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Transcriptionally Targeted Nonviral Gene Transfer Using a β-Catenin/TCF-Dependent Promoter in a Series of Different Human Low Passage Colon Cancer Cells

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Abstract: Nonviral transfections of six low passage human colon cancer cell lines using the artificial β-catenin/TCF-dependent promoter CTP4 demonstrated a high promoter activity which was 1000- to 70000-fold higher than in HeLa control cells. Luciferase gene expression levels obtained with CTP4 in epithelial-like tumor cell cultures were only slightly lower than with the strong viral CMV promoter/enhancer, whereas in less differentiated tumor cultures CTP4 expression levels exceeded the CMV expression levels up to 28-fold. Three cell lines representing different morphology typical of the original tumors, more differentiated epithelial-like (COGA-5), piled-up (COGA-12), and poorly differentiated rounded-up (COGA-3), were selected for further investigation. Gene transfer was optimized using lipopolyplex formulation of cationic lipid DOSPER and polycation PEI25br. Lipopolyplexes enabled up to 1300-fold or 400-fold higher luciferase expression compared to the corresponding lipoplexes or polyplexes, respectively. Lipopolyfection of an interleukin-2 (IL-2) gene expression construct driven by the CTP4 promoter resulted in very high levels of up to 95 ng of secreted IL-2 per 10^5 cells and 24 h. The lipopolyplexes were also able to transfect multicellular spheroids that mimic the three-dimensional structure of real tumors.

Keywords: Wnt signaling pathway; β-catenin; colorectal cancer; nonviral gene therapy; lipopolyfection; multicellular spheroids

Introduction

Colorectal cancer is one of the most frequent cancers in the western hemisphere.[1] The standard treatment of this disease is surgery complemented by chemotherapeutic drugs.[2] However, despite advances in therapeutic strategies, the median five-year survival from colon cancer still remains unsatisfactory. Gene therapy represents a new approach to cancer therapy, which could provide a powerful alternative to conventional treatment.[3] Nonviral gene transfer using either cationic lipids (lipoplexes) or cationic polymers (polyplexes) has been widely used for gene delivery both in vitro and in vivo.[4−6] However, despite significant improve-

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ments in lipoplex and polyplex formulation, many obstacles must still be overcome[7−10] for efficient and target-cell specific transfection to be achieved in vivo.

Tumor specific gene expression can be achieved by tumor specific transcription mediated through specific promoters (so-called "transcriptional targeting").[11] A common problem with such tumor specific promoters, however, is that they typically generate low levels of gene product and thus limited scope for therapeutic effects.[12] Lipinski and co-workers[13] recently developed a synthetic tumor specific promoter, "CTP4", that is both highly tumor specific and highly active (i.e., comparable in activity to the human cytomegalovirus immediate early enhancer/promoter) in a range of tumor cell lines with constitutively activated Wnt signaling.[13] The key structural feature of this promoter is that it contains ten binding sites for the HMG box protein T cell factor (Tcf4) and is activated in cells containing Tcf4 and nuclear β-catenin. The latter protein is very tightly regulated in normal cells. In a range of tumors, however, in particular colorectal tumors, mutations in the adenomatous polyposis coli (APC) tumor suppressor gene result in nuclear accumulation of β-catenin through the failure of APC-mediated degradation.[14] Accumulation of β-catenin can also result from mutations within the β-catenin gene itself.

In the initial characterization of the CTP4 promoter, the poorly differentiated SW480 cell line was the only colorectal cancer cell line evaluated.[13] In the current work, to evaluate the characteristics of CTP4 on a broader range of relevant colorectal tumors, we extended the studies by using a recently established panel of low passage human colon cancer cell lines that still closely resemble the phenotypes of their corresponding original tumor cells.[15]

We report on the activity of the promoter CTP4 in six low passage colon cancer cell lines which exhibit heterogeneous properties and morphologies.[15] Two of these lines exhibited epithelial-like morphology (COGA-1, COGA-5,), two lines exhibited piled-up morphology (COGA-5L, COGA-12), and another two cell lines exhibited a rounded-up morphology (COGA-2, COGA-3). These cell lines retain the morphologies characteristic of the respective original tumor cells and are characterized for a range of mutations, for example, those known to mediate oncogenic and tumor-suppressive effects.[15] For control purposes, two established cell lines, HeLa (noncolorectal) and the standard colorectal cell line SW480, were used in our investigation. Gene transfer was performed using newly optimized DOSPER/PEI lipopolyplexes[16] and was compared with the results for the corresponding lipoplexes and polyplexes. Finally, we investigated the efficiency of these transcriptionally targeted lipopolyplexes in the transfection of multicellular spheroids.

Experimental Section

Transfection Reagents and Chemicals. The plasmids pCMV-Luc ,[17] pCTP4-Luc ,[13] pEGFP-N1, pEGFP-LG-CTP4, pGShIL-2tet,[18,19] and pCTP4-hIL-2 were used for transfection. pEGFP-N1 was purchased from Clontech Laboratories, Inc. (Palo Alto, CA). pEGFP-LG-CTP4 was constructed by substitution of the CMV promoter of pEGFP-N1 by the CTP4 promoter from pCTP4-Luc.[13] First, a fragment containing the CMV promoter was removed from pEGFP-N1 by cleaving the plasmid with *Ase*I and *Eco*47III. The

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cohesive ends originating from the digestion by *Ase*I were converted to blunt ends by Klenow polymerase prior to religation of the vector, leading to the plasmid pEGFP-N1-0. Finally a *Sac*I−*Bgl*II digested fragment of the pCTP4-Luc plasmid, containing the CTP4 promoter, was inserted into the *Sac*I−*Bam*HI restriction sites of the MCS of the pEGFP-N1-0 plasmid. pCTP4-hIL-2 was constructed by replacement of the luciferase gene of pCTP4-Luc against the hIL-2 gene from pGShIL-2tet. The luciferase gene was removed by digestion with *Bgl*II and *Bam*HI, and the *Bgl*II−*Not*I digested hIL-2 gene from pGShIL-2tet was inserted into the dephosphorylated vector. Both the vector and the insert were converted to blunt ends prior to insertion using Klenow polymerase. All enzymes were obtained from Promega (Mannheim, Germany). DOSPER (1,3-dioleoyloxy-2-(6-carboxy-spermyl)-propylamid) was obtained from Roche (Mannheim, Germany). Branched PEI (PEI25br) with an average molecular weight of 25 kDa was obtained from Sigma-Aldrich (Vienna, Austria) and was used as a 1 mg/mL stock solution, neutralized with HCl. Cell culture media, antibiotics, and fetal calf serum (FCS) were purchased either from Invitrogen (Karlsruhe, Germany) or Gibco BRL (Eggenstein, Germany). The human IL-2 ELISA kit was purchased from Bender MedSystems (Vienna, Austria).

Cell Cultures. All cultured cells were grown at 37 °C in a 5% CO_2 humidified atmosphere. HeLa (ATCC CCL-2, cervix epithelial adenocarcinoma, noncolorectal cells) and SW480 (ATCC CCL-228, human colorectal adenocarcinoma cells with APC mutation) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% serum. Human low passage colon carcinoma cells COGA-1, COGA-2, COGA-3, COGA-5, COGA-5L, and COGA-12[15] were cultured in RPMI 1640 medium containing 10% serum. All cells were seeded in 96-well, 24-well, or 12-well plates 24 h prior to gene transfer using $1-2 \times 10^4$ cells (96-well) or $1-2 \times 10^5$ cells (24- and 12-well) per well, respectively.

Multicellular Spheroid Culture. Multicellular spheroids were generated as previously described,[20] using the liquid overlay technique. Briefly, 24-well culture plates (Nalge Nunc International, Naperville, IL) were coated with 300 μL of 1% SeaPlague agarose (Biozym, Hess, Germany) in serum-free growth medium. Cells from a single-cell suspension were added at 10^5 cells per well in a total volume of 1 mL of growth medium with 2% serum. Multicellular spheroids were allowed to form over 48 h or 96 h.

Formation of Transfection Complexes. Lipopolyplexes were prepared as described before.[16] First, plasmid DNA was diluted in HEPES-buffered saline (HBS) at DNA concentrations ranging from 2.5 to 20 μg/mL. Immediately, PEI25br was added at optimized N/P (describes the molar ratio of PEI nitrogen/DNA phosphate) ratio of 8/1[16] and incubated at room temperature for 5−10 min. DOSPER was diluted in a similar manner in a separate tube, added to the DNA/PEI25br precomplexes at the optimized w/w ratio of DOSPER/DNA of 8/1,[16] and incubated at room temperature for 30−40 min.

Lipoplexes were prepared as described before.[21] In brief, cationic lipid DOSPER and plasmid DNA were diluted at the same ratio as described above in HBS in separate tubes, mixed together, and incubated for 30−40 min at room temperature. Polyplexes were prepared in the same way[22] at the same molar ratio as described above.

Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) containing complexes was prepared according to the manufacturer's instructions. Briefly, plasmid DNA and Lipofectamine 2000 at a DNA (in μg)/Lipofectamine 2000 (in μL) ratio of 2:3 were diluted in separate tubes in Opti-MEM (Invitrogen, Karlsruhe, Germany) at a DNA concentration of 20 μg/mL, mixed together, and incubated for 30 min at room temperature.

Gene Transfer to Monolayer Cultures. For the luciferase assays $(1-2) \times 10^4$ cells were seeded in 96-well plates, and for the IL-2 detection $(1-2) \times 10^5$ cells were seeded in 24-well or 12-well plates. For analysis of enhanced green fluorescent protein (EGFP), $(1-2) \times 10^5$ cells were seeded in 12-well plates 24 h prior transfection. The growth medium was removed and replaced with 50 μL (96-well), 200 μL (24-well), or 400 μL (12-well) of serum-free medium in the case where Lipofectamine 2000 was used for transfection or medium containing 10% FCS in the cases where lipopolyplexes, lipoplexes, or polyplexes were used. Transfection complexes (96-well, 20 μL, 0.1 μg of plasmid DNA; 24-well, 50 μL, 0.25−1 μg of DNA; 12-well, 100 μL, 0.25−1 μg of DNA) were then added dropwise to each well. Four hours following incubation at 37 °C/5% CO_2, transfection medium was replaced by 100 μL (96-well), 600 μL (24-well), or 1 mL (12-well) of fresh growth medium. Gene transfer was performed in triplicate of at least two independent experiments.

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Gene Transfer to Multicellular Spheroids. Multicellular spheroids were grown in 1 mL of medium containing 2% serum. Transfection was performed directly in the growth medium to avoid disturbance of the spheroids. Forty-eight hours after spheroid formation 700 μL of growth medium was removed from each well and lipopolyplex formulation, diluted in a small volume (50 μL), was added to the multicellular spheroid cultures. Transfection medium was not exchanged after transfection.

Luciferase Assay. Twenty-four hours following gene transfer, medium was removed and the cells were washed with phosphate-buffered saline. The cells were then lysed with 50 μL of lysis buffer containing 25 mM tris(hydroxymethyl)aminomethane, pH 7.8, 2 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol, 10% glycerol, and 1% Triton X-100, and 30 min later the luciferase activity was measured using a Lumat LB9507 instrument (Berthold, Bad Wildbad, Germany) as described recently.[23] In brief, luciferase light units were recorded from an aliquot of the cell lysate with 10 s integration after automatic injection of freshly prepared luciferin using the luciferase assay system (Promega, Mannheim, Germany). Luciferase activity was measured in triplicate, and the relative light units (RLU) were determined per 1×10^4 cells. 10^7 light units corresponds to 2 ng of recombinant luciferase (Promega, Mannheim, Germany).

Flow Cytometric Analysis of EGFP Expression. Forty-eight hours after transfection cells were harvested after incubation with trypsin/EDTA solution and kept on ice until analysis. The DNA stain propidium iodide (PI) (Sigma-Aldrich, Taufkirchen, Germany) was added to the cell suspension at 1 μg/mL to discriminate between viable and dead cells. The number of dead cells and EGFP-positive cells was quantified using a Cyan MLE flow cytometer (Dako-Cytomation, Copenhagen, Denmark). PI and EGFP fluorescence were excited at 488 nm. Emission of PI fluorescence was detected using a 613 $\pm$ 20 nm bandpass filter. Dead cells were excluded by gating PI-positive cells by forward scatter versus PI fluorescence. Emission of EGFP was detected using a 530 $\pm$ 40 nm bandpass filter and a 613 $\pm$ 20 nm bandpass filter to analyze EGFP-positive cells by diagonal gating.[24] To exclude cell debris and doublets, cells were appropriately gated by forward versus side scatter and pulse width, and 2×10^4 gated events per sample were collected.

Epifluorescence Microscopy. Living or 4% PFA (paraformaldehyde) fixed cell imaging of EGFP expressing cells was performed 48 h after transfection using an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss Axiocam camera. Light was collected through a 5×0.12 or a 10×0.25 numerical aperture objective (Carl Zeiss, Jena, Germany). EGFP fluorescence was excited using a 470 $\pm$ 20 nm bandpass filter, and emission was collected using a 540 $\pm$ 25 nm bandpass filter. Digital image recording and image analysis were performed with the Axiovision 3.1 software (Carl Zeiss, Jena, Germany).

Confocal Laser Scanning Microscopy of Multicellular Spheroids. Multicellular spheroids were transferred to Lab-Tek 8 chambered coverglasses (Nalge Nunc International, Naperville, IL) and fixed in 4% PFA for 30−60 min. For counterstaining cells were incubated with DAPI (4′,6′-diamidino-2-phenylindole) at a concentration of 1 μg/mL in phosphate-buffered saline (PBS) for 15 min. Imaging of EGFP expression of transfected multicellular spheroids was performed 48 h after transfection using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss, Jena, Germany) equipped with a UV and an argon laser delivering light at 364 and 488 nm, respectively. Light was collected through a 10×0.3 NA objective (Carl Zeiss, Jena, Germany). DAPI fluorescence was excited with the 364 nm line; emission was collected using a 385 nm long-pass filter. Excitation of EGFP fluorescence was achieved by using the 488 nm line, with the resulting fluorescent wavelengths observed using a 505 nm long-pass filter. No signal overspill between the individual fluorescence channels was observed. An optical section thickness of 10 μm was chosen. Digital image recording and image analysis were performed with the LSM 5 software, version 3.0 (Carl Zeiss, Jena, Germany).

Cryosections of Multicellular Spheroids. Multicellular spheroids were transferred to a 48-well plate prior to fixation in 4% PFA for 2 h at 4 °C. Subsequently the multicellular spheroids were incubated in 30% sucrose (in water) over night at 4 °C. The fixed multicellular spheroids were then embedded in tissue freezing medium (Leica Microsystems, Nussloch, Germany) and frozen at −20 °C. Cryosections were made using a Leica CM3050S cryostat (Leica Microsystems, Nussloch, Germany) with a section thickness of 10 μm. Sections were transferred to SuperFrost microscope slides (Menzel, Braunschweig, Germany) prior to analysis by epifluorescence microscopy.

Human IL-2 ELISA. At 24 h after transfection growth medium was replaced by fresh medium. At 48 h after transfection the supernatants were collected and stored at −80 °C until IL-2 ELISA was performed. Human IL-2 expression was determined using a human IL-2 ELISA kit (Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions.

Results

Gene Expression Efficiency of the CTP4 Promoter in a Panel of Low Passage Human Colon Cancer Cell Lines. The relative activities of the tissue-specific CTP4 promoter and the strong, constitutively active viral CMV promoter were compared in six low passage colon cancer cell lines by transient transfection experiments (Figure 1) using the luciferase gene expression plasmids pCTP4-Luc and pCMV-

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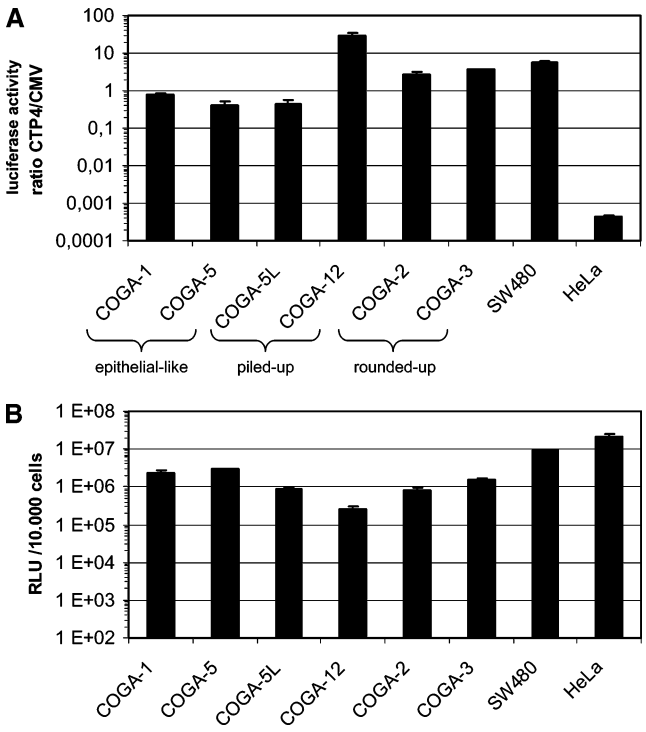

Figure 1. Ratios of luciferase expression by the CTP4 promoter in comparison to the CMV immediate early enhancer/promoter in low passage colon cancer cell lines of different phenotypic morphologies (A). COGA-1 and COGA-5 cells are representatives for epithelial-like morphology, COGA-5L and COGA-12 cells for piled-up morphology, and COGA-2 and COGA-3 cells for rounded-up morphology. Colorectal SW480 cells and noncolorectal HeLa cells were used as controls. pCMV-Luc or pCTP4-Luc (0.05 μg per 10^4 cells) were used for transfection using Lipofectamine 2000. The luciferase activities obtained by the CMV promoter are shown in (B). The values are representative means of triplicates of at least two independent experiments.

Luc and Lipofectamine 2000 transfection reagent. These cell lines exhibit various morphologies characteristic of the respective original tumor-derived cultures and contain a range of oncogenic and tumor-suppressive mutations.[15] SW480 and HeLa cells were used as CTP4-permissive and nonpermissive control cells, respectively. Figure 1A shows CTP4 promoter-mediated transfection levels normalized by the CMV promoter-mediated expression. Figure 1B displays the luciferase expression levels obtained with the CMV promoter construct; the differences reflect different gene delivery efficiencies. In the noncolorectal control cell line HeLa, the CTP4 promoter was 2300-fold less active than CMV (Figure 1A, right bar). In sharp contrast, in colorectal carcinoma cells the CTP4 promoter activities were 1000- to 70000-fold higher than in HeLa cells and within the range (0.4- to 28-fold) of the strong CMV promoter. In the more differentiated cell lines with epithelial-like morphology (Figure 1A, left two bars), CTP4 was only slightly less active than the CMV promoter (0.8- and 0.4-fold in COGA-1 and COGA-5, respectively). CTP4 activity was 3−4-fold higher than CMV in the poorly differentiated rounded-up cell lines COGA-2 and COGA-3. A comparable result was observed with the control colorectal cell line SW480. Tumor cell cultures with intermediate "piled-up" morphology (COGA-5L and -12) show differing expression levels. For COGA-5L, expression levels equal those in COGA-5 (which is derived from the same patient), whereas COGA-12 showed very high CTP4 promotor activity, 28-fold higher than CMV. Taken together, the above data confirm the known high efficiency and specificity of the CTP4 promoter in established tumor cell lines and extend these findings to a series of low passage human colorectal cancer cell lines which more faithfully reflect the properties of primary human tumors.

Transfection of Selected Low Passage Colon Cancer Cell Lines with Transcriptionally Targeted Lipopolyplexes. We next compared the relative activities of the CTP4 and CMV promoters in the low passage lines COGA-3, COGA-5, and COGA-12 using an optimized lipopolyplex formulation of DOSPER and PEI25br for gene transfer (transcriptionally targeted lipopolyplexes). In addition, the results were compared with the corresponding individual lipoplex and polyplex formulations. COGA-3, COGA-5, and COGA-12 each represent one morphological category of the low passage lines. The SW480 and HeLa cell lines were again used for control purposes (Figure 2). The CTP4 promoter was significantly more active than CMV in the cell lines SW480, COGA-3, and COGA-12, independent of the transfection formulation (11-fold, 10-fold, and 20-fold, respectively; Figure 2D,A,C). In contrast, CMV was up to 950-fold more active than CTP4 in HeLa cells (Figure 2E). In the COGA-5 line CTP4 displayed medium activity approximately 2-fold lower than CMV (Figure 2B).

In all cell lines tested the gene transfer efficiencies of lipopolyplexes were significantly higher than those of the corresponding lipoplexes or polyplexes. The expression levels obtained by lipopolyplex formulations were up to 1300-fold higher than with the corresponding lipoplexes of DOSPER and up to 100-fold higher than the corresponding polyplexes of PEI25br (Figure 2).

Influence of Plasmid DNA Concentration on CTP4- and CMV-Mediated Gene Expression Levels. We next determined whether the observed differences in gene expression mediated by the CTP4 and CMV promoters were influenced by the amount of plasmid DNA used for transient transfection. SW480 cells were transfected with either 0.1 μg or 0.05 μg of DNA, using lipopolyplex, lipoplex, and polyplex formulations respectively. As expected, luciferase expression levels were generally lower with 0.05 μg of transfected DNA than with 0.1 μg of DNA (Figure 3). However, the difference in CTP4 and CMV-mediated reporter gene expression was more pronounced when lower amounts of DNA were used. It was also dependent on the transfection formulation used. With 0.05 μg of plasmid DNA CTP4-mediated luciferase expression was 10-fold higher than with the CMV promoter with lipopolyplexes, and 85-fold and 14-fold higher with lipoplexes and polyplexes respectively. In contrast, with 0.1 μg of transfected DNA these

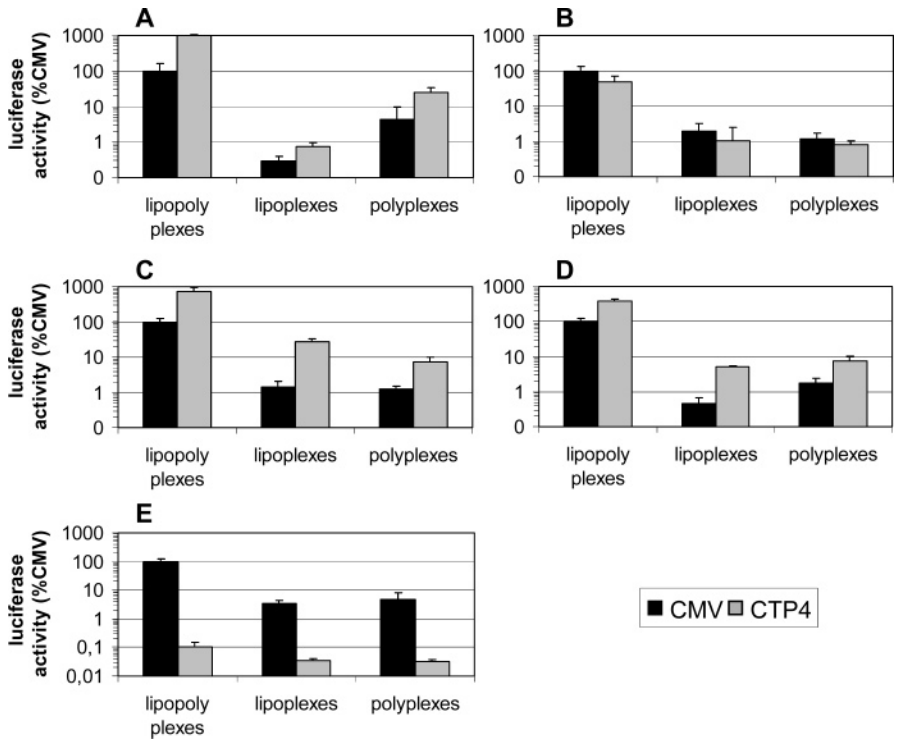

Figure 2. Luciferase expression by the CTP4 promoter in comparison to the CMV immediate early enhancer/promoter using lipopolyplex, lipoplex, and polyplex formulations for gene transfer in COGA-3 (A), COGA-5 (B), COGA-12 (C), SW480 (D), and HeLa cells (E). pCMV-Luc or pCTP4-Luc (0.1 μg per 10^4 cells) was used for transfection. Luciferase activities in all transfections are shown as a percentage of the activity of the CMV promoter using lipopolyplexes. The activity of 100% corresponds to 2×10^5 RLU in panel A, 7.2×10^5 RLU in panel B, 6.2×10^5 RLU in panel C, 9.6×10^7 RLU in panel D, and 4.2×10^6 RLU in panel E per 1×10^4 cells used for transfection. Values are means $\pm$ SE of triplicates.

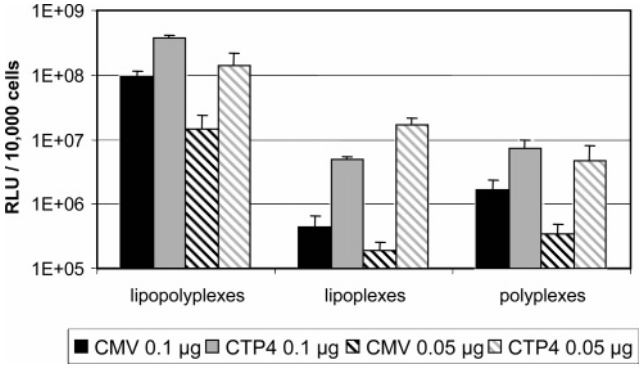

Figure 3. Influence of the dose of transfected plasmid DNA on the luciferase expression levels on cells using different formulations. Indicated amounts of pCMV-Luc or pCTP4-Luc were used for transfection of 1×10^4 SW480 cells. Values are means $\pm$ SE of triplicates.

differences in expression were 4-fold in the case of lipopolyplexes, 11-fold with lipoplexes, and 4-fold with polyplexes (Figure 3).

Percentage of Transfected Cells with or without Transcriptionally Targeted Gene Expression. To eliminate the formal possibility that the higher level of luciferase reporter gene expression observed with the CTP4 promoter compared to CMV was the result of enhanced gene transfer efficiency rather than increased protein expression per cell, the percentage of transfected cells was determined by flow cytometric analysis of EGFP expression.

The colon cancer cell lines SW480, COGA-3, COGA-5, and COGA-12 were transfected with plasmids containing the EGFP gene under the control of the CMV or the CTP4 promoters respectively using lipopolyplex, lipoplex, and polyplex formulations. As shown in Figure 4 the number of transfected cells was not dependent to a significant degree on the promoter construct used.

The highest percentage of transfected cells was obtained with lipopolyplexes (up to 15% in the low passage cell lines and up to 40% in SW480 cells) (Figure 4). Lower percentages of EGFP-positive cells were obtained in all cell lines both with lipoplexes (up to 5% in the low passage cell lines and up to 7% in SW480 cells) and with polyplexes (up to 0.4% in the low passage cell lines and up to 1% in SW480 cells) (Figure 4).

Transfection of Multicellular Spheroids with Transcriptionally Targeted Lipopolyplexes. The preceding experiments were all performed with cells in monolayer culture. We next investigated the efficiency of lipopolyplex-mediated transfection of multicellular spheroids. Multicellular spheroids are model cell systems which mimic the three-dimensional structure of in vivo tumors, an important

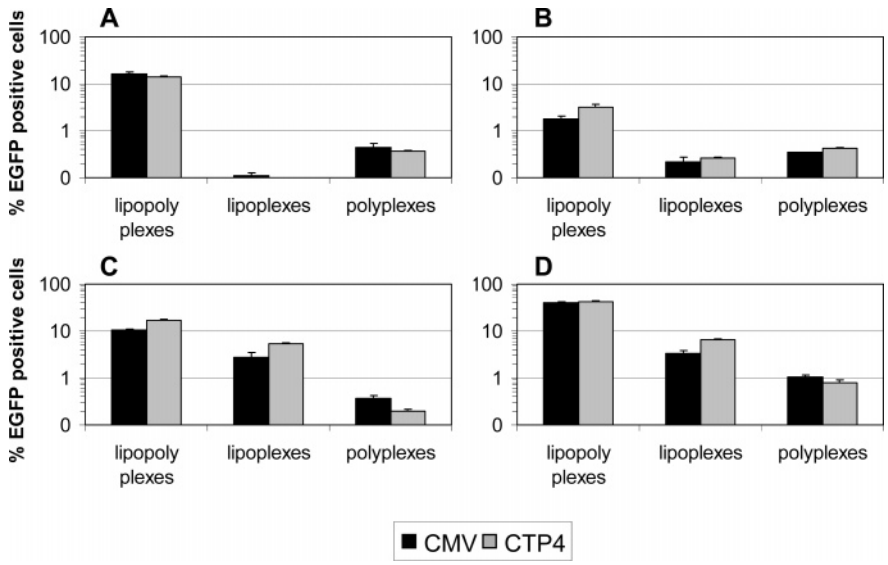

Figure 4. Percentage of EGFP-positive COGA-3 (A), COGA-5 (B), COGA-12 (C), and SW480 (D) cells following gene transfer using different formulations in combination with plasmids containing the EGFP gene under control of the CMV or CTP4 promoter. For transfections, 0.5 of μg pCMV-EGFP or pCTP4-LG-EGFP per 1 $\times$ 10^5 cells were used. The mean percentages of EGFP-positive cells $\pm$ SE of duplicates are shown.

parameter neglected by traditional monolayers. Spheroids of cell line COGA-12 were transfected 96 h after formation with lipopolyplexes containing 0.25 μg of the CMV-EGFP or CTP4-EGFP expression plasmids. EGFP-positive cells were monitored by epifluorescence microscopy 48 h after transfection (Figure 5A,B). Both promoters led to significant EGFP expression, without visible differences in the number and intensity of EGFP-positive cells. Interestingly, only the upper part of the multicellular spheroids which were exposed to the growth medium exhibited EGFP fluorescence (Figure 5C), while the lower part facing the agarose coating on the bottom of the culture plate did not (Figure 5D).

Confocal laser scanning microscopy of DAPI counterstained, EGFP-transfected multicellular spheroids revealed that predominantly cells located between the surface and about 60 μm depth were transfected (Figure 6). DAPI also stained only the outer cell layers of the multicellular spheroids. As the depth of penetration of confocal laser scanning microscopy is limited, additionally cryosections of the transfected multicellular spheroids were prepared. These confirmed that only cells at the surface of the multicellular spheroids exhibited EGFP fluorescence (data not shown).

Colorectal Cancer Specific Expression of Immune Stimulatory IL-2. To investigate also the therapeutic advantages of the CTP4 promoter in comparison to the CMV promoter, selected cell lines (COGA-12 and SW480) were transfected with lipopolyplexes containing the human interleukin-2 (IL-2) gene under the control of the CMV or the CTP4 promoter. Each plasmid was applied in two different concentrations (0.25 μg and 1 μg of plasmid DNA per 1 $\times$ 10^5 cells). When using 0.25 μg of DNA the CMV promoter led to secretion of 4 ng of IL-2 per 10^5 COGA-12 cells, and to secretion of 0.3 ng of IL-2 per 10^5 SW480 cells 48 h following transfection (Figure 7). The use of the CTP4 promoter enhanced the IL-2 production up to 350-fold in SW480 cells (95 ng of IL-2 per 10^5 cells) compared to the IL-2 expression levels achieved by the CMV promoter. The level of secreted IL-2 could be further enhanced by increasing the amount of plasmid DNA. As shown in Figure 7A using 1 μg of plasmid DNA, up to 27 ng of IL-2 per 10^5 COGA-12 cells was detected independent of which promoter was used. In SW480 cells CMV promoter-driven IL-2 gene also demonstrated high expression at 1 μg of DNA (18 ng of IL-2 per 10^5 cells) (Figure 7B). Interestingly, when the CTP4 promoter was used in SW480 cells the highest level of IL-2 was already achieved with 0.25 μg of DNA (95 ng of IL-2 per 10^5 cells). In SW480 cells, no further enhancement was measured by higher DNA concentrations in the case of the CTP4 promoter, while in COGA-12 cells the highest level of IL-2 was observed using 1 μg of DNA for transfection. The IL-2 expression was also investigated in multicellular spheroids of COGA-12. Spheroids were transfected with lipopolyplexes containing 0.5 μg of pGS-hIL2-tet (CMV promoter) or pCTP4-hIL-2 (CTP4 promoter). Two days after transfection, pGS-hIL2-tet led to secretion of 0.5 $\pm$ 0.2 ng of IL-2 per spheroid in 48 h and pCTP4-hIL-2 to 1.4 $\pm$ 0.2 ng of IL-2 per spheroid in 48 h (data not shown).

Discussion

Cancer specific gene expression is an important factor for the development of cancer gene therapy systems. In more than 70% of human colon tumors mutations leading to nuclear β-catenin accumulation are found.[14] Therefore, a β-catenin/TCF-dependent promoter should be highly specific for expression in most colon cancer cells. Such a promoter, CTP1, was recently developed by Lipinski et al.[25] This promoter was further optimized for its specificity, resulting in the artificial CTP4 promoter which is highly effective in the SW480 colon cancer cell line and other tumor cell types.[13]

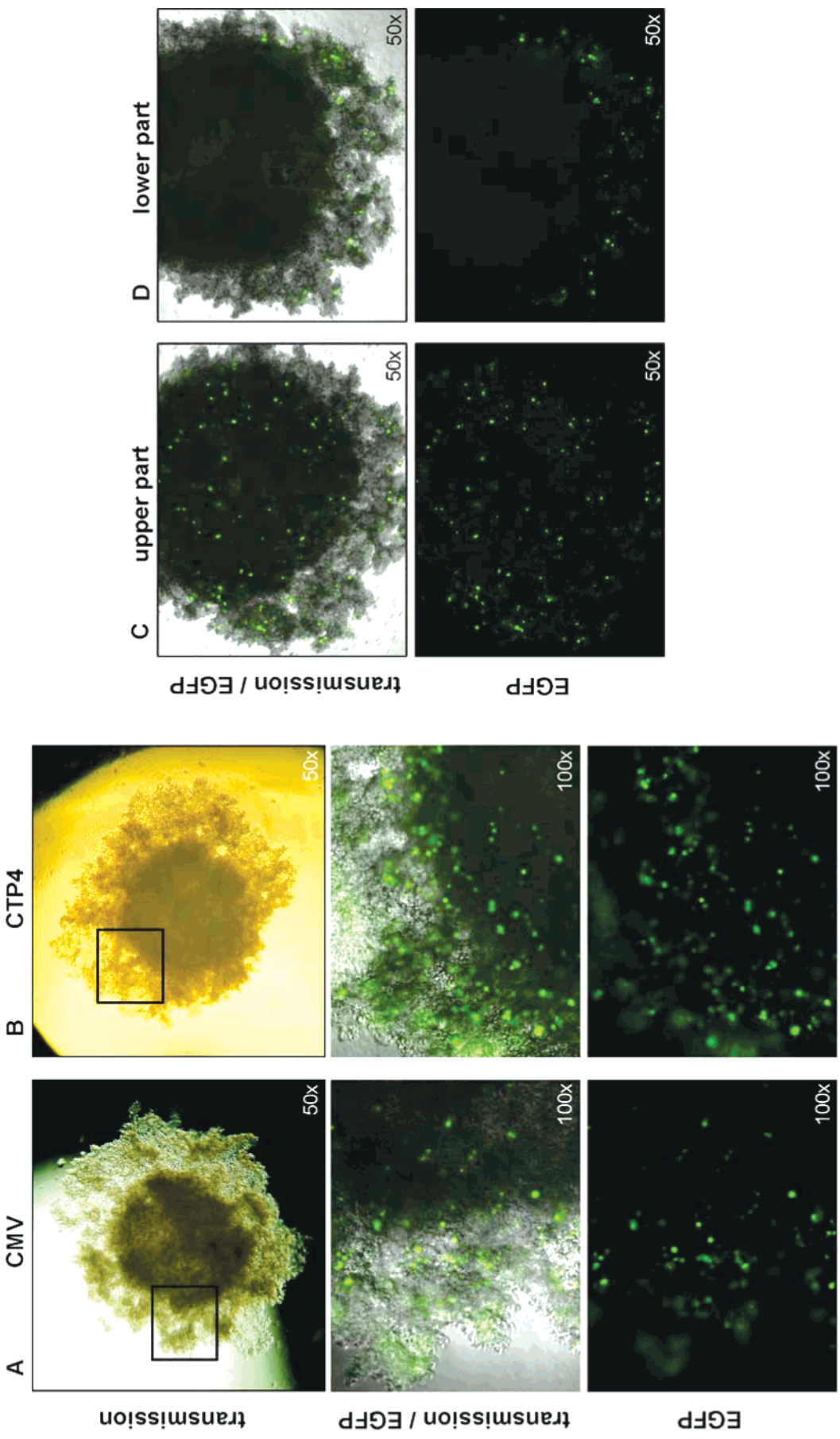

Figure 5. Comparison of EGFP expression controlled by (A) the CMV and (B) the CTP4 promoter in multicellular spheroids. Spheroids of COGA-12 cells were transfected with 0.25 μg of pEGFP-N1 (A) or pEGFP-LG-CTP4 (B). Forty-eight hours after transfection multicellular spheroids were fixed in 4% PFA and analyzed by epifluorescence microscopy. The top panels show transmission light images of whole multicellular spheroids. The squares mark the respective areas of the multicellular spheroids that are displayed below as magnified overlays of transmission light and epifluorescence images (middle panels) and epifluorescence images (lower panels). Panel C shows higher EGFP expression in the upper, accessible part of a pEGFP-N1 transfected COGA-12 spheroid as compared to the lower part of the same spheroid (D).

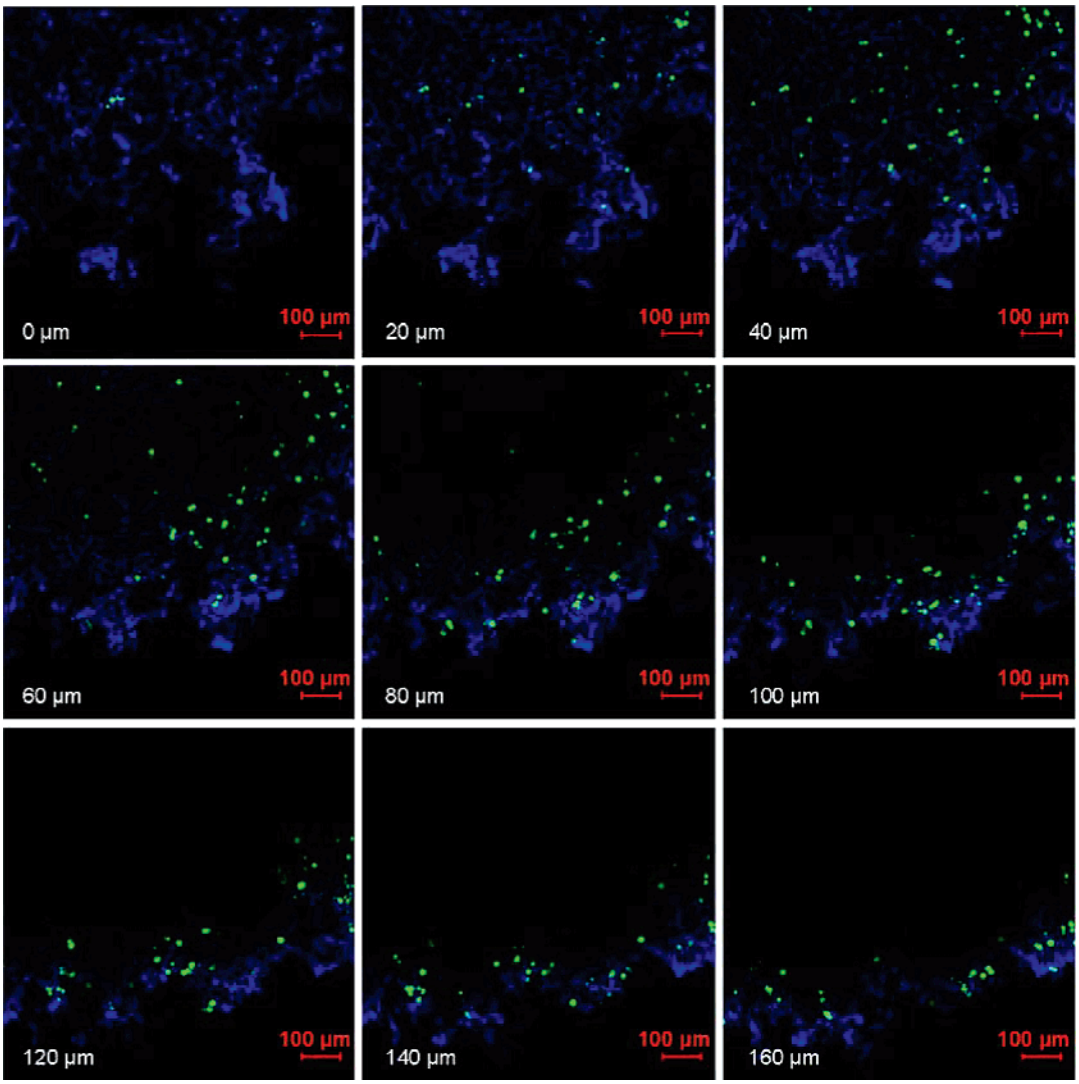

Figure 6. Distribution of EGFP transfected cells within a multicellular spheroid of COGA-12 cells using lipopolyplexes for gene transfer. Spheroids were transfected with 0.25 μg of pEGFP-N1. Forty-eight hours later spheroids were fixed in 4% PFA and counterstained with DAPI. Confocal laser scanning microscopy stack images were taken every 20 μm moving from the outside to the inside of the spheroid. Overlays of EGFP and DAPI fluorescence images are shown. DAPI stained cell layers indicate the surface of the spheroid.

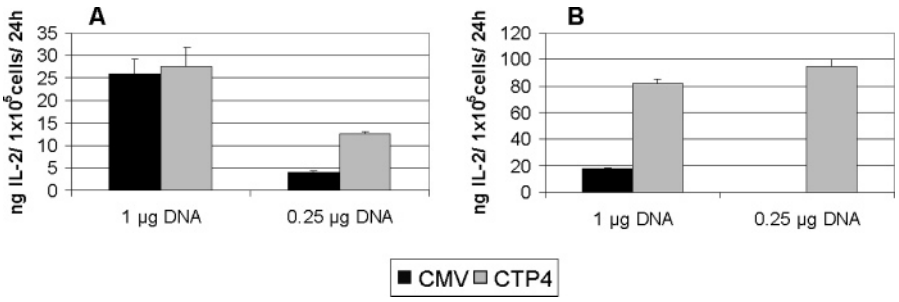

Figure 7. Interleukin-2 (IL-2) production in COGA-12 (A) and SW480 cells (B) transfected with lipopolyplexes using the CTP4 promoter or the CMV promoter. Transfection was performed using 0.25 μg and 1 μg of pGShIL-2tet or pCTP4-hIL-2 per 1 $\times$ 10^5 cells. Twenty-four hours after transfection the growth medium was replaced by fresh medium, 48 h following gene transfer the supernatants were collected, and the level of secreted human IL-2 per 1 $\times$ 10^5 in 24 h was determined using an IL-2 ELISA kit. Values are means of duplicates.

In the current work we extend the validation of the CTP4 promoter by using a panel of well-characterized human low passage colon cancer cultures reflecting the diverse properties of real patient tumors.[15] Despite the morphological diversity

of the different tumors, CTP4 displayed high promoter activities in all six low passage colon cancer cell lines similar to that observed in the SW480 colorectal cell line, reaching or even exceeding the activity of the strong, constitutively active viral CMV promoter (Figure 1). In noncolorectal HeLa cells, however, the CTP4 promoter was >1000-fold less effective than the CMV promoter, in agreement with published results by Lipinski and co-workers.[13]

Differences of CTP4 promoter activity within the colon carcinoma cultures could be correlated with morphological differences of tumors and molecular pathogenetic differences in the Wnt signaling pathway. In particular, cell lines COGA-1 and COGA-5, with differentiated epithelial-like morphology and normal β-catenin colocation with E-cadherin at the plasma membrane,[15] display lower CTP4 promoter activity than the poorly differentiated "rounded-up" cell lines SW480 or COGA-2 and COGA-3, displaying also aberrant nuclear location of β-catenin.[15] COGA-5L and COGA-12, with intermediate "piled-up" morphology and elevated but diffusely distributed β-catenin,[15] show differing expression levels. COGA-12 showed very high CTP4 promotor activity. For COGA-5L, expression levels were lower and equal to the levels in COGA-5 which is derived from the primary tumor of same patient. Other mutations associated with the Wnt signaling pathway[26] may be involved in the activity of the CTP4 promoter. For example, APC protein that is involved in the degradation of β-catenin is inactivated in the cell lines COGA-5 and COGA-12, while it is not mutated in the cell line COGA-3.[15] In the latter cell line, however, β-catenin carries a mutation which prevents its degradation.

Although the specificity and efficiency of promoter systems like CTP4 is intriguing, practical application for gene therapy is hampered by poor intracellular delivery of gene vectors. A novel nonviral gene delivery approach combines cationic lipids and polycations for DNA complexation into lipopolyplexes.[27−29] We previously demonstrated that lipopolyplexes of the cationic lipid DOSPER and the polycation PEI25br significantly enhanced gene transfer efficiency compared to corresponding lipoplexes of DOSPER and polyplexes of PEI25br.[16] These lipopolyplexes were applied for the following evaluation of the CTP4 promoter. Three cell lines were chosen as representatives for epithelial-like (COGA-5), piled-up (COGA-12), and rounded-up (COGA-3) morphologies.[15] The transcriptionally targeted lipopolyplexes mediated an up to 1300-fold higher luciferase expression compared to the corresponding lipoplexes and polyplexes. Experiments using the reporter gene EGFP demonstrated that also the percentage of EGFP-positive cells was significantly higher (up to 40%) when lipopolyplexes were used for transfection compared to the corresponding lipoplexes or polyplexes (Figure 4). Within the lipopolyplex groups, similar percentages of transfected cells were found regardless of whether the CTP4 or the CMV promoter was used. This indicates that the enhanced transgene expression levels obtained by the CTP4 promoter compared to the CMV promoter are largely due to higher transcriptional activity achieved by the CTP4 promoter and cannot be attributed to differences in the gene delivery process.

The high potential of transcriptionally targeted lipopolyplexes as an improved nonviral gene transfer system was further demonstrated by the observation that these complexes provided also the ability to efficiently transfect multicellular spheroids (Figure 5). The percentage of EGFP-positive cells obtained with lipopolyplexes containing the EGFP gene under control of the CMV promoter or the CTP4 promoter was equal. Confocal microscopy and cryosectioning, however, demonstrated that only cells located near the surface of the spheroids were transfected (Figure 6). This finding strongly suggests that the gene transfer complexes are able to diffuse only to a limited distance into the compact spheroid structure. Possible reasons include sticking of the positively charged lipopolyplexes to the surface cell layer or/and polyplex aggregation which prevents further diffusion into the spheroid. Shielding the surface charge of the particle, for example by PEGylation,[30] might be a possible remedy for overcoming this barrier.

For the case that only a limited percentage of tumor cells can be transfected, therapeutic genes should be selected which mediate biological bystander effects also on nontransfected tumor cells. Transcriptionally targeted lipopolyplexes might therefore be useful for therapeutic strategies including tumor-specific expression of immune stimulatory genes such as interleukin-2.[31] Therefore, we investigated the expression levels of IL-2 after transfection with lipopolyplexes (Figure 7). Both the CMV and the CTP4 promoter mediated very high levels of IL-2 protein (up to 95 ng of IL-2 per 1×10^5 cells in 24 h) both in COGA-12 cells and SW480 cells. Despite the reduced accessibility of spheroids, pronounced IL-2 expression levels were also observed upon transfection of COGA-12 cell spheroids (1.4 ng per spheroid with the CTP4 promoter, 0.5 ng with the CMV promoter). When

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lower amounts of DNA were used for IL-2 transfection, the CTP4 promoter was up to 350-fold more effective than the CMV promoter. Comparable results were achieved with luciferase expression vectors (Figure 3). The benefit of the CTP4 promoter over the CMV promoter was clearly visible when using lower DNA doses, where the DNA concentration and not the cellular gene expression machinery is the limiting factor. Therefore, there could be a clear in vivo benefit resulting from significantly higher levels of therapeutic gene expression compared to CMV-driven expression. Especially higher levels of cytokine could determine if the patient's immune system is able to induce an anticancer immune response enabling a systemic response to all metastatic tumor deposits.

In conclusion, the use of transcriptionally targeted lipopolyplexes that combine efficient lipopolyplex formulations for delivery with tumor specific CTP4 promoter-driven expression might be a powerful approach for the treatment of colon cancer. The CTP4 promoter was found to be active in a broad range of human colon cancer cell cultures and also in three-dimensional multicellular spheroids. As shown with luciferase, EGFP, and IL-2 gene expression constructs, the CTP4 promoter enables expression levels comparable with or even higher than the strong but unspecific CMV transcription cassette.

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