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Chemosensitizing tumor cells by targeting the Fanconi anemia pathway with an adenovirus overexpressing dominant-negative FANCA

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Fanconi anemia (FA) is a rare genetic disorder characterized by bone-marrow failure and cellular hypersensitivity to crosslinking agents, including cisplatin. Here, we studied the use of the FA pathway as a possible target for cancer gene therapy with the aim to sensitize tumor cells for cisplatin by interfering with the FA pathway. As proof-of-principle, FA and non-FA lymphoblast-derived tumors were grown subcutaneously in *scid* mice and treated with two different concentrations of cisplatin. As predicted, the antitumor response was considerably improved in FA tumors. An adenoviral vector encoding a dominant-negative form of FANCA, FANCA600DN, was generated that interfered with endogenous FANCA–FANCG interaction resulting in the disruption of the FA pathway as illustrated by disturbed FANCD2 monoubiquitination. A panel of cell lines, including non-small-cell lung cancer cells, could be sensitized approximately two- to three-fold for cisplatin after Ad.CMV.FANCA600DN infection that may increase upon enhanced infection efficiency. In conclusion, targeting the FA pathway may provide a novel strategy for the sensitization of solid tumors for cisplatin and, in addition, provides a tool for examining the role of the FA pathway in determining chemoresistance in different tumor types.

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Chemotherapy remains one of the most effective cancer treatments today, although the effectiveness of the great variety of chemotherapeutic agents in the clinic is often hampered by chemoresistance of the tumor cells and dose-limiting toxicity in normal tissues. For patients with advanced non-small-cell lung cancer (NSCLC), chemotherapy is a standard treatment, which significantly improves the quality of life; unfortunately, only a small amount of these patients will benefit from the therapy. Novel strategies to improve the efficacy of chemotherapy are warranted and cancer gene therapy provides one novel approach.[1] Adenoviral vectors are popular for the selective delivery of therapeutic genes at the tumor site; for example, restoring the expression of defective tumor-suppressor genes such as p53 showed some therapeutic activity in a subset of patients with advanced NSCLC in a phase I clinical trial.[2] The use of dominant-negative (DN) genes that upon expression disrupt essential drug sensitivity pathways in infected cells provides another strategy for cancer therapy. For example, Rosenberg and co-workers described an example of this method in which the overexpression of a truncated form of xeroderma pigmentosum A (XPA) resulted in the sensitization of the NSCLC cell line A549 to cisplatin and UV light.[3]

In this study, we have explored the Fanconi anemia (FA) pathway as a possible target for the sensitization of cancer cells to chemotherapy. FA is a rare autosomal recessive disorder clinically characterized by a variety of congenital abnormalities, early childhood hematological defects and high predisposition to cancer.[4,5] Moreover, cells derived from FA patients show at least a 10-fold increase in sensitivity to crosslinking agents, such as mitomycin C, cisplatin and diepoxybutane, as well as an increased level of spontaneous chromosomal aberrations.[6,7] So far, 11 distinct FA complementation groups have been identified (A–C, D1, D2, E–G, I–J, L) and currently eight FA genes have been cloned.[8–10] These genes encode proteins whose precise molecular functions remain unclear.[10–15] The FA proteins are known to interact with each other in a complex network, designated the FA pathway, which plays an important role in the normal cellular response to crosslinking agent-induced

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damage and in maintaining genome stability (for recent reviews, see D'Andrea and Grompe[9] and Bogliolo et al[16]). The FANCA, FANCC, FANCE, FANCF, FANCG and FANCL proteins have been shown to form a nuclear complex that is required for the monoubiquitination of the FANCD2 protein. The modification of FANCD2 is a key downstream step in the FA pathway, since any disruption of the complex abrogates FANCD2 monoubiquitination.[10,17,18] However, mutations in FANCD1/BRCA2 gene and the putative FANCJ gene do not affect the monoubiquitination of FANCD2, suggesting that these proteins may act in a parallel independent step or downstream the FANCD2 protein.[19] The *FANCA* gene is the most commonly mutated gene in FA patients, and previous work showed that the interaction between FANCA and FANCG proteins is essential for the FA pathway to function.[13,14] Moreover, the overexpression of carboxyl-deleted mutant forms of FANCA with an intact FANCG binding domain that is localized in the amino-terminal part of the protein was able to sensitize HeLa cervical tumor cells, likely due to the disruption of the endogenous functional FA complex.[20]

Here, we examined whether the FA pathway could be a target for the sensitization of tumor cells, in particular, NSCLC cells by using an adenoviral vector overexpressing an FANCA DN form. First, we show that a disrupted FA pathway results in an improved response towards cisplatin treatment in a subcutaneous (s.c.) xenograft transplantation model in *scid* mice using normal and FA-C lymphoblasts. Subsequently, we demonstrate that the adenovirus-mediated overexpression of a truncated FANCA variant in NSCLC cell lines causes a modest sensitization to cisplatin that is accompanied by a disruption of the endogenous FANCA–FANCG complex and by a reduction of monoubiquitination of FANCD2. This study shows that the FA pathway can be a target for the sensitization of solid tumor cells for chemotherapy with cisplatin, although the degree of sensitization with Ad.CMV.FANCA600DN is limited by the infection efficiency achieved and likely also by other known or unknown molecular mechanisms that may affect cisplatin sensitivity and/ or the FA pathway in NSCLC cells.

Materials and methods

Cell culture

HeLa cervix carcinoma cells, NCI-H460 and A549 NSCLCs and HepG2 hepatoma cells were obtained from the American Type Culture Collection (Manassas, VA). Human embryonic kidney 293 cells were used for constructing and growing replication-deficient recombinant adenovirus. Lymphoblastoid cell lines used were HSC93, HSC536, VU143, HSC72 and EUFA867 cells representing normal, FA-C, FA-G, FA-A and an unclassified FA group, respectively. HeLa cells were cultured in DMEM; HEK293 and A549 in F12-supplemented DMEM, and H460 and the lymphoblastoid cell lines in RPMI 1640 supplemented with 1% sodium pyruvate. The media were supplemented with 10% heat-inactivated fetal calf serum and antibiotics and were all obtained from Invitrogen BV.

Plasmids and adenoviral vectors

The pCDNA3-FANCA600DN construct has been described previously.[20] The FANCA600DN fragment was ligated into pShuttleCMV[21] digested with *Hind*III/*Xba*I, yielding the construct pShuttleCMV-FANCA600DN. To produce the final recombinant adenoviral vectors, pShuttleCMV-FANCA600DN was linearized with *Pme*I and subsequently cotransformed into *Escherichia coli* BJ5183 cells with the adenoviral backbone plasmid pAdEasy-1.[21] Recombinants were selected by kanamycin resistance and confirmed by restriction endonuclease analysis. Recombinant plasmids were linearized with *Pac*I and transfected into 293 cells using Lipofectamine Plus method (Invitrogen BV) according to the manufacturer's guidelines. Recombinant replication-deficiency adenoviruses were prepared and further propagated on 293 cells for CsCl gradient purification according to standard procedures. Ad.GFP was generated by homologous recombination in 293 cells, as described previously.[22] Particle titers from all viruses were determined by absorbance measurements at 260 nm, and functional PFU (plaque-forming units) titers were determined by limiting-dilution plaque titration on 293 cells according to standard procedures. All studies were performed with a single batch of Ad.CMV.FANCA600DN and Ad.GFP.

Fluorescence microscopy analysis

A total of 5×10^5 cells were seeded onto coverslips in 12-well plates. The next day, Ad.CMV.FANCA600DN virus was added to A549 and H460 cells at 300 PFU/cell, and at 200 PFU/cell for HeLa cells, and washed away after 4 hours. At 48 hours after infection, the medium was aspirated and cells were washed three times with phosphate-buffered saline (PBS). The immunostaining procedure was carried out at room temperature. Cells were fixed with 3.7% formaldehyde in PBS for 30 minutes, washed with PBS, permeabilized with 0.2% Triton in PBS for 10 minutes and washed with PBS again. Following a blocking step with 3% normal rabbit serum (Dako, Glostrup, Denmark) in PBS for 1 hour, an affinity purified guinea-pig antibody directed against the amino-terminus of FANCA was diluted 1:250 in blocking solution and applied 1 hour for the detection of FANCA600DN protein. After washing with PBS, cells were incubated with fluorescein-conjugated rabbit anti-guinea-pig secondary antibody (Dako, Denmark) dilution 1:300 for 45 minutes. Finally, coverslips were rinsed three times with PBS and mounted onto microscope slides with Vectashield (Vector Laboratories, Inc., Burlingame, CA). Hoechst 33342 (Sigma, St Louis, MO) was used to counter stain the cell nuclei. Slides were examined under UV light on an inverted Leica DMIRB/E fluorescence microscope (Leica Heidelberg, Heidelberg, Germany). Images ($\times 400$ magnification) were collected using Leica Q500MC Quantimet software V01.01 (Leica Cambridge Ltd, Cambridge, UK).

Immunoprecipitation and Western blotting

For immunoprecipitation, 4×10^6 cells were infected with Ad.CMV.FANCA600DN or Ad.GFP for 4 and 48 hours later, cells were harvested and disrupted in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet NP-40, 1 mM Pefabloc, 0.1% Aprotinine, 0.1% Pepstatine, 0.1% Leupeptine). Lysates were clarified by centrifugation at 14,000 rpm in a cold microcentrifuge. Protein concentrations were determined using a BCA protein assay (Pierce, Rockford, IL). The amount of protein was normalized to 1 mg for each immunoprecipitation, except for the lymphoblastoid cells for which 10^7 cells were used. Extracts were incubated with guinea-pig serum against FANCG (amino acids (AA) 480–622)[13] at 4°C overnight and antibody-bound proteins were collected with protein A–agarose beads (Invitrogen BV). The beads were washed three times with lysis buffer and resuspended in sample buffer. Samples were separated on a 7.5% SDS-polyacrylamide gel and transferred onto a Polyscreen® PVDF membrane (Perkin-Elmer Life Sciences Inc.). For Western analysis of FANCA/FANCA600DN expression, equal amounts of protein (20 μg) were separated on a Novex 7% Tris-Acetate gels (Invitrogen BV) and transferred onto a Polyscreen® PVDF membrane. Membranes were either incubated with the rabbit FANCA antibody 89[13] (IP) or an affinity-purified guinea-pig antibody against FANCA (AA 1–271) (WB) at a dilution of 1:500 or 1:700, respectively, in 2% dry milk in TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween). After washing with TBST, membranes were incubated with peroxidase-conjugated goat anti-guinea-pig or goat anti-rabbit immunoglobulins (Dako, Denmark) diluted 1:5000 in 2% dry milk in TBST.

For detecting FANCD2 monoubiquitination, Novex 3–8% Tris-Acetate gels (Invitrogen BV) were used. In brief, 3.5×10^6 cells either mock-infected or infected with Ad.GFP/Ad.CMV.FANCA600DN at the same PFUs/cell as described above, and treated with 10 μM cisplatin overnight when indicated. Equal amounts of protein (50 μg) were loaded on gel, except for control HSC93 and EUFA867 cells, where 5×10^5 cells were used. PVDF membranes were incubated with a purified FANCD2 rabbit antibody (against AA 1–292) diluted at 1:1000 in 2% dry milk in TBST. Membranes were washed and developed by using the ECL (Amersham, Braunschweig, Germany) or Lumilight Plus (Roche Diagnostics) Western blotting analysis system. Quantitative analysis of FANCD2-S/-L bands was carried out using Quantity One quantification software (BioRad Laboratories Ltd, UK).

Cell viability assay

HepG2 cells were seeded in 96-well plates at a density of 4000 cells/well; and HeLa, H460 and A549 cells were seeded in 24-well plates at a density of 40,000 cells/well, which allowed higher infection efficiency. The next day, cells were infected with the adenoviral vectors at the indicated PFU/cell in quadruplicate, and after another 24 hours increasing concentrations of cisplatin (Platosin®, PCH Pharmachemie, Haarlem, The Netherlands) were added to the cells. The optimal amount of PFU/cell was determined for each cell line based on the highest obtainable infection efficiency in the absence of toxicity. Cells were monitored and when showing signs of chemotherapy-induced cell death, which occurred at different time points depending on the cell line used (HepG2, HeLa and H460, day 6 p.i., A549, day 8 p.i.), the culture media were removed and replaced by 100 or 200 μl of 10% WST-1 reagent (Roche Diagnostics, Mannheim, Germany) in the culture medium, in 96- or 24-well plates, respectively. Cells were incubated at 37°C for 30 minutes to 1 hour, and absorbance was measured at a wavelength of 450 nm. Results depicted display the ratio of WST-1 conversion in the treated cells relative to the untreated cells that was set at 100%. Data are represented as the average $\pm$ SD of a quadruplicate experiment.

Xenograft tumor model

Two groups of three female CB.17/IcrHanHsd-*scid* mice (Harlan Nederland, Horst, The Netherlands) were injected at both flanks of the body with 10^7 HSC93 (wild-type) or 10^7 HSC536 (FA-C lymphoblastoid cell line) cells.[23] When tumors reached 200 mm^3, animals were killed and tumors were removed, washed and prepared in small pieces for the generation of tumors in mice for further experiments. Mice were divided into six groups with each group containing six animals: three groups were implanted in both flanks with HSC93-derived tumor pieces, whereas the three other remaining groups received the HSC536-derived pieces. Animals were treated when tumors reached an average volume over 100 mm^3. Tumor volumes differences between the different groups were not significant at starting point of the treatment. Treatment was with PBS alone (control) or 2 and 5 mg/kg cisplatin; the first dose was applied by intravenous (i.v.) injection in the tail vein followed by a second dose administered by intraperitoneal (i.p.) injection 7 days later. Animals were hosted in macrolon cages type 3, and received food and water *ad libitum*. Mice were euthanized when the tumor volume exceeded 1500 mm^3 or whenever the animals showed any symptoms of illness. Statistical analysis of differences in antitumor effect and in body weight changes was performed using Student's *t*-test. Differences were considered significant when $P < .01$.

Results

FA lymphoblast-derived tumors display enhanced sensitivity to cisplatin

In order to investigate whether the disruption of the FA pathway in tumors will lead to the predicted improved antitumor response to cisplatin, we set up a lymphoblast-derived tumor model in *scid* mice. For this purpose, the FA-C lymphoblastoid cell line HSC536 and the wild-type cell line HSC93 were used to generate tumors with either a disrupted or functional FA pathway, respectively. Both types of tumors were treated with PBS as control and two different concentrations of cisplatin, 5 and 2 mg/kg cisplatin, and treatment started when tumors had a

volume of approximately 100 mm^3 (see also Materials and methods). As shown in Figure 1, both FA- and normal lymphoblast-derived tumors grew rapidly after PBS treatment and mice were killed between days 11 and 14 when tumor volumes exceeded 1500 mm^3 or due to the occurrence of open wounds, often observed in HSC536-derived tumors, which together with a more than 10% weight loss formed the end points in this experiment. At day 11, both HSC93-derived tumors treated with 5 and 2 mg/kg cisplatin showed significant difference in tumor volume ($P<.001$) and the overall relative survival of both treated groups was extended to 20–25 days. The FA cell-derived tumors displayed an enhanced response to both 5 and 2 mg/kg cisplatin treatment, when compared to the PBS group ($P<.001$). The relative survival time was around 60 days, doubling the survival time achieved in the non-FA tumors, and the higher dose of cisplatin was more effective than the lower dose at suppressing tumor growth at later time points (see day 56, $P<.01$). As an indication of the side effects provoked by the chemotherapy, we also monitored the weight loss of the animals during the experiment and, as expected, animals that

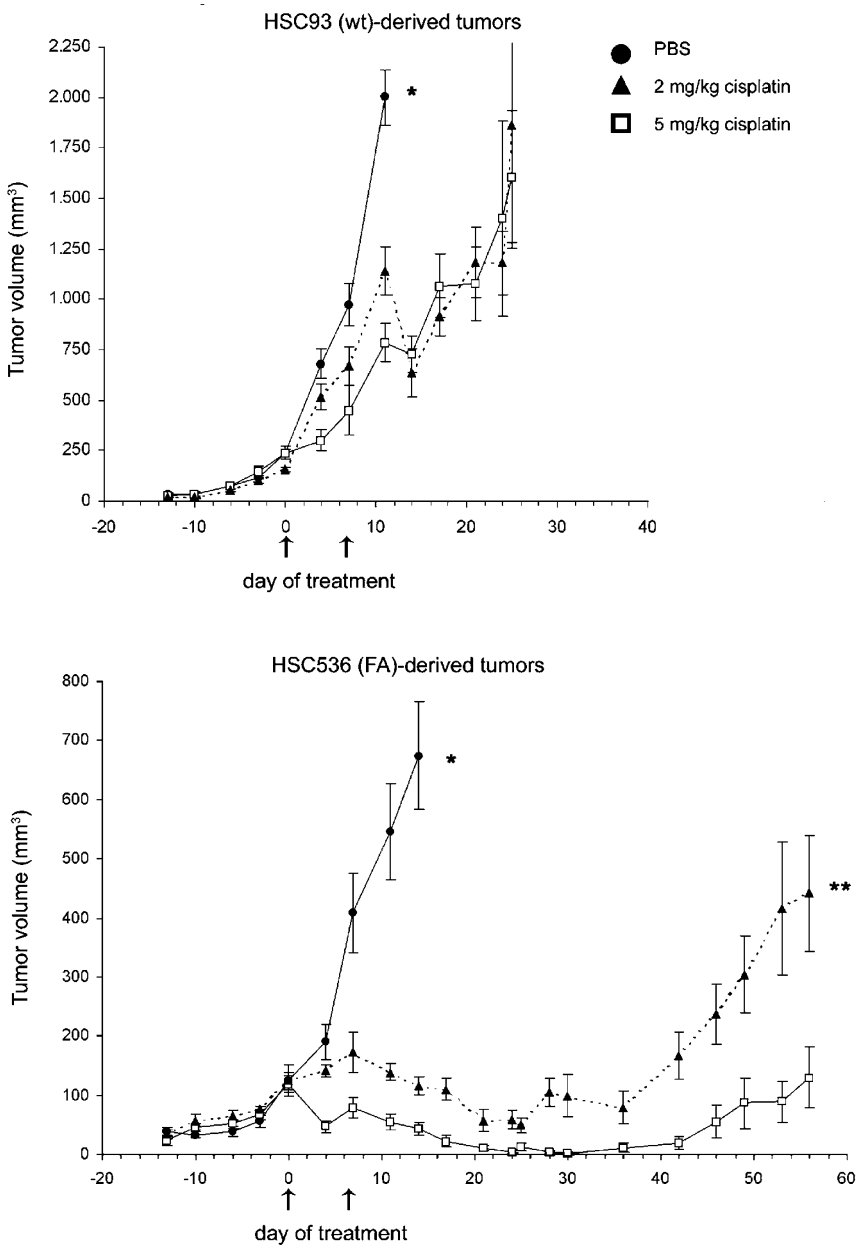

Figure 1 Antitumor effect of cisplatin in FA- and non-FA lymphoblast-derived tumors. HSC536 (FA) and HSC93 (wild-type) tumors were grown as s.c. xenografts on *scid* mice. Tumor growth was followed in time, with day 0 being the start of cisplatin treatment. Arrows indicate the days of cisplatin treatment at days 0 and 7. Data are represented as the mean $\pm$ SD of the tumor volume of the living animals in each group at the time points indicated. *$P<.001$; **$P<.01$.

received the lower dose of cisplatin showed a comparable tumor growth delay response without suffering the weight loss the animals treated with higher dose did, an indication of an enhanced response with minimized side effects (data not shown). As anticipated, this experiment demonstrates that tumors with a disrupted FA pathway are more sensitive to the antitumorigenic effect of cisplatin.

Construction and characterization of Ad.CMV.FANCA600DN

As a strategy to disrupt the FA pathway in tumor cells, a cDNA encoding a truncated FANCA protein encompassing the first 600 amino acids was cloned into an adenoviral vector. Ad.CMV.FANCA600DN was characterized by determining FANCA600DN protein expression by Western blotting using an antibody that recognizes the N-terminal part of the protein in extracts derived from the human NSCLC cell lines H460 and A549, and HeLa cervix carcinoma (Fig 2a). Interestingly, the expression of the 65 kDa FANCA600DN protein correlated with a decreased expression of the endogenous FANCA protein. This is probably caused by the disruption of the interaction between endogenous FANCA and FANCG, which is necessary to stabilize FANCA.[24] Furthermore, immunofluorescence staining of H460, A549 and HeLa cells infected with Ad.CMV.FANCA600DN confirmed the expression of the truncated protein in both the nucleus and cytoplasm, with the major fraction present in the nucleus (Fig 2b).

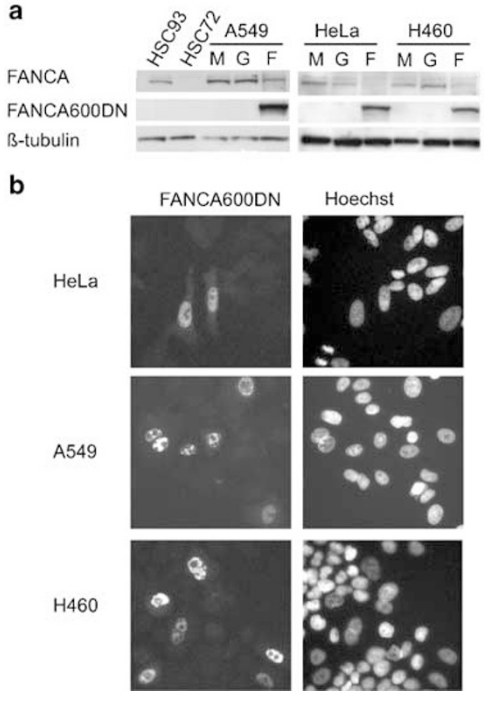

Figure 2 Adenovirus-mediated expression of FANCA600DN. (**a**) Western blot analysis showing the expression of endogenous FANCA (165 kDa) and FANCA600DN protein (65 kDa) in A549, H460 and Hela cells infected with 300 and 200 PFU/cell (MOI), respectively, of Ad.CMV.FANCA600DN and Ad.GFP, used as a negative control. M: mock-infected cells; G: Ad.GFP-infected cells; F: Ad.CMV.FANCA600DN-infected cells. β-tubulin expression was used as a loading control. (**b**) Confirmation of FANCA600DN expression by immunofluorescence microscopy (left panel). Nuclei (right panel) were counterstained with Hoechst 33342.

FANCA600DN protein sequesters endogenous FANCG

To confirm whether overexpressed FANCA600DN serves as an alternative interaction interface for FANCG, we examined FANCA–FANCG complex formation in Ad.CMV. FANCA600DN- and control Ad.GFP-infected H460, A549 and Hela cells in immunoprecipitation experiments. After pulling down FANCG, endogenous FANCA was found to be associated with FANCG in uninfected and Ad.GFP-infected cells. However, the cells infected with Ad.CMV.FANCA600DN showed a clear decrease in the intensity of the band representing endogenous FANCA, indicating that FANCA600DN can compete with FANCA for FANCG binding (Fig 3). The detection of co-precipitating FANCA600DN, as well as FANCG, was hampered by its comigration with the immunoglobulin heavy-chain band of the antibody used to pull down FANCG (data not shown).

Effective disruption of the FA pathway

To monitor FA pathway disruption, we assessed FANCD2 monoubiquitination that is considered to represent a downstream step in the FA pathway indicating the functionality of the other FA proteins forming a complex with ubiquitin ligase activity.[18] In untreated HeLa, H460 and A549 cells, the two isoforms of FANCD2 could be detected by Western blotting, the upper one representing the monoubiquitinated form, designated FANCD2-L and the lower one FANCD2-S (see Fig 4). In untreated cells, FANCD2-S is more abundant (FANCD2-S/-L ratio $\sim$3, as described in Materials and methods), while cisplatin treatment induces FANCD2-L accumulation (FANCD2-S/-L ratio $\sim$1). Upon infection with Ad.CMV.FANCA600DN, HeLa cells showed a reduction in cisplatin-induced accumulation of FANCD2-L when compared to mock- and Ad.GFP-infected cells (FANCD2-S/-L ratio $\sim$1.5). As expected, the formation of FANCD2-L was not completely blocked likely caused by limiting infection efficiencies (see also below and Table 1). This

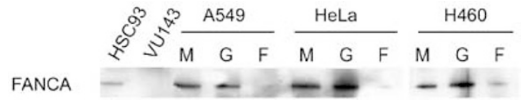

Figure 3 Ad.CMV.FANCA600DN-mediated disruption of the endogenous FANCA–FANCG complex by sequestering FANCG. Immunoprecipitation of the FANCA–FANCG complex from extracts derived from A549, H460 and HeLa cells after Ad.CMV.FANCA600DN infection at 300 PFU/cell in A549 and H460 cells, and 200 PFU/cell in HeLa cells. After FANCG pull down, co-precipitating FANCA was determined by Western blotting. As a negative control, Ad.GFP virus was employed, and as control for FANCG immunoprecipitation HSC93 (wt) and VU143 (FANCG negative), lymphoblasts were used. M: mock-infected cells; G: Ad.GFP-infected cells; F: Ad.CMV.FANCA600DN-infected cells.

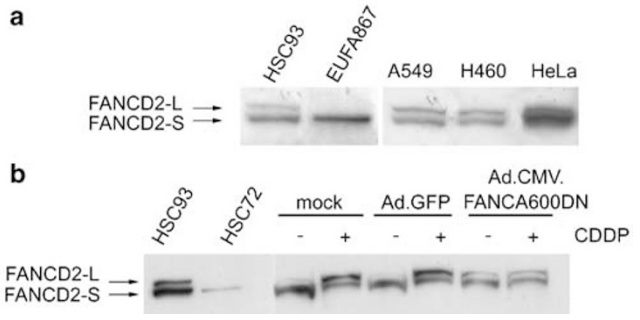

Figure 4 FA pathway functioning in cancer cells as determined by FANCD2 monoubiquitination and the effect of FANCA600DN overexpression. (**a**) FANCD2-S (nonubiquitinated) and FANCD2-L (monoubiquitinated) expression in untreated HeLa, H460 and A549 cells. (**b**) Cisplatin-induced (10 μM) FANCD2-L accumulation is decreased after Ad.CMV.FANCA600DN infection in HeLa cells, when compared with mock- and Ad.GFP-infected cells. As controls for FANCD2 monoubiquitination, HSC93 (wt), EUFA867 (unclassified FA patient) and HSC72 (FA-A patient) lymphoblasts were used.

Table 1 Infection efficiency and cisplatin sensitization by Ad.CMV.FANCA600DN *vs* Ad.GFP

Cell line	Infection efficiency (%)	IC50 (μM) (Ad.GFP)	IC50 (μM) (Ad.FANCA600DN)	Sensitization effect
HepG2	60–65	1.8	0.7	2.5
HeLa	20–30	6.5	3.2	2
H460	35–40	14	8	1.8
A549	40	13.6	6.6	2.1

finding further confirms that FANCA600DN is effective in interfering with the FA pathway when assessed by FANCD2-monoubiquitination.

Ad.CMV.FANCA600DN-dependent tumor cell sensitization

To test the sensitizing effect of the overexpressed DN FANCA variant, H460, A549, HeLa and HepG2 (hepatocarcinoma) cells were infected with Ad.CMV.FANCA600DN and the control virus Ad.GFP. The PFU/cell used for the different cell lines varied and was based on the amount that showed the highest infection efficiency in the absence of viral toxicity as determined in titration experiments (not shown). Figure 5 shows that both HepG2 and HeLa, along with the NSCLC cell lines H460 and A549, could be sensitized for cisplatin by approximately two-fold, when compared to Ad.GFP-infected cells. As determined by immunofluorescence microscopy (see Figure 2b), the percentage of infected cells was typically 30–60%, with the highest infection efficiency seen in HepG2 (60–65%) (not shown), as hepatocytes are known to express high levels of the adenovirus entry receptor CAR.[25,26] In addition, the

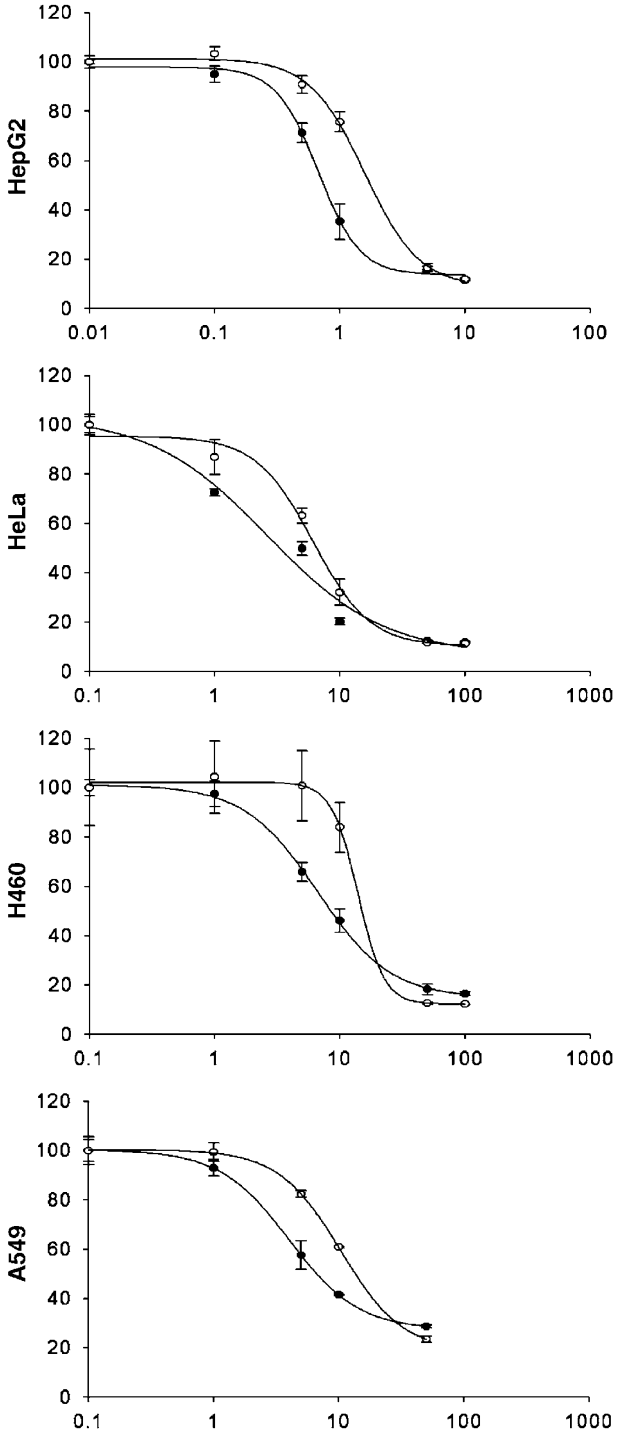

Figure 5 Sensitizing effect of Ad.CMV.FANCA600DN for cisplatin in several cell types. Cytotoxicity curves of cisplatin-treated cells infected with Ad.CMV.FANCA600DN (●) and Ad.GFP (○). HepG2, HeLa, A549 and H460 cells were infected with 100, 200 and 300 PFU/cell (A549 and H460), respectively. The mean and SD of a representative experiment is shown.

infection efficiency of the NSCLC cancer cell lines used was also assessed by determining the percentage of GFP expression after Ad.GFP infection, confirming the infection efficiencies in the range of 30–40% (see Table 1).

Since a possible bystander effect of FANCA600DN overexpression can be neglected one can assume that, at 100% infection efficiency levels, the total sensitization achieved ranges approximately between four- and seven-fold.

Discussion

Resistance of tumor cells and the occurrence of dose-limiting toxicities form the most important restrictions to the success of chemotherapy. Therapeutic approaches that will lead to the selective sensitization of tumor cells may overcome these limitations and broaden the therapeutic window. As an approach to achieve this, we addressed the possibility of using the FA pathway in cancer cells as a target to induce sensitivity for a potent class of anticancer agents, the crosslinking agents. In this study, cisplatin is used rather than other crosslinking agents such as mitomycin C because of its superior therapeutic effect in many human tumor types, including lung cancer cells. First, we demonstrated the favorable characteristics of a tumor with a defective FA pathway versus one with a functional pathway in a xenograft mice model. FA-lymphoblast-derived tumors showed a clear improved antitumor response after cisplatin treatment when compared with tumors from normal lymphoblasts. Moreover, a low concentration of cisplatin (2 mg/kg) showed a strong antitumor response in the FA tumors, thus illustrating that the FA pathway can be a valuable target for improving the curative potential of cisplatin. Subsequently, a gene therapy approach was developed, employing an adenovirus, to interfere with the FA pathway in solid tumor cells. A DN carboxyl-terminal truncated FANCA variant that we previously showed to be able to sensitize HeLa cells[20] was inserted in an adenoviral vector and characterized for its ability to interfere with the FA pathway. FA pathway disruption was demonstrated in different ways: first, in FANCG pull-down experiments FANCA600DN competed with endogenous FANCA binding; second, Ad.FANCA600DN-infected cells expressing high levels of FANCA600DN showed a decrease in endogenous FANCA expression determined by Western blotting that can be explained by the previously reported finding that FANCA is stabilized in the FA complex;[24] and third, Ad.FANCA600DN-infected Hela cells showed an approximately 60% reduction in cisplatin-dependent FANCD2 monoubiquitination. Subsequently, the sensitizing potential of the virus was examined in a panel of tumor cell lines. The cell lines tested could be sensitized two to three-fold for cisplatin, although the sensitization effect can be assumed to be higher (ranging from four- to seven-fold) when corrected for infection efficiency. Whether this increase in sensitivity translates into an improved antitumor response in mice models remains to be determined. Depending on the outcome of such experiments, it is currently too early to predict the possible therapeutic benefit in a clinical setting. Interesting in this respect is a recent report in which it was proposed that a defective FA pathway, through methylation of the FANCF promoter, may explain the relative high sensitivity of ovarian tumors to cisplatin in the clinic and, moreover, that its often-occurring onset of resistance during treatment may be due to demethylation of the FANCF promoter.[27] Whether this assumption is correct remains to be demonstrated and the Ad.FANCA600DN virus could be a helpful tool in such studies. However, several other cellular factors can be hypothesized to interfere with FANCA600DN-dependent sensitization, including factors that might act at the same level or downstream FANCD2 (such as the gene product defective in the newly discovered FANCJ complementation group[8]) and the BRCA1 and BRCA2 proteins.[28–32] Alternatively, other drug resistance mechanisms may compensate for virus-induced disruption of the FA pathway such as increased nucleotide excision repair (NER) or mismatch repair.

Regardless of the above, our current study indicates that in cervical, hepatoma and NSCLC cell lines Ad.FANCA600DN infection results in sensitization towards cisplatin. Whether gene therapy strategy will be effective remains to be studied further. A clear limitation of the Ad.FANCA600DN approach is the limited infection efficiency of the virus, especially when taking into account the *in vivo* structure of tumors that strongly reduces the penetration of a nonreplicating adenovirus and thus antitumor activity. No significant bystander effect can be expected from FANCA600DN overexpression apart from perhaps a virus-induced immune response *in vivo*. An alternative is to make use of conditionally replicating adenoviruses (CRAds) that can specifically replicate in tumor cells, thus facilitating the spreading of an inserted therapeutic gene.[33] However it has to be shown whether insertion of an FANCA600DN expression cassette in CRAds has additional therapeutic value; our preliminary studies indicate no such benefit (unpublished data). Currently, we are examining the sensitizing effect of Ad.CMV.FANCA600DN in tumor cells that have acquired different drug resistance mechanisms, which will give valuable information on the relationship between the FA pathway and other drug resistance mechanisms and the sensitivity to cisplatin.

In conclusion, Ad.CMV.FANCA600DN is a useful tool to determine the applicability of targeting the FA pathway as a novel chemosensitizing strategy and as a tool to understand the molecular basis of the functioning of the FA pathway in varying cancer cell types and in relation to other drug resistance pathways.

Acknowledgments

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