(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 October 2010 (21.10.2010)

PCT

(10) International Publication Number
WO 2010/118832 A2

(51) International Patent Classification:
A61K 38/17 (2006.01) *A61K 48/00* (2006.01)

(21) International Application Number:
PCT/EP2010/002128

(22) International Filing Date:
29 March 2010 (29.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09075182.7 17 April 2009 (17.04.2009) EP

(71) Applicant *(for all designated States except US)*: **FRAUNHOFER GESELLSCHAFT ZUR FÖRDERUNG DER ANGEWANDTEN FORSCHUNG E. V.** [DE/DE]; Hansastraße 27c, 80686 München (DE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **BORLAK, Jürgen** [DE/DE]; Arpker Str. 12 G, 31275 Lehrte / lmmensen (DE). **LEHNER, Frank** [DE/DE]; Rehbocksweg 2, 30916 lsemhagen (DE). **KLEMPNAUER, Jürgen** [DE/DE]; Alter Winkel 10, 31515 Steinhude (DE).

(74) Agent: **BAUMBACH, Fritz**; Robert-Rössle-Strasse 10, 13125 Berlin (DE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/118832 A2

(54) Title: INHIBITION OF THE LIVER ENRICHED PROTEIN FOXA2 FOR THE TREATMENT OF COLORECTAL LIVER METASTASES

(57) Abstract: The invention is directed to the use of substances decreasing the expression or activity of Foxa2 and/or increasing the expression or activity of HNF6 in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases. Areas of application are the life sciences and the pharmaceutical industry. In one aspect, the invention provides a medicament and medication, compositions, and substances, and the use of said compositions and substances for preventing and treating liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases. The invention is based on the surprising finding that siRNA-mediated gene silencing recovers HNF6 activity in Colorectal adenocarcinoma cells and that transfection of a plasmid encoding HNF6 significantly reduces the proliferation of both Colorectal adenocarcinoma cells and Human hepatocellular liver carcinoma cells and results in cell cycle arrest of said cells. In particular, the invention therefore relates to a composition that - decreases or inhibits the expression or activity of Foxa2 and/or increases the expression or activity of HNF6 for use in the treatment of tumor cells and as a medicament (or for the use as a medicament, respectively) for the prevention or treatment of liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, more particular for preventing or treating colorectal liver metastases, or for the prevention or treatment of colorectal adenocarcinoma or for the prevention or treatment of hepatocellular carcinoma.

Inhibition of the liver enriched protein FOXA2 for the treatment of colorectal liver metastases

The invention is directed to the use of substances decreasing the expression or activity of Foxa2 and/or increasing the expression or activity of HNF6 in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases. Areas of application are the life sciences and the pharmaceutical industry.

Colorectal cancer is the second leading cause of cancer death in the world. Nearly 800,000 new cases are diagnosed each year, and approximately 500,000 deaths have been estimated annually for the US alone (1, 2). As of today, the molecular causes of metastatic spread of tumor cells are unknown, and research is needed to improve an understanding of disease to enable novel treatment opportunities.

The aim of the present invention is thus to make available a medicament and medication, compositions, and substances, and the use of said compositions and substances for preventing and treating liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases.

To this end, the implementation of the actions and embodiments as described in the claims provides appropriate means to fulfill these demands in a satisfying manner.

Thus, the invention in its different aspects and embodiments is implemented according to the claims.

The invention is based on the surprising finding that siRNA-mediated gene silencing recovers HNF6 activity in Colorectal adenocarcinoma cells and that transfection of a plasmid encoding HNF6 significantly reduces the proliferation of both Colorectal adenocarcinoma cells and Human hepatocellular liver carcinoma cells and results in cell cycle arrest of said cells.

In a first aspect, the invention therefore relates to a composition that

- decreases or inhibits the expression or activity of Foxa2 and/or
- increases the expression or activity of HNF6

for use in the treatment of tumor cells and as a medicament (or for the use as a medicament, respectively) for the prevention or treatment of liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, more particular for preventing or treating colorectal liver metastases, or for the prevention or treatment of colorectal adenocarcinoma or for the prevention or treatment of hepatocellular carcinoma.

In one preferred embodiment, the medicament according to the invention comprises a composition that decreases or inhibits the expression or activity of Foxa2 and is used for the prevention or treatment of liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases.

In another preferred embodiment, the medicament according to the invention comprises a composition that increases the expression or activity of HNF6 and is used for the treatment of colorectal adenocarcinoma cells or of hepatocellular carcinoma cells, or of colorectal adenocarcinoma or hepatocellular carcinoma, respectively.

Within the context of the invention, colorectal adenocarcinoma in particular refers to epithelial colorectal adenocarcinoma and colorectal adenocarcinoma cells in particular refer to epithelial colorectal adenocarcinoma cells.

Preferably, the medicament or medication according to the invention further comprises a pharmaceutically acceptable carrier and/or recipient and/or diluent.

In particular, the medicament or medication comprises a composition that decreases or inhibits the expression or activity of the gene Foxa2 or of its gene product and/or increases the expression or activity of the gene HNF6 or of its gene products in a mammal preferably in a human being or a mouse, more preferably in a human being.

The genes according to the invention concern genes of mammalia, preferably genes of the genome of mus musculus or homo sapiens, in particular the respective genes of homo sapiens are preferred.

Preferably, the medicament or medication according to the invention comprises a pharmaceutically effective amount of a composition or of a vector as detailed below.

Within the context of the present invention an antisense composition is provided, in particular for use in the treatment of tumor cells, preferably of primary or secondary liver malignancies, more preferably of colorectal liver metastases, wherein the antisense composition comprises a nucleotide sequence complementary to a coding or ORF sequence of Foxa2.

The term "Foxa2" according to the invention is in particular directed to the coding sequence of the gene Foxa2. Also, the term "gene Foxa2" according to the invention is in particular directed to the coding sequence of the gene Foxa2.
The term "HNF6" according to the invention is in particular directed to the coding sequence of the gene HNF6.

In this regard, the term "coding sequence" is directed to the portion of an mRNA which actually codes for a protein. Within the context of Foxa2 the term "coding sequence" or "ORF" according to the invention is in particular directed to residues 186-1577 of the polynucleotide sequence as set forth in SEQ ID NO: 1. The term "nucleotide sequence complementary to a coding sequence" in particular is directed to an oligonucleotide compound, preferably RNA or DNA, more preferably DNA, which is complementary to a portion of an mRNA, in particular to residues 186-1577 of the polynucleotide sequence as set forth in SEQ ID NO: 1, and which hybridizes to and prevents translation of the mRNA. Preferably, the antisense DNA is complementary to the 5' regulatory sequence or the 5' portion of the coding sequence of said mRNA.
It is preferred that the antisense composition comprises a nucleotide sequence containing between 10-40 nucleotides , preferably 12 to 25 nucleotides, and having a base sequence effective to hybridize to a region of processed or preprocessed mammalian, preferably human or murine, more preferably human mRNA. In

particular, it is preferrend if the antisense composition comprises a nucleotide sequence containing between 10-40 consecutive nucleotides, preferably 12 to 25 consecutive nucleotides, from the sequence of residues 666-2056 as set forth in SEQ ID NO: 2.

In particular, the composition comprises a nucleotide sequence effective to form a base-paired heteroduplex structure composed of mammalian, preferably human or murine, more preferably human RNA transcript and the oligonucleotide compound, whereby this structure is characterized by a Tm of dissociation of at least 45°C.

Further, an siRNA composition is provided, in particular for use in the treatment of tumor cells, preferably of primary or secondary liver malignancies, more preferably of colorectal liver metastases, wherein the siRNA composition comprises an siRNA reducing or preferably inhibiting the expression of Foxa2.

The present invention employs siRNA for use in modulating the level of protein presence in the cell. SiRNA oligonucleotides directed to Foxa2 specifically hybridize nucleic acids encoding the gene product of Foxa2 and interfere with gene expression of Foxa2.

Preferably, the siRNA composition comprises siRNA (double stranded RNA) that corresponds to the nucleic acid ORF sequence of the gene product coded by mammalian, preferably human or murine, more preferably human Foxa2 or a subsequence thereof; wherein the subsequence is 19, 20, 21, 22, 23, 24, or 25 contiguous RNA nucleotides in length and contains sequences that are complementary and non-complementary to at least a portion of the mRNA coding sequence.

In one aspect, the invention concerns the use of an siRNA composition in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, wherein the siRNA composition comprises a siRNA forming a double stranded duplex which consists of 15-30 base pairs and wherein said duplex comprises a (first) RNA sequence complementary to 15-30 consecutive nucleotides of SEQ ID No:1, in particular complementary to 15-30

consecutive nucleotides from the sequence of residues 187-1577 as set forth in SEQ ID No:1

In one embodiment, the siRNA composition according to the invention comprises

- a first (antisense) RNA sequence or RNA strand, wherein said first sequence or first strand consists of 15-30 consecutive nucleotides of SEQ ID NO: 2, preferably of 15-30 consecutive nucleotides from the sequence of residues 666-2056 as set forth in SEQ ID No:2, and
- a second (sense) RNA sequence or RNA strand, wherein said second sequence or second strand consists of 15-30 consecutive residues of SEQ ID NO. 1, preferably of 15-30 consecutive nucleotides from the sequence of residues 187-1577 as set forth in SEQ ID No:1.

In another embodiment, the siRNA composition according to the invention comprises a siRNA forming a double stranded duplex which consists of 15-30 base pairs and wherein said duplex comprises a nucleotide sequence complementary to 15-30 consecutive nucleotides of a Foxa2 sequence selected from the group consisting of the sequences SEQ ID NOs:8-11 .

In a particular preferred embodiment, the siRNA composition according to the invention comprises a RNA strand consisting of 45-300 nucleotides, wherein said strand comprises

- a first (antisense) RNA sequence consisting of 15-30 consecutive nucleotides of SEQ ID NO: 2, preferably of 15-30 consecutive nucleotides from the sequence of residues 666-2056 as set forth in SEQ ID No:2, and
- a second (sense) RNA sequence consisting of 15-30 consecutive residues of SEQ ID NO: 1, preferably of 15-30 consecutive nucleotides from the sequence of residues 187-1577 as set forth in SEQ ID No:1, wherein the first RNA sequence is preferably located downstream of the first RNA sequence, and
- a third RNA sequence connecting the first and the second RNA sequence and consisting of 1-100 nucleotides, which preferably form a loop and/or a hairpin structure, and wherein the second RNA sequence comprises a nucleotide sequence complementary to 15-30 nucleotides of the first RNA sequence.

A further embodiment concerns the siRNA composition according to the invention, wherein the first RNA sequence is selected from the group consisting of SEQ ID NOs: 5-9 and/or wherein the second RNA sequence is selected from the group consisting of SEQ ID NOs: 10-13, and wherein preferably the combination of SEQ ID NO: 6 and SEQ ID NO:10 or the combination of SEQ ID NO: 7 and SEQ ID NO 11 or the combination of SEQ ID NO: 8 and SEQ ID NO 12 or the combination of SEQ ID NO: 9 and SEQ ID NO 13 is selected.

In yet another embodiment, the siRNA composition according to the invention comprises a first RNA strand consisting of a sequence selected from the SEQ ID NOs: 5-9 and/or comprises a second RNA strand consisting of a sequence selected from the SEQ ID NOs 10-13 strand, and wherein preferably the combination of SEQ ID NO: 6 and SEQ ID NO:10 or the combination of SEQ ID NO: 7 and SEQ ID NO 11 or the combination of SEQ ID NO: 8 and SEQ ID NO 12 or the combination of SEQ ID NO: 9 and SEQ ID NO 13 is selected.

In another aspect the invention is also directed to a vector comprising

- a promoter and
- a DNA sequence operably linked to said promoter, wherein the DNA sequence encodes a RNA strand consisting of 45-300 nucleotides, wherein said strand comprises a first (antisense) RNA sequence consisting of 15-30 consecutive nucleotides of SEQ ID NO: 2, preferably of 15-30 consecutive nucleotides from the sequence of residues 666-2056 as set forth in SEQ ID No:2, and comprises a second (sense) RNA sequence, preferably located downstream of the first RNA sequence, consisting of 15-30 consecutive residues of SEQ ID NO: 1, preferably of 15-30 consecutive nucleotides from the sequence of residues 187-1577 as set forth in SEQ ID No:1, and comprises a third RNA sequence connecting the first and the second RNA sequence and consisting of 1-100 nucleotides, which preferably form a loop and/or a hairpin structure, and wherein the second RNA sequence comprises a nucleotide sequence complementary to 15-30 nucleotides of the first RNA sequence,

and wherein the promoter is preferably a suitable promoter for gene expression in cells of colorectal liver metastases, in particular the Foxa2 promoter.

According to the invention it is in particular understood that the Foxa2 promoter is a nucleotide sequence comprising the Foxa2 binding site having the sequence TTTGTTTGTTTG.

Within the context of the invention the term "vector" refers to any DNA molecule usable as a vehicle to transfer foreign genetic material (transgene) into a cell, in particular refers to plasmids, viruses, artificial chromosomes, and cosmids. In particular the term "vector" is directed to a vector for the transcription of the transgene, e.g. encoding the siRNA or other nucleotide sequences as described herein, in the target cell.

The term "operably linked" according to the invention means that the promoter drives expression of the transgene, e.g. of the siRNA or the nucleotide sequences as described herein, in the target cell.

The nucleotide sequences and siRNA according to the invention may be prepared by any standard method for producing a nucleotide sequence or siRNA, such as by recombinant methods, in particular synthetic nucleotide sequences and siRNA is preferred.

Furthermore, an antibody composition is provided, wherein the antibody composition comprises a pharmaceutically effective amount of an antibody or fragment thereof that specifically binds to a polypeptide encoded by the gene Foxa2, in particular to a polypeptide having the sequence as set forth in SEQ ID NO:3.

Within the inventive context, antibodies are understood to include monoclonal antibodies and polyclonal antibodies and antibody fragments (e.g., Fab, and $F(ab')_2$) specific for one of said polypeptides. Polyclonal antibodies against selected antigens may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats.

Preferably, monoclonal antibodies are used in the antibody compositions of the invention which may be readily generated using conventional techniques (see Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A

Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are incorporated herein by reference).

Further, a polypeptide composition (also termed as polypeptide composition (1)) is provided, wherein the polypeptide composition comprises a polypeptide coded by the sequence of the gene HNF6, preferably coded by the polynucleotide sequence consisting of the sequence set forth in SEQ ID NO:12 .
Preferably, the polypeptide composition comprises a peptide coded by the entire ORF/coding sequence of HNF6. Such peptides include isolated polypeptides comprising an amino acid sequence which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, most preferably at least 97-99% identity, in particular 100 % identity, to the entire amino acid sequence of the gene product of HNF6, in particular to amino acid sequence consisting of the sequence set forth in SEQ ID NO:13.

Within the context of HNF6 the term "coding sequence" or "ORF" according to the invention is in particular directed to the sequence residues 1-1398 of the polynucleotide sequence set forth in SEQ ID NO: 12.

Polypeptides of the present invention can be prepared in any suitable manner. Such polypeptides include isolated naturally occurring polypeptides, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by a combination of these methods. Means for preparing such polypeptides are well understood in the art.

A further aspect of the invention concerns the use of a composition that decreases or inhibits the expression or activity of the gene Foxa2 or of its gene product and/or increases the expression or activity of the gene HNF6 or of its gene products for the preparation of a medicament or medication, preferably for the preparation of a medicament or medication for preventing, treating, or ameliorating liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases.

In particular, an antisense composition, an siRNA composition, an antibody composition or the polypeptide composition (1) as detailed herein, or a combination thereof, is used for the preparation of the medicament or medication.

In one embodiment, a nucleotide composition (also termed as nucleotide composition (1)) is used for the preparation of said medicament or medication, wherein the nucleotide composition comprises a nucleotide sequence of the gene HNF6.

The invention is thus also directed to the use of a nucleotide composition comprising a nucleotide sequence of HNF6 in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells.

The nucleotide composition particularly comprises a nucleic acid being from about 20 base pairs to about 100,000 base pairs in length, wherein the nucleotide sequence is included. Preferably the nucleic acid is from about 50 base pairs to about 50,000 base pairs in length. More preferably the nucleic acid is from about 50 base pairs to about 10,000 base pairs in length. Most preferred is a nucleic acid from about 50 pairs to about 4,000 base pairs in length. The nucleotide sequence can be a gene or gene fragment that encodes a protein, an oligopeptide or a peptide. Preferably, the nucleotide sequence of the present invention may comprise a DNA construct capable of generating a gene product coded by HNF6 and may further include an active constitutive or inducible promoter sequence.

In particular the nucleotide composition comprises a nucleotide sequence encoding a polypeptide which has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, to the amino acid sequence of the gene product of HNF6, in particular to the amino acid sequence consisting of the sequence set forth in SEQ ID 13.

In this regard, nucleotide sequences coding for polypeptides which have at least 97% identity are highly preferred, whilst those with at least 98-99% identity are more preferred, and those with at least 99% identity are most preferred. In particular, it is preferred if the nucleotide sequence encodes a polypeptide with 100 % identity to the

entire amino acid sequence of the gene product of HNF6, in particular to the amino acid sequence consisting of the sequence set forth in SEQ ID 13.

In particular, the nucleotide composition comprises a DNA sequence that has at least 70% identity, preferably at least 80% identity, more preferably at least 90% identity, yet more preferably at least 95% identity, to the ORF (or coding sequence, respectively) of one of HNF6 over the entire coding region. Within the context of HNF6 the term "ORF" or "coding sequence" according to the invention is in particular directed to the polynucleotide sequence consisting of the sequence set forth in SEQ ID NO: 12. In this regard, nucleoetide sequences which have at least 97% identity are highly preferred, whilst those with at least 98-99% identity are more highly preferred, and those with at least 99% identity are most highly preferred. In particular, it is preferred if the nucleotide sequence encodes a DNA sequence that has 100 % identity to the entire ORF of HNF6 over the entire coding region.

In one embodiment, the nucleotide composition according to the invention comprises a DNA sequence which hybridizes to the complementary strand of the polynucleotide sequence consisting of the sequence set forth in SEQ ID NO: 12 and encodes a protein having the biological activity of HNF6, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells.

In a preferred embodiment, the nucleotide composition according to the invention comprises a DNA sequence encoding a protein having the sequence as set forth in SEQ ID NO: 13.

In a particular preferred embodiment, the nucleotide composition according to the invention comprises a DNA sequence consisting of the sequence set forth in SEQ ID NO:12.

In a further aspect the invention is also directed the use of a vector in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, wherein the vector comprises a promoter and a DNA

sequence operably linked to said promotor, and wherein the DNA sequence is the DNA sequence or the nucleotide sequence comprised by the nucleotide composition described herein.

In particular, said DNA sequence is selected from the group consisting of

- the DNA sequences which hybridize to the complementary strand of the polynucleotide sequence consisting of the sequence set forth in SEQ ID NO: 12 and encode a protein having the biological activity of HNF6,
- the DNA sequences encoding a protein having the sequence as set forth in SEQ ID NO: 13,
- the DNA sequence consisting of the sequence set forth in SEQ ID NO:12

and said promoter is preferably selected from the group consisting of

- a suitable promoter for gene expression in cells of colorectal liver metastases, in particular the Foxa2 promoter,
- a suitable promoter for gene expression in colon cells, in particular a promoter of a colon specific gene selected from the group consisting of CA1 (carbonic anhydrase 1), CEA (carcinoembryonic antigen), and Mucin1,
- suitable promoter for gene expression in liver cells, in particular the HBV core promoter or a promoter of a liver specific gene selected from the group consisting of Albumin, Phosphoenolpyruvatcarboxykinase and Ornithintranscarbamylase.

The nucleotide composition or vector according to the invention may further comprise an enhancer element and/or a promoter located 5' to and controlling the expression of said therapeutic nucleotide sequence or gene. The promoter is a DNA segment that contains a DNA sequence that controls the expression of a gene located 3' or downstream of the promoter. The promoter preferably is the DNA sequence to which RNA polymerase specifically binds and initiates RNA synthesis (transcription) of that gene, typically located 3' of the promoter.

Preferably, a pharmaceutically effective amount of one of the compositions specified above or a pharmaceutically effective amount of a combination thereof is used, in particular furthermore a pharmaceutically acceptable carrier and/or recipient and/or diluent is used for the preparation of the medicament or medication.

Preferably, one or more of said genes and/or their gene products is incubated with a compound to be tested and changes in the expression of said genes and/or derived sequences and/or the function of said gene products are determined. In particular, the antisense composition, the siRNA composition, the antibody composition, the nucleotide composition (1) or the polypeptide composition (1), as detailed herein, or a combination thereof, is used as test compound.

More preferably, said use for screening and identifying drugs comprises the steps of: (1) contacting a test cell expressing, preferably overexpressing, at least one of said genes with a test compound; (2) detecting the expression level of said gene; and (3) determining the compound that suppresses said expression level compared to a normal control level of said gene as an inhibitor of said gene.

According to the invention it is particularly preferred, if the test cell is a Colorectal adenocarcinoma cell, in particular a CACO-2 cell, or, more preferably a human hepatocellular liver carcinoma cell, in particular a HepG2 cell.

In particular, a compound that enhances the expression or activity of HNF6 is identified by the inventive use comprising the steps of : (1) contacting a test cell expressing, in particular overexpressing, Foxa2 with a test compound; (2) detecting the expression level of Foxa2; and (3) determining the compound that decreases said expression level compared to a normal control level of said gene as an enhancer of HNF6.

Within this context, it is particularly preferred that the use comprises the steps of : (1) contacting a test compound with a polypeptide encoded by Foxa2; (2) detecting the binding activity between the polypeptide and the test compound; and (3) selecting a compound that binds to the polypeptide.

More specifically, said use comprises the steps of (a) contacting a test compound with a polypeptide encoded by the selected gene, in particular encoded by Foxa2; (b) detecting the biological activity of the polypeptide of step (a); and (c) selecting a compound that suppresses the biological activity of the polypeptide encoded by the

gene Foxa2 in comparison with the biological activity detected in the absence of the test compound,
and/or enhances the biological activity of the polypeptide encoded by the polynucleotide HNF6
in comparison with the biological activity detected in the absence of the test compound. Advantegeously, cell proliferation is detected as biological activity, or any other biological activity of the cell related to carcinogenesis or tumorigenesis, in particular the presence of tumor markers known in the art, is detected.

In a further preferred embodiment the use comprises the steps of : (1) contacting a test compound with a cell into which a vector comprising the transcriptional regulatory region, in particular a promoter as mentioned herein, of one or more of the selected genes, in particular of Foxa2 and/or HNF6, and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced; (2) measuring the activity of said reporter gene; and (3) selecting a compound that reduces the expression level of said reporter gene when the selected gene is an up-regulated gene selected from the group consisting of Foxa2.
and/or that enhances the expression level of said reporter gene when the selected gene is a down-regulated gene selected from the group consisting of HNF6, as compared to a control.

More particular, the use comprises drugs, in particular a test compound and/or a medicament or medication as specified above, wherein the drugs regulate the expression of one or more of said genes and/or the function of one or more of said gene products and/or their derived molecules, and said drugs are used for the production of means for preventing, treating, or ameliorating liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases.

In one embodiment of the use for screening and identifying drugs DNA and/or related molecules encoding one or more of said gene products and/or derived structures are used, and/or one or more polypeptides, peptides and/or derived molecules having the function of one or more of said gene products, are used.

As a conclusion, restoring HNF6 activity, in particular by using a composition or vector as claimed in one of the claims 4-20, is used according the invention to prevent disease progression and growth of colorectal liver metastases.

In summary, inhibition of Foxa2, in particular by using a composition or vector as claimed in one of the claims 4-11 or 20, preferably by said method, is used according to the invention for the prevention or treatment colorectal liver metastases.

Further scope of applicability of the present invention will become apparent from the detailed description given hereinafter. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description:

Recently, the regulation of some major hepatic nuclear factors in primary human colon cancer and colorectal liver metastases has been reported (3). HNF6 expression was found to be absent in healthy colon or primary colon cancer, but abundant expression of unacetylated HNF6 was observed in nuclear extracts of colorectal liver metastases. However, unacteylated HNF6 was unable to bind to DNA and genes regulated by this factor. Because of its known interaction with HNF6, expression of FOXA2 was investigated, which was found to be highly upregulated in colorectal liver metastases. There is evidence for HNF6 to serve as a coactivator thereby enhancing FOXA2 transcription, but FOXA2 represses HNF6 transcription and genes targeted by this transcription factor.
Based on own initial results and of findings reported by others on the role of liver-enriched transcription factors in growth and differentiation of carcinomas it was wished to probe for the role of FOXA2 in the regulation of HNF6 activity in colorectal liver metastases (4-6). Therefore the consequences of functional knockdown of FOXA2 on HNF6 DNA binding activity in the human colon cancer cell line Caco-2 were studied. Also, the role of HNF6 on cell cycle regulation in the human hepatoma cell line HepG2 was investigated. Overall, the study aimed for an improved understanding of an inhibitory crosstalk between FOXA2 and HNF6 in metastasizing colon cancer.

Materials and methods

Cell culture

Caco-2 cells and HepG2 cells were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were cultured as described by Lampen et al. (Caco-2 cells) and Wilkening et al. (HepG2 cells) (7, 8).

RNA isolation and cDNA synthesis

RNA was isolated with the RNeasy Mini Kit (Quiagen) according to the manufacturer's recommendation and cDNA synthesis was carried out as recently reported (3).

Quantitative PCR analysis with the Roche Light Cycler System

Real time PCR was done with the LightCycler® according to the manufacture's recommendation (Roche Diagnostics, Penzberg, Germany) with oligonucleoitides previously reported (3). SYBR® Green I was used as a fluorescent dye to determine the amplified PCR product after each cycle. The length of PCR products was checked by gel electrophoresis. Gene expression of FOXA2 , C/EBPalpha, CYP51, HSP105B and HNF6 was determined with primers reported in (3) in a standard PCR reaction containing 50 ng of DNA, 4.0 mM MgCl2 and 2 µl of LightCycler DNA Master hybridisation mixture (LightCycler DNA Master Hybridization Probes, Roche Diagnostics Inc) in a total volume of 20 µl. The reaction was started with a denaturation step at 95^{0}C for 20 seconds and amplification was performed for 50 cycles denaturation (95^{0}C for 0 seconds; ramp rate 20^{0}C per second), annealing (58^{0}C for 8 seconds, ramp rate 20^{0}C per second) and extension (72^{0}C for 18 seconds, ramp rate 20^{0}C per second). In the case of the mitochondrial ATPase the reaction was started with a denaturation step at 95 ^{0} C for 20 seconds and amplification was performed for 50 cycles of denaturation (95^{0}C for 0 seconds; ramp rate 20^{0}C per second), annealing (55^{0}C for 8 seconds, ramp rate 20^{0}C per second) and extension (72^{0}C for 18 seconds, ramp rate 20^{0}C per second). PCR products were identified by monitoring DNA melting curves in the glas capillary. At the end of each extension phase fluorescence was observed and used for quantitative measurements within the linear range of amplification yielding calculated concentrations as relative units. Exact quantification was achieved by serial dilution with cDNA produced from total RNA extracts using serial dilution steps. The obtained

values were divided by those of mitochrondrial ATPase to obtain expression values relative to the housekeeping gene.

Design of EMSA-oligos for HNF6 binding sites in promotor sequences of human genes

Known binding sites of HNF6 (ONECUT1) were collected from the TRANSFAC database, which is a database on gene regulation (www.biobase.de). It collects data on transcription factors and their binding sites in promoters and enhancers of eukaryotic genes. The search was done with TRANSFAC release 9.4. Additionally, to retrieve promoters of human genes TRANSPro release 2.1 was used. The HNF6 matrix (M00639; V$HNF6_Q6) was employed and the search was based on 13 known binding sequences for HNF6. The design for the oligo probes for HNF6 and FOXA2 was optimized as reported previously (3).

Annealing of synthetic oligonucleotides and [32P] labeling

Oligonucleotides representing a high affinity consensus HNF6 binding site were chosen. Oligonucleotides were annealed at a concentration of 19.2 $pM \cdot \mu L^{-1}$ in 200 mM Tris (pH 7.6), 100 mM $MgCl_2$ and 500 mM NaCl at 80 °C for 10 min, then cooled slowly to room temperature overnight and stored at 4 °C. Annealed oligonucleotides were diluted to 1:10 in Tris-EDTA buffer (1 mM EDTA, 10 mM Tris, pH 8.0) and labelled using [32P] ATP (Amersham Biosciences Europe GmbH, Freiburg, Germany, 250 μCi, 3,000 $Ci \cdot mM^{-1}$) and T4 polynucleotide kinase (New England Biolabs GmbH, Frankfurt am Main, Germany). End-labelled probes were separated from unincorporated [32P] ATP with a Microspin G-25 Column (Amersham Biosciences Europe GmbH, Freiburg, Germany) and eluted in a final volume of 100 μL.

Electrophoretic mobility shift assay (EMSA)

The procedure for EMSA was adapted from a previously described method (9). Briefly, 5 μg of CaCo2 nuclear extract were incubated with the binding buffer consisting of 25 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 34 mM KCl, 2 mM DTT, 2 mM Pefablock (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μL aprotinin (2.2 $mg \cdot mL^{-1}$, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), 50 ng poly (dI-dC) and 80 ng bovine serum albumin (PAA Laboratories GmbH, Cölbe, Germany).

The binding reaction was carried out for 20 min on ice, and free DNA and DNA-protein complexes were resolved on a 6% polyacrylamide gel. For supershift studies, a specific HNF6 and/or HNF4alpha antibody (Santa Cruz Biotechnology Inc., Heidelberg, Germany) was added to the reaction mix 10 min before addition of the labelled probe. In the case of NGN3, no commercial antibody is available. Thus, a competition assay at x100 and x500-fold access of unlabeled oligonucelotide probe specific to NGN3 was used. Gels were blotted to Whatman 3 MM paper, dried under vacuum, exposed to imaging screens (Imaging Screen-K, Bio-Rad Laboratories GmbH, Munich, Germany) for autoradiography overnight at room temperature and analysed using a phosphor imaging system (Molecular Imager FX pro plus; Bio-Rad Laboratories GmbH, Munich, Germany) and the Quantity One Version 4.2.2 software (Bio-Rad Laboratories GmbH, Munich, Germany).

Transfection of HNF6 into Caco-2 cells and the HepG2 human hepatoma cell line

To confirm the proposed inhibitory cross-talk of FOXA2 and HNF6 the colon carcinoma cell line Caco-2 cells and the human hepatoma cell line HepG2 were transfected with a HNF6 containing plasmid. In the case of the Caco-2 cells the expression plasmid was transfected with Lipofectamine 2000 (Invitrogen), a cationic-lipid transfection reagent according to the manufacture's recommendation, whereas in the case of HepG2 cells a retroviral transfection of plasmids was carried out as originally described by Soneoka et al. (10).

Briefly, two vectors were received from CE Pierreux to enable efficient transfection of HNF6 (Hormone and Metabolic Research Unit, Institute of Cellular Pathology and Université Catholique de Louvain, Brussels, Belgium). HNF6 was cloned into (a) V-831, pCMV-MCS (Stratagene), a mammalian expression vector containing a multiple cloning site (MCS), CMV promoter, and other elements for high-level gene expression; (b) V-894, pIRES2-EGFP (Clontech, discontinued), which contains the internal ribosome entry site (RES; 1,2) of the encephalomyocarditis virus (ECMV) between the MCS and the enhanced green fluorescent protein (eGFP) coding region. This permitted both the gene of interest (cloned into the MCS) and the eGFP gene to be translated from a single bicistronic mRNA. Plasmid DNA was isolated using the Maxiprep endotoxin-free kit (Qiagen). The DNA was precipitated with EtOH at 4000 rpm for 90 min and 2x washings at 4000 rpm for 60 min. The HNF6 insert was

confirmed by RLFP with *Bam*HI/*Eco*RI for V-894 and *Bam*HI/*Xho*I for V-831. The product was approximately 1.6 kb after restriction enzyme digestion.

Cells (200,000 per 35-mm well) were seeded in a six-well culture plate containing 2 ml per well of Dulbeco's modified Eagles medium (DMEM) with fetal calf serum (end conc. 8.7 %), glutamine (end conc. 2x), and penicillin/streptomycin (end conc. 2 x). Cells were grown at 37 °C in a humidified 5% CO_2/approx. 95% air atmosphere. The medium was prepared as follows: 500 ml DMEM + 50 ml of 10% FCS + 12 ml of 100x L-glutamine and 12 ml of 100x penicillin/streptomycin.

Prior to transfection (1 h), DMEM medium was removed and cells were washed with PBS. The medium was then replaced with Opti-MEM I medium, a versatile chemically defined medium formulated to significantly reduce the amount of serum required for cultivating mammalian cells *in vitro*. It is a modification of Eagle's Minimal Essential Medium, buffered with HEPES and sodium bicarbonate, and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine or GlutaMAX, trace elements, and growth factors. The protein level is minimal (15 mg/ml), with insulin and transferrin being the only protein supplements. Phenol red is included at a reduced concentration as a pH indicator. Transfection was carried out using Lipofectamine 2000 (Invitrogen), a cationic-lipid transfection reagent according to the manufacture's recommendation. Efficiency of transfection was assed qualitatively by fluorescent microscopy of which an example is given in Fig. 1a.

In the case of HepG2, the HNF6 containing plasmid (see above) was cloned into the pczCFG5.1MCS vector, which is one necessary component of the three-vector transduction system. In order to produce replication-deficient retroviral particles genes encoding the structural and envelope proteins, as well as the HNF6 coding plasmid were transfect into the human embryonic kidney 293T packaging cells. Then, the viral particles derived from the HEK293T cell line were used to transfect HNF6 into HepG2 cells.

Small-interference FOXA2 RNA (siRNA)-mediated knockdown in Caco-2

Caco-2 cells were cultured to 70-80% of confluence and were transfected with 3 different FOXA2 siRNA probes (see FOXA2 Stealth^{Tm} (Invitrogen) as originally designed by Invitrogen. These probes were used according to the manufacture's recommendations and allowed verification of phenotypic changes as well as control of off-target effects. Transfection efficency was controlled by the Block-iT^{TM} Alexa

Fluor® Red Fluorescent Oligo (Invitrogen). This red-labeled dsRNA oligomer is designed for use in RNAi experiments to facilitate assessment and optimization of dsRNA oligonucleotides delivery into mammalian cells by use of cationic lipids (Lipofectamine 2000). In Fig. 2 images of individual FOXA2 siRNA probes transfections and of BLOCK-IT Alexa Fluor Red Fluorescent Oligo as well as a negative control are depicted. Notably, Caco-2 cells were incubated with the various FOXA2 siRNA oligonucleotide for 48 h to down regulate FOXA2 expression. FITC-labeled scrambled siRNA (Control-FITC block-it fluorescent Oligo #2013, Invitrogen, Germany) was used as a negative and transfection control.

Measurement of RNAi activity

Quantitative RT-PCR was applied to determine the gene expression of FOXA2 after siRNA knock-down and of HNF6 and genes regulated by this factor, i.e. C/EBPalpha, HSP105B and CYP51 using the oligionucleotide probes and the protocol described above.

Cell cycle and cell proliferation assay

Cells were plated in 96-well microtiter plates at a density of 5000 cells/well 24 h prior to treatment. Cell cycle and cell proliferation were measured using the CycleTest Plus Reagent and the BrdU labeling kit according to the manufacture's recommendations (see below).

Cell cycle analysis

The effects of HNF6 recovery on the cell cycle were studied using flow cytometry analysis. Cells were plated in six-well sterile plastic plates at a density of 10^5 -2 x 10^5 cells/well and were allowed to attach for 24 h. Then cells were collected by trypsinization and DNA staining was performed with the CellTest Plus Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). According to the manufacturer's instruction cells were washed with a buffer solution containing sodium citrate, sucrose, and dimethyl sulfoxide (DMSO). Then cells were incubated according to a three-step sequence: a) 10 min at room temperature with solution A containing trypsin in a spermine tetrahydrochloride detergent buffer (to digest cell membranes and cytoskeleton); b) 10 min at room temperature with solution B containing a trypsin inhibitor and ribonuclease A in citrate-stabilizing buffer

with spermine terahydrochloride (to inhibit the trypsin activity and to digest RNA); c) 15 min in the refrigerator with solution C containing propidium iodide and spermine tetrahydrochloride in citrate-stabilizing buffer. Analysis was performed using a FACScan (Becton Dickinson GmbH Immunozytometrische Systeme, Heidelberg, Germany), and data analysis was carried out with CELLQuest software, while cell cycle distribution was determined using the Modifit software (Verity Software House, Inc.)

BrdU cell proliferation assay

BrdU incorporation was measured using the BrdU Cell Proliferation Assay (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Cells were labeled with BrdU (1:100) for the last 4 h of incubation. Cells were washed, fixated, and incubated with mouse anti-BrdU antibody (1:100; 100 µl/well) for 1 h at room temperature. Antibody labeling was detected by secondary peroxidase-coupled goat-anti-mouse antibody (1:1000, 100 µl/well; 30 min at room temperature). After washing, peroxidase substrate was added for 15 min. The peroxidase reaction was stopped by adding 100 ml 2.5N sulfuric acid, and absorbance was measured using dual wavelengths of 450 and 595 nm.

Results

Recovery of HNF6 expression in Caco-2 cell cultures

Initially, studies were carried out with the human colon adenocarcinoma cell line Caco-2. Unlike colorectal liver metastases, primary colon cancer and Caco-2 cells do not express detectable levels of HNF6 protein. Therefore an HNF6-containing plasmid was employed and its DNA binding activity was studied upon transfection. The expression permitted imaging of HNF6 (cloned into the MCS) by fluorescence microscopy. As shown in Fig. 1A it was possible to successfully transfect Caco-2 cells with HNF6. To further probe for HNF6 binding activity EMSA band shift assays were performed. As depicted in Fig. 1C and unlike controls HNF6 nuclear protein expression and DNA binding activity were observed. Notably, expression level of HNF6 in transfected Caco-2 cells was comparable to that of human liver, i.e. the positive control.

siRNA-mediated functional knockdown of FOXA2

Evidence from the own laboratory and other investigators suggests a regulatory loop of FOXA2 with HNF6 (3, 11). Essentially, HNF6 functions as a coactivator protein to potentiate the transcriptional activity of FOXA2 (11). Furthermore, it was shown that a C/EBPα-HNF6 protein complex stimulates HNF6 and FOXA2 transcriptional activity through recruitment of the CBP coactivator protein (12).

To further probe for an inhibitory FOXA2-HNF6 crosstalk a small-interference RNA-mediated knockdown of FOXA2 in Caco-2 cells was carried out at a confluency of about 70%. As shown in Fig. 2A a statistically significant nearly 80% knockdown of FOXA2 gene expression was achieved and reduced FOXA2 protein expression was observed, albeit at different levels when individual experiments were compared. Indeed, a total of 6 individual experiments were carried out and in 4 out of 6 experiments the data was robust and reliable suggesting that only some probes are efficient in silencing FOXA2 gene expression. Fig. 2B depicts the results of FOXA2 gene expression of three independent experiments. Also, HNF6 gene expression in Caco-2 cell cultures transfected with FOXA2 siRNA probes was studied (see Fig. 2B). Notably, functional knockdown of FOXA2 resulted in a significant 6-fold increase in HNF6 gene expression. The efficiency of the functional knock down by various FOXA2 siRNA probes is shown in Fig. 2C while in untreated Caco-2 cells FOXA2 gene expression was nearly twice that of HNF6 (see Fig. 2D), even though HNF6 transcripts were not translated into protein. Furthermore, the DNA binding of HNF6 after functional knock down of FOXA2 was investigated. As shown in Fig. 1D nuclear protein binding to an optimized HNF6 probe was significantly reduced when FOXA2 and HNF6 antibodies were used concomitantly. This suggests binding of FOXA2 to a HNF6 optimized oligonucleotide probe. Notably, only a faint band was seen in band shift assays with FOXA2 alone, while the HNF6 antibody was able to shift the band significantly.

Based on the study of Odom et al. (13), who employed a CHIP-chip protocol to identify HNF6 target genes, three genes targeted by this factor were selected. As shown in Fig. 3 siRNA-mediated functional knockdown of FOXA2 resulted in a 3-fold, 4-fold, and 8-fold increase in gene expression of HSP105B, CYP51, and C/EBPα, respectively.

Cell cycle and BrdU labeling experiments with the Caco-2 and HepG2 cell lines

As compared to the empty vector HNF6 transfection caused a highly significant cell cycle arrest in the G2/M and the G1 phase in Caco-2 and HepG2 cells, respectively. Likewise, cell proliferation was significantly reduced by 80% and 50% in HNF6-transfected Caco-2 and HepG2 cells (see Fig. 4).

Discussion

The study aimed for an improved understanding of a role of FOXA2 and HNF6 in secondary liver malignancies. Specifically, the human colon carcinoma and hepatoma cell lines Caco-2 and HepG2 enabled to address mechanistically the role of FOXA2 on HNF6 activity. HNF6 was therefore transfected into Caco-2 and HepG2 cells. The transfected protein was stable and DNA binding of HNF6 was observed as evidenced by electromobility band shift assays. Notably, the same optimized oligonucleotide probes were used to investigate DNA binding of HNF6 as reported in an initial study on human colorectal liver metastases (3). In this study no DNA binding of HNF6 was observed with extract of nuclear proteins isolated from colorectal metastatic liver tumors, even though abundant expression of the HNF6 protein was seen. In fact, HNF6 DNA binding was selectively abrogated through lack of posttranscriptional acetylation (3). In the present study transfection of the HNF6 protein in Caco-2 cell cultures was evidenced to result in HNF6 DNA binding activity (see Fig. 1C). Note, no HNF6 DNA binding activity was observed with control Caco-2 cell cultures (see Fig. 1C). Because of the presumed inhibitory crosstalk between FOXA2 and HNF6 the consequences of functional knockdown of FOXA2 on HNF6 gene expression was investigated. As shown in Fig. 2B an approximately 6-fold increase in HNF6 gene expression was determined for FOXA2 siRNA-transfected Caco-2 cell cultures. Thus, FOXA2 knockdown recovers HNF6 activity. HNF6 gene transcription was evidenced to be increased upon siRNA-mediated functional knockdown of FOXA2. Likewise, gene expression of HNF6-regulated genes, notably, HSP105B, CYP51, and C/EBPα is demonstrated to be significantly upregulated upon siRNA-mediated functional knockdown of FOXA2. Notably, functional knockdown of FOXA2 induced transcriptional regulation of C/EBPα, a transcription factor that causes arrests of cell proliferation through direct inhibition of Cdk2 and Cdk4 (14). Consequently, C/EBPα links HNF6 to cell cycle regulation. Here, inhibition of FOXA2

is shown to stimulate HNF6 activity, as evidenced by cell cycle analysis and BrdU cell proliferation assays, all of which demonstrates inhibition of growth, i.e. cell cycle arrest at the G1 and the G2/M phase (see Fig. 4). However, the role of FOXA2 in the regulation of HNF6 activity remains controversial. Some investigators suggest HNF6 to function as a coactivator protein to potentiate the transcriptional activity of FOXA2 (15), whereas others report HNF6 function to be independent of FOXA2 (16). In an initial study FOXA2 and HNF6 were reported to be key regulators in human colorectal liver metastases. HNF6 DNA binding was found to be selectively abrogated as a result of impaired HNF6 acetylation and interaction with FOXA2. In line with the clinical study HNF6 protein is now reported to be below the level of detection in Caco-2 cell cultures, even though expression of HNF6 mRNA could be evidenced but was approximately half of that observed for FOXA2. It is of considerable importance that siRNA-mediated functional knockdown of FOXA2 resulted in transcriptional activation of HNF6 and of genes targeted by this factor. The findings with the human colon cancer cell line Caco-2 agreed well with previous studies on the human hepatoma HepG2 cell line co-transfected with HNF6 or its deletion mutants as well as FOXA1, FOXA2, or FOXA3 TATA-luciferase reporter constructs (15). Also, within the context of the invention, HNF6 was transfected into HepG2 cells. This resulted in cell cycle arrest in the G1 phase. Likewise, cell proliferation was significantly reduced in the BrdU labeling assay, therefore confirming an important inhibitory role of HNF6 in the regulation of cell cycle progression and cell proliferation. Overall, results from the human Caco-2 and HepG2 cells agreed well. Importantly, FOXA2 protein was strongly induced in human colorectal liver metastases and the findings of the present study are highly suggestive for an inhibitory crosstalk of FOXA2 and HNF6 in colorectal liver metastases. There is a report to suggest HNF6 activity to be independent of FOXA2 (16) and in this conditional FOXA2 knockout mouse model targeted expression of HNF6 genes appeared to be independent of the presence of FOXA2. In the clinical study, however, HNF6 was not expressed in healthy or cancerous colon, but was abundantly expressed in nuclear extracts of colorectal liver metastatic tissue. Nonetheless, HNF6 DNA binding activity was selectively abrogated in colorectal liver metastases. Thus, HNF6 appeared to be detrimental to malignantly transformed cells. Further evidence stems from siRNA-mediated functional knockdown of FOXA2, which recovered HNF6 activity and caused cell cycle arrest.

Overall, HNF6 stimulated C/EBPα-dependent transcription (12) and resulted in an approximately 6-fold increased in C/EBPα gene expression in transfected Caco-2 cell cultures. A functional link between recovery of HNF6 activity and C/EBPα dependent cell cycle regulation was established by cell cycle analysis and BrdU labeling assay. The fact that HNF6 gene expression was increased as a result of FOXA2 siRNA-mediated functional knockdown evidences an inhibitory crosstalk between FOXA2 and HNF6 but there is conclusive evidence for C/EBPα to bring about growth arrest by inhibiting Cdk2 and Cdk4 (14).

In conclusion, FOXA2 is reported to inhibit HNF6 activity and of genes targeted by this transcription factor. Also, siRNA mediated knockdown of FOXA2 is demonstrated to increase transcriptional activation of HNF6 and of genes targeted by this factor. Recovery of HNF6 activity is demonstrated to result in cell cycle arrest in human tumor cells. The study within the context of the invention demonstrates a significant role of FOXA2 in colorectal liver metastases, which makes FOXA2 and HNF6 an interesting target in the therapy of colorectal liver metastases.

In a nutshell, to better understand their proposed inhibitory crosstalk the consequences of functional knockdown of FOXA2 on HNF6 and C/EBPα activity was investigated in the human colon Caco-2 and HepG2 carcinoma cell lines. Specifically, siRNA-mediated gene silencing of FOXA2 repressed transcript expression by > 80%. This resulted in a statistically significant 6-, 3-, 4-, and 8-fold increase in mRNA expression of HNF6 and of genes targeted by this protein, e.g. HSP105B, CYP51, and C/EBPα, as determined by qRT-PCR. Thus, functional knockdown of FOXA2 recovered HNF6 activity. Furthermore, with nuclear extracts of Caco-2 cells no HNF6 DNA binding was observed, but expression of HNF1α, FOXA2, FOXA3, and HNF4α protein was abundant. A plasmid encoding HNF6 was therefore transfected into Caco-2 cells and a retroviral vector was employed to transfect HNF6 into the human hepatoma HepG2 cell. This resulted in abundant HNF6 protein expression with DNA binding activity being recovered as determined by EMSA. By flow cytometry the consequences of HNF6 expression on cell cycle regulation in transfected cells were studied. Essentially, HNF6 inhibited cell cycle progression in the G2/M and G1 phase in Caco-2 and HepG2 cell lines, respectively. Furthermore, in these cell lines proliferation was reduced by 80% and 50% as determined by BrdU labeling assay.

In conclusion, functional knockdown of FOXA2 recovered HNF6 activity and inhibited growth of tumor-cells. Thus, FOXA2 represents a novel therapeutic target in primary and secondary liver malignancies.

References:

1. Berrino F, De Angelis R, Sant M, et al. Survival for eight major cancers and all cancers combined for European adults diagnosed in 1995-99: results of the EUROCARE-4 study. The lancet oncology 2007 Sep;8(9):773-83.

2. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA: a cancer journal for clinicians 2007 Jan-Feb;57(1):43-66.

3. Lehner F, Kulik U, Klempnauer J, Borlak J. The hepatocyte nuclear factor 6 (HNF6) and FOXA2 are key regulators in colorectal liver metastases. Faseb J 2007 May;21(7):1445-62.

4. Hayashi Y, Wang W, Ninomiya T, Nagano H, Ohta K, Itoh H. Liver enriched transcription factors and differentiation of hepatocellular carcinoma. Mol Pathol 1999 Feb;52(1):19-24.

5. Kishimoto T, Kokura K, Ohkawa N, et al. Enhanced expression of a new class of liver-enriched b-Zip transcription factors, hepatocarcinogenesis-related transcription factor, in hepatocellular carcinomas of rats and humans. Cell Growth Differ 1998 Apr;9(4):337-44.

6. Xu L, Hui L, Wang S, et al. Expression profiling suggested a regulatory role of liver-enriched transcription factors in human hepatocellular carcinoma. Cancer research 2001 Apr 1;61(7):3176-81.

7. Lampen A, Bader A, Bestmann T, Winkler M, Witte L, Borlak JT. Catalytic activities, protein- and mRNA-expression of cytochrome P450 isoenzymes in intestinal cell lines. Xenobiotica; the fate of foreign compounds in biological systems 1998 May;28(5):429-41.

8. Wilkening S, Stahl F, Bader A. Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. Drug Metab Dispos 2003 Aug;31(8):1035-42.

9. Niehof M, Streetz K, Rakemann T, et al. Interleukin-6-induced tethering of STAT3 to the LAP/C/EBPbeta promoter suggests a new mechanism of transcriptional regulation by STAT3. The Journal of biological chemistry 2001 Mar 23;276(12):9016-27.

10. Soneoka Y, Cannon PM, Ramsdale EE, et al. A transient three-plasmid expression system for the production of high titer retroviral vectors. Nucleic Acids Res 1995 Feb 25;23(4):628-33.

11. Rausa FM, 3rd, Hughes DE, Costa RH. Stability of the hepatocyte nuclear factor 6 transcription factor requires acetylation by the CREB-binding protein coactivator. The Journal of biological chemistry 2004 Oct 8;279(41):43070-6.

12. Yoshida Y, Hughes DE, Rausa FM, 3rd, et al. C/EBPalpha and HNF6 protein complex formation stimulates HNF6-dependent transcription by CBP coactivator recruitment in HepG2 cells. Hepatology (Baltimore, Md 2006 Feb;43(2):276-86.

13. Odom DT, Zizlsperger N, Gordon DB, et al. Control of pancreas and liver gene expression by HNF transcription factors. Science 2004 Feb 27;303(5662):1378-81.

14. Wang H, Iakova P, Wilde M, et al. C/EBPalpha arrests cell proliferation through direct inhibition of Cdk2 and Cdk4. Mol Cell 2001 Oct;8(4):817-28.

15. Rausa FM, Tan Y, Costa RH. Association between hepatocyte nuclear factor 6 (HNF-6) and FoxA2 DNA binding domains stimulates FoxA2 transcriptional activity but inhibits HNF-6 DNA binding. Molecular and cellular biology 2003 Jan;23(2):437-49.

16. Rubins NE, Friedman JR, Le PP, Zhang L, Brestelli J, Kaestner KH. Transcriptional networks in the liver: hepatocyte nuclear factor 6 function is largely independent of Foxa2. Molecular and cellular biology 2005 Aug;25(16):7069-77.

Titles and legends to figures

Figure 1: HNF6 protein expression and DNA binding activity in the human colon carcinoma cell line Caco-2.
A. HNF6 plasmid-transfected into Caco-2 cells; B. empty vector control; C. HNF6 DNA binding activity with nuclear extracts isolated from HNF6-transfected Caco-2 cells; D. HNF6 DNA binding activity with nuclear extracts isolated from HNF6-transfected Caco-2 cells and band shift assays with FOXA2 or a combination of HNF6 and FOXA2

Figure 2: Functional knockdown of FOXA2 in the human colon carcinoma cell line Caco-2.

A. Caco-2 cells transfected with FOXA2 siRNA; B. FOXA2 and HNF6 gene expression after functional knockdown of FOXA2 in three independent experiments; C. FOXA2 gene expression in Caco-2 cells transfected with FOXA2 siRNA; D. Gene expression of HNF6 and FOXA2 in untreated Caco-2 cells

The RNAi probes according the Figure 2 correspond to
probe 1: SEQ ID Nos 5 and 9
probe 2: SEQ ID Nos 6 and 10
probe 3: SEQ ID Nos 4 and 8

The sequences as set forth in SEQ ID Nos 7 and 11 correspond to a further RNAi probe not shown in Figure2.

Figure 3: Gene expression of FOXA2 and HNF6 target genes HSP105B, CYP51 and C/EBPα in the human carcinoma cell line Caco-2 after siRNA-mediated functional knockdown of FOXA2. Results represents the mean of n=3 individual experiments.

Figure 4: Cell cycle and BrdU labeling in Caco-2 and HepG2 cells.
A. Cell cycle of Caco-2 and HNF6-transfected Caco-2 cells
B. BrdU labeling of Caco-2 and HNF6-transfected Caco-2 cells
C. Cell cycle of HepG2 and HNF6-transfected HepG2 cells
D. BrdU labeling of HepG2 and HNF6-transfected HepG2 cells

The features of the invention being disclosed in the preceding description and the subsequent claims can be of importance both singularly and in arbitrary combination for the implementation of the invention in its different embodiments.

Claims:

1. A medicament for the prevention or treatment of liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells, comprising a composition that decreases or inhibits the expression or activity of Foxa2 and/or
increases the expression or activity of HNF6.

2. Medicament according to claim 1 for the prevention or treatment of liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases, comprising a composition that decreases or inhibits the expression or activity of Foxa2.

3. Medicament according to claim 1 for the treatment of colorectal adenocarcinoma cells or of hepatocellular carcinoma cells, comprising a composition that increases the expression or activity of HNF6.

4. SiRNA composition, wherein the siRNA composition reduces or inhibits the expression of Foxa2, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases.

5. SiRNA composition, in particular according to claim 4, comprising a siRNA forming a double stranded duplex which consists of 15-30 base pairs and wherein said duplex comprises a RNA sequence complementary to 15-30 consecutive nucleotides of SEQ ID No:1, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases.

6. SiRNA composition according to any one of claims 4-5, comprising a first RNA sequence consisting of 15-30 consecutive nucleotides of SEQ ID NO: 2 and comprising a second RNA sequence consisting of 15-30 consecutive residues

of SEQ ID NO. 1, wherein the second RNA sequence comprises a nucleotide sequence complementary to 15-30 nucleotides of the first RNA sequence.

7. SiRNA composition according to any one of claims 4-6, forming a double stranded duplex which consists of 15-30 base pairs and wherein said duplex comprises a nucleotide sequence complementary to 15-30 consecutive nucleotides of a Foxa2 sequence selected from the group consisting of the sequences SEQ ID NOs:8-11 .

8. SiRNA composition according to any one of claims 4-7, comprising a RNA strand consisting of 31-300 nucleotides, wherein said strand comprises a first RNA sequence consisting of 15-30 consecutive nucleotides of SEQ ID NO: 2 and a second RNA sequence consisting of 15-30 consecutive residues of SEQ ID NO: 1 and a third RNA sequence connecting the first and the second RNA sequence and consisting of 1-100 nucleotides, and wherein the second RNA sequence comprises a nucleotide sequence complementary to 15-30 nucleotides of the first RNA sequence.

9. SiRNA composition according to any one of claims 4-8, wherein the first RNA sequence is selected from the group consisting of SEQ ID NOs: 5-9 and/or wherein the second RNA sequence is selected from the group consisting of SEQ ID NOs: 10-13, and wherein preferably the combination of SEQ ID NO: 6 and SEQ ID NO:10 or the combination of SEQ ID NO: 7 and SEQ ID NO 11 or the combination of SEQ ID NO: 8 and SEQ ID NO 12 or the combination of SEQ ID NO: 9 and SEQ ID NO 13 is selected.

10. SiRNA composition, in particular according to any one of claims 4-9, comprising a first RNA strand consisting of a sequence selected from the SEQ ID NOs: 5-9 and/or comprising a second RNA strand consisting of a sequence selected from the SEQ ID NOs 10-13 strand, and wherein preferably the combination of SEQ ID NO: 6 and SEQ ID NO:10 or the combination of SEQ ID NO: 7 and SEQ ID NO 11 or the combination of SEQ ID NO: 8 and SEQ ID NO 12 or the combination of SEQ ID NO: 9 and SEQ ID NO 13 is selected.

11. Vector for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, comprising a promoter and a DNA sequence encoding the RNA strand according to claim 8, wherein the DNA sequence is operatively linked to the promoter, and wherein the promoter is preferably a suitable promoter for gene expression in cells of colorectal liver metastases, in particular the Foxa2 promoter.

12. Antisense composition, wherein the antisense composition comprises a nucleotide sequence complementary to a coding sequence of Foxa2 , for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases.

13. Antibody composition, wherein the antibody composition comprises a pharmaceutically effective amount of an antibody or fragment thereof that binds to a polypeptide encoded by Foxa2, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases.

14. Polypeptide composition, wherein the polypeptide composition comprises a polypeptide coded by the sequence of HNF6, in particular a polypeptide having the sequence SEQ ID NO: 14, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells.

15. Nucleotide composition comprising a nucleotide sequence of HNF6, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells.

16. Nucleotide composition, in particular according to claim 15, comprising a DNA sequence which hybridizes to the complement of SEQ ID NO: 12 and encodes

a protein having the biological activity of HNF6, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases , or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells.

17. Nucleotide composition according to any one of claims 15-16, comprising a DNA sequence encoding a protein of the sequence SEQ ID NO: 13.

18. Nucleotide composition according to any one claims 15-17, comprising the DNA sequence of SEQ ID NO:12.

19. Vector, comprising a promoter and a DNA sequence according to any one of claims 15-18, for use in the treatment of tumor cells, in particular of primary or secondary liver malignancies, preferably of colorectal liver metastases, or for the treatment of colorectal adenocarcinoma cells or hepatocellular carcinoma cells, wherein the DNA sequence is operatively linked to the promoter, and wherein the promoter is preferably a suitable promoter for gene expression in cells of colorectal liver metastases, in particular the Foxa2 promoter, or wherein the promoter is a suitable promoter for gene expression in colon cells, in particular a promoter of a colon specific gene selected from the group consisting of CA1, CEA and Mucin1 or wherein the promoter is a suitable promoter for gene expression in liver cells, in particular the HBV core promoter or a promoter of a liver specific gene selected from the group consisting of Albumin, Phosphoenolpyruvatcarboxykinase and Ornithintranscarbamylase.

20. Composition or vector according to one of the claims 4-19, wherein the gene is a human or murine gene.

21. Medicament according to any one of claims 1-3, wherein the composition is a composition as claimed in one of the claims 4-10, 12-18 or 20,
or wherein the composition comprises a vector according to one of the claims 11 or 19 .

22. Medicament according to claim 2, wherein the composition is a composition as claimed in one of the claims 4-10 or 20, or wherein the composition comprises a vector according to claims 11.

23. Medicament according to claim 3, wherein the composition is a composition as claimed in one of the claims 12-18 or 20, or wherein the composition comprises a vector according to claims 19

24. Use of a composition that decreases or inhibits the expression or activity of Foxa2 and/or increases the expression or activity of HNF6 for the preparation of a medicament, in particular a medicament according to one of the claims 1-3 or 21-23.

25. Use of an antisense composition for the preparation of a medicament, wherein the antisense composition comprises a nucleotide sequence complementary to a coding sequence of Foxa2, in particular a medicament according to one of the claims 1-2 or 21-22.

26. Use of an siRNA composition for the preparation of a medicament, wherein the siRNA composition reduces or inhibits the expression of Foxa2, in particular a medicament according to one of the claims 1-2 or 21-22.

27. Use of an antibody composition for the preparation of a medicament, wherein the antibody composition comprises a pharmaceutically effective amount of an antibody or fragment thereof that binds to a polypeptide encoded by Foxa2, in particular a medicament according to one of the claims 1-2 or 21-22.

.

28. Use of a nucleotide composition for the preparation of a medicament , in particular a medicament according to one of the claims 1, 3, 21 or 22, wherein the nucleotide composition comprises a nucleotide sequence of HNF6.

29. Use of a polypeptide composition for the preparation of a medicament, in particular a medicament according to one of the claims 1, 3, 21 or 22, wherein the polypeptide composition comprises a polypeptide coded by the sequence of HNF6.

30. Use according to one of the claims 24-29, wherein the gene is a human or murine gene.

31. Use according to one of the claims 24-30 for the preparation of a medicament for preventing, treating, or ameliorating liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases.

32. Use of the gene Foxa2 and/or its gene product to screen for and to identify drugs against liver metastases, in particular metastases in the liver made up of or derived from non-hepatic tumor cells, preferably colorectal liver metastases, preferably for identifying a compound that inhibits the expression of Foxa2, comprising the steps of : (1) contacting a test cell expressing said gene with a test compound; (2) detecting the expression level of said gene; and (3) determining the compound that suppresses said expression level compared to a normal control level of said gene as an inhibitor of said gene.

33. Use according to claim 32 for identifying a compound that enhances the expression or activity of a HNF6.

34. Use according to one of the claims 32-33, wherein the compound is an siRNA reducing or inhibiting the expression of Foxa2.

35. Use according to one of the claims 32-34, wherein the compound a nucleotide sequence complementary to a coding sequence of Foxa2.

36. Use according to one of the claim 32-35, wherein the compound is an antibody or fragment thereof that binds to a polypeptide encoded by Foxa2.

37. Use as claimed in one of the claims 32-36, comprising the steps of : (1) contacting a test compound with a polypeptide encoded by Foxa2; (2) detecting the binding activity between the polypeptide and the test compound; and (3) selecting a compound that binds to the polypeptide.

38. Use as claimed in one of the claims 32-37, comprising the steps of : (a) contacting a test compound with a polypeptide encoded by Foxa2;
(b) detecting the biological activity of the polypeptide of step (a); and (c) selecting a compound that suppresses the biological activity of the polypeptide encoded by Foxa2 in comparison with the biological activity detected in the absence of the test compound,
and/or enhances the biological activity of the polypeptide encoded by HNF6 in comparison with the biological activity detected in the absence of the test compound.

39. Use as claimed in claim 38, wherein said biological activity is cell proliferation.

40. Use according to one of the claims 32-39, comprising the steps of: (1) contacting a test compound with a cell into which a vector comprising the transcriptional regulatory region of one or more of the selected genes, in particular of Foxa2 and/or HNF6, and a reporter gene that is expressed under the control of the transcriptional regulatory region has been introduced; (2) measuring the activity of said reporter gene; and (3) selecting a compound that reduces the expression level of said reporter gene when the selected gene Foxa2
and/or that enhances the expression level of said reporter gene when the selected gene is HNF6.

41. Restoring HNF6 activity, in particular by using a composition or vector as claimed in one of the claims 12-20, to prevent disease progression and growth of colorectal liver metastases.

42. Inhibition of Foxa2, in particular by using a composition or vector as claimed in one of the claims 4-11 or 20, preferably by the method according to claim 41, for the prevention or treatment colorectal liver metastases.

Figure 1

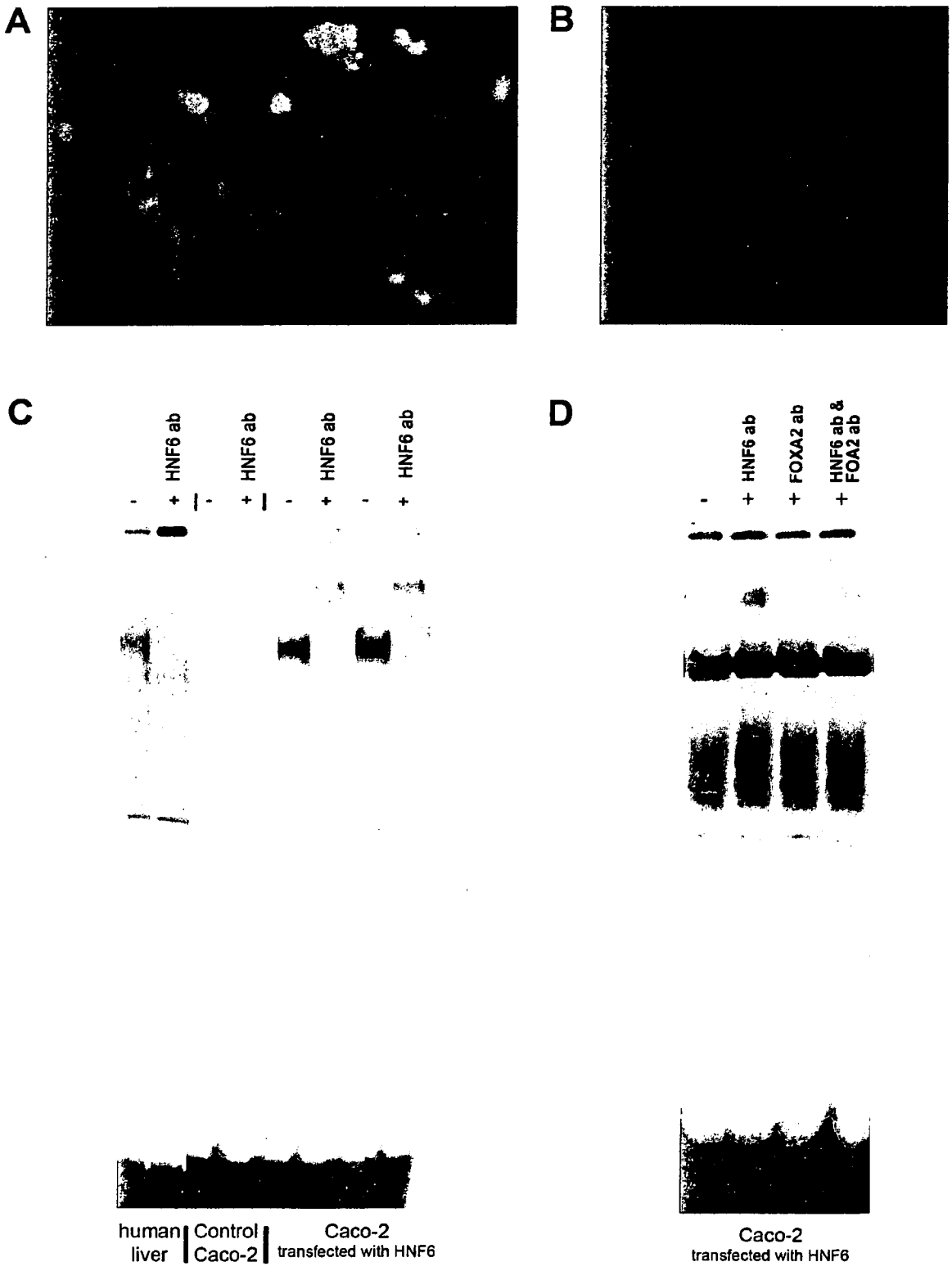

A
B
C
HNF6 ab
HNF6 ab
HNF6 ab
HNF6 ab
- + - + - + - +
human liver
Control Caco-2
Caco-2 transfected with HNF6
D
HNF6 ab
FOXA2 ab
HNF6 ab & FOA2 ab
- + + +
Caco-2 transfected with HNF6

Figure 2

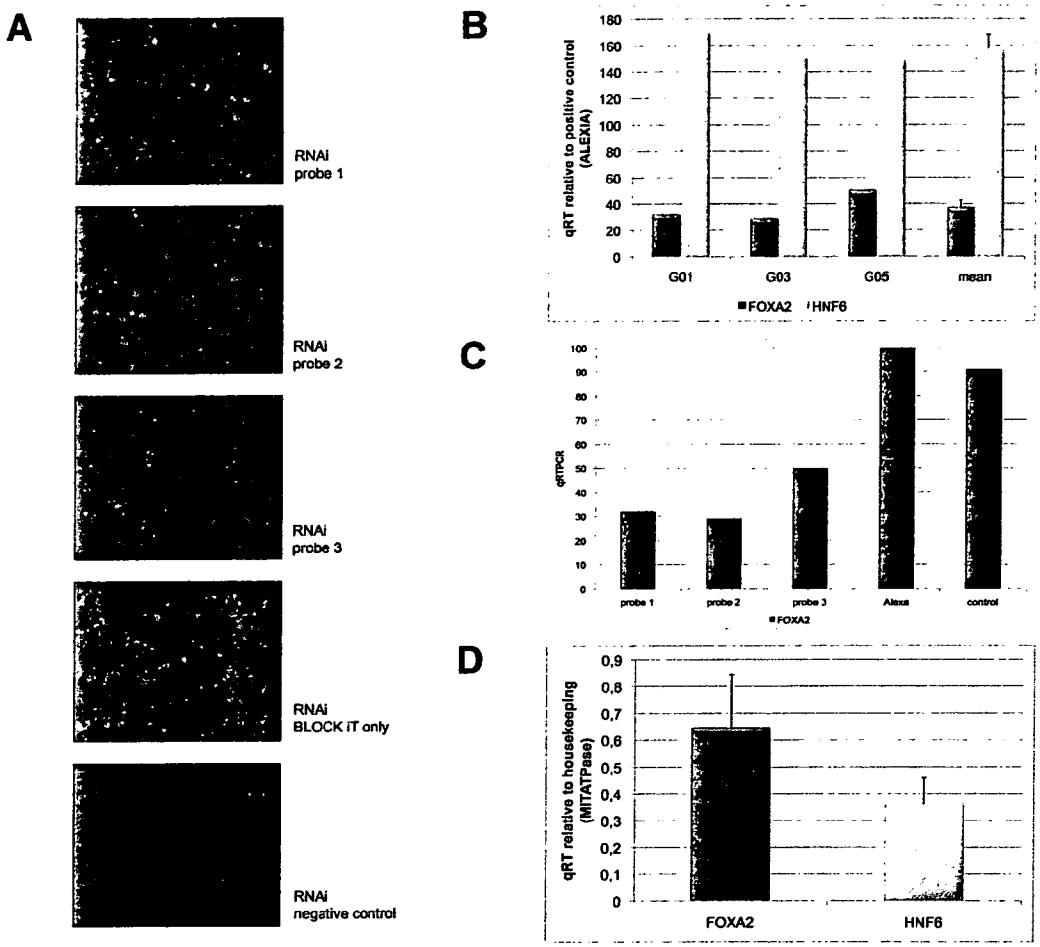
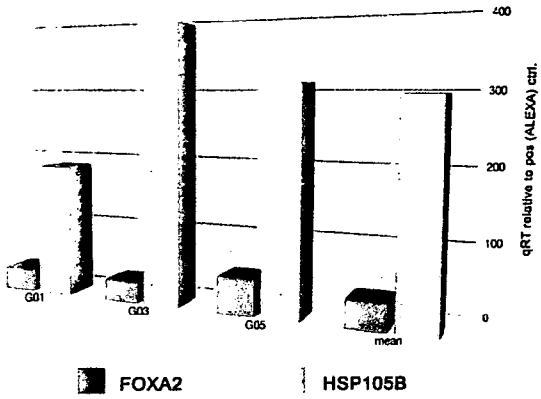
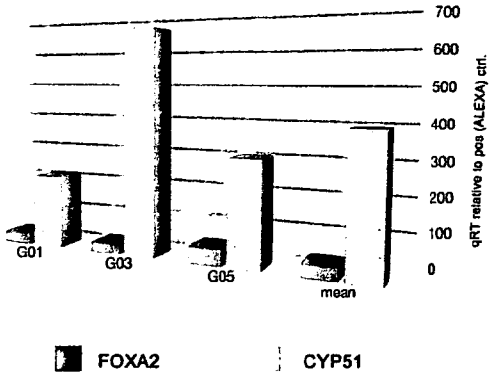

A
RNAi probe 1
RNAi probe 2
RNAi probe 3
RNAi BLOCK iT only
RNAi negative control
B
qRT relative to positive control (ALEXIA)
180
160
140
120
100
80
60
40
20
0
G01
G03
G05
mean
FOXA2
HNF6
C
qRTPCR
100
90
80
70
60
50
40
30
20
10
0
probe 1
probe 2
probe 3
Alexa
control
FOXA2
D
qRT relative to housekeeping (MITATPase)
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
FOXA2
HNF6

Figure 3


400
300
200
100
0
qRT relative to pos (ALEXA) ctrl.
G01
G03
G05
mean
FOXA2
HSP105B


700
600
500
400
300
200
100
0
qRT relative to pos (ALEXA) ctrl.
G01
G03
G05
mean
FOXA2
CYP51

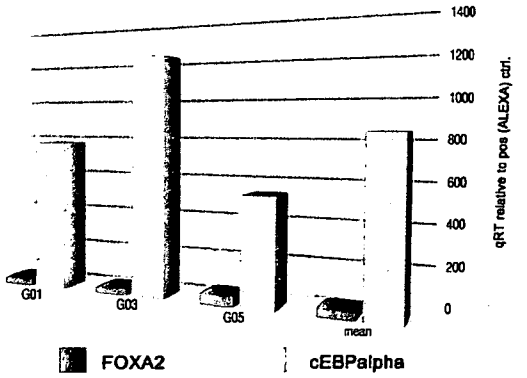
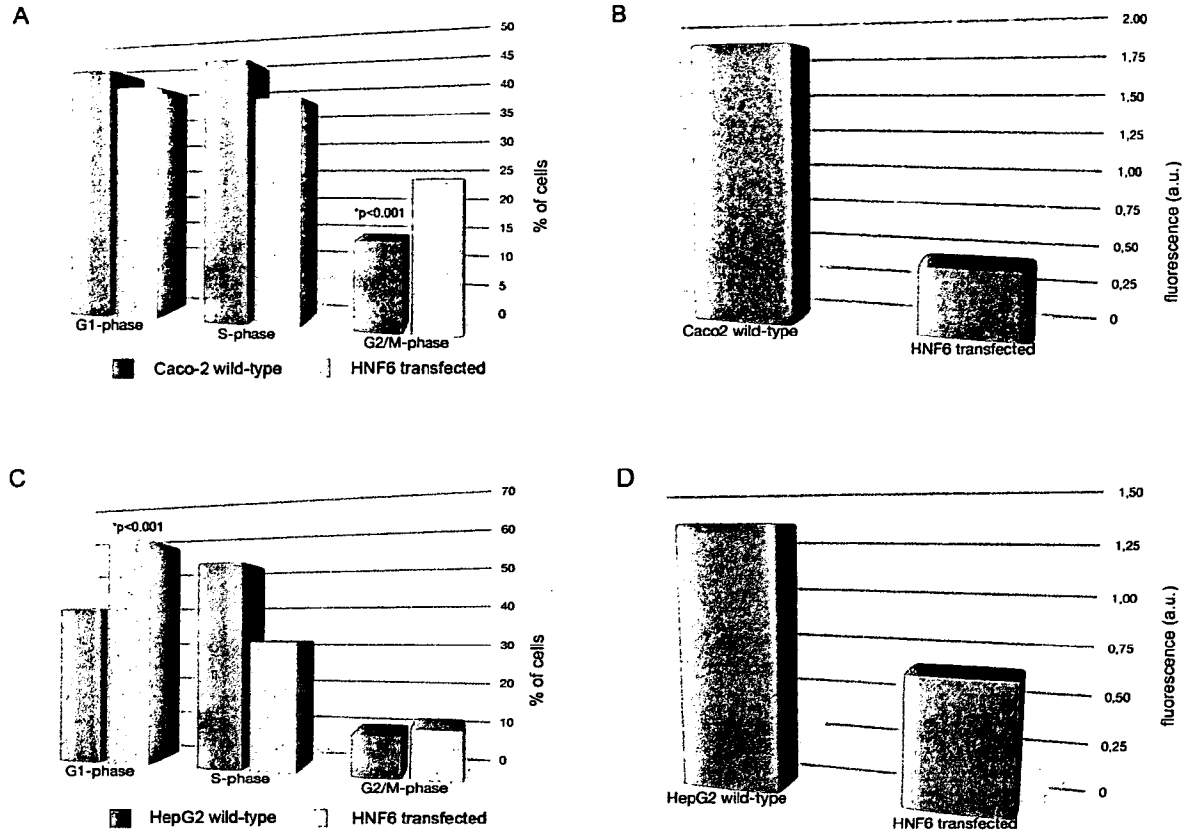

1400
1200
1000
800
600
400
200
0
qRT relative to pos (ALEXA) ctrl.
G01
G03
G05
mean
FOXA2
cEBPalpha

Figure 4


A
50
45
40
35
30
25
20
15
10
5
0
% of cells
*p<0.001
G1-phase
S-phase
G2/M-phase
Caco-2 wild-type
HNF6 transfected
B
2.00
1,75
1,50
1,25
1,00
0,75
0,50
0,25
0
fluorescence (a.u.)
Caco2 wild-type
HNF6 transfected
C
70
60
50
40
30
20
10
0
% of cells
*p<0.001
G1-phase
S-phase
G2/M-phase
HepG2 wild-type
HNF6 transfected
D
1,50
1,25
1,00
0,75
0,50
0,25
0
fluorescence (a.u.)
HepG2 wild-type
HNF6 transfected