Hillen W, Ciliberto G, Palombo F. 2002. Tight control of gene expression by a helper-dependent adenovirus vector carrying the rtTA2(s)-M2 tetracycline transactivator and repressor system. Gene Ther 9:1415–1421.

Sekharam M, Zhao H, Sun M, Fang Q, Zhang Q, Yuan Z, Dan HC, Boulware D, Cheng JQ, Coppola D. 2003. Insulin-like growth factor 1 receptor enhances invasion and induces resistance to apoptosis of colon cancer cells through the Akt/Bcl-x(L) pathway. Cancer Res 63:7708–7716.

Sleigh MJ. 1987. Differential regulation of viral and cellular genes in F9 mouse embryonal carcinoma cells. Nucleic Acids Res 15:9379–9395.

Terry NH, White RA. 2001. Cell cycle kinetics estimated by analysis of bromodeoxyuridine incorporation. Methods Cell Biol 63:355–374.

Teschendorf C, Warrington KH, Jr., Siemann DW, Muzyczka N. 2002. Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. Anticancer Res 22:3325–3330.

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci USA 97:7963–7968.

van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H. 2002. The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111: 241–250.

van Roessel P, Brand AH. 2002. Imaging into the future: Visualizing gene expression and protein interactions with fluorescent proteins. Nat Cell Biol 4:E15–E20.

Wang WS, Chen PM, Hsiao HL, Ju SY, Su Y. 2003. Overexpression of the thymosin beta-4 gene is associated with malignant progression of SW480 colon cancer cells. Oncogene 22:3297–3306.

Welman A, Burger MM, Hagmann J. 2000. Structure and function of the C-terminal hypervariable region of K-Ras4B in plasma membrane targetting and transformation. Oncogene 19:4582–4591.

Windham TC, Parikh NU, Siwak DR, Summy JM, McConkey DJ, Kraker AJ, Gallick GE. 2002. Src activation regulates anoikis in human colon tumor cell lines. Oncogene 21:7797–7807.

Xu R, Janson CG, Mastakov M, Lawlor P, Young D, Mouravlev A, Fitzsimons H, Choi KL, Ma H, Dragunow M, Leone P, Chen Q, Dicker B, During MJ. 2001. Quantitative comparison of expression with adeno-associated virus (AAV-2) brain-specific gene cassettes. Gene Ther 8:1323–1332.

Zhu Z, Zheng T, Lee CG, Homer RJ, Elias JA. 2002. Tetracycline-controlled transcriptional regulation systems: Advances and application in transgenic animal modeling. Semin Cell Dev Biol 13:121–128.