Comparative Proteomics of Colon Cancer Stem Cells and Differentiated Tumor Cells Identifies BIRC6 as a Potential Therapeutic Target*[S]

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Patients with liver metastases from colon carcinoma show highly variable responses to chemotherapy and tumor recurrence is frequently observed. Therapy-resistant cancer stem cells have been implicated in drug resistance and tumor recurrence. However, the factors determining therapy resistance and tumor recurrence are poorly understood. The aim of this study was to gain insight into these mechanisms by comparing the proteomes of patient-derived cancer stem cell cultures and their differentiated isogenic offspring.

We established colonosphere cultures derived from resection specimens of liver metastases in patients with colon cancer. These colonospheres, enriched for colon cancer stem cells, were used to establish isogenic cultures of stably differentiated nontumorigenic progeny. Proteomics based on one-dimensional gel electrophoresis coupled to nano liquid chromatography tandem MS was used to identify proteome differences between three of these paired cultures. The resulting data were analyzed using Ingenuity Pathway Software.

Out of a total data set of 3048 identified proteins, 32 proteins were at least twofold up-regulated in the colon cancer stem cells when compared with the differentiated cells. Pathway analysis showed that "cell death " regulation is strikingly different between the two cell types. Interestingly, one of the top-up-regulated proteins was BIRC6, which belongs to the class of Inhibitor of Apoptosis Proteins. Knockdown of BIRC6 sensitized colon cancer stem cells against the chemotherapeutic drugs oxaliplatin and cisplatin.

This study reveals that differentiation of colon cancer stem cells is accompanied by altered regulation of cell death pathways. We identified BIRC6 as an important mediator of cancer stem cell resistance against cisplatin and oxaliplatin. Targeting BIRC6, or other Inhibitors of Apoptosis Proteins, may help eradicating colon cancer stem cells. ***Molecular & Cellular Proteomics 10: 10.1074/mcp.M111.011353, 1–11, 2011.***

Treatment of colorectal cancer patients with chemotherapy is characterized by highly divergent tumor responses, but tumor recurrence is almost always observed. Therefore, chemotherapy is not considered to be a curative modality in the treatment of colorectal cancer (1). Tumor recurrence may be because of the presence of therapy-resistant, genetically distinct tumor subclones. These subclones may either be pre-existent or may be generated as a direct result of the chemotherapy itself. More recently, it has been suggested that therapy resistance and subsequent tumor recurrence could be mediated by the "cancer stem cell " fraction of colorectal tumors. Cancer stem cells make up only a few percent of the total tumor cell mass, but are uniquely endowed with tumor-initiating capacity (2–5).

Interestingly, normal intestinal stem cells have been identified as the cell-of-origin of intestinal tumors (6, 7). Cancer stem cells may therefore be transformed descendants of normal tissue stem cells. Although normal stem cells give rise to differentiated cells lacking tissue-regenerating capacity, cancer stem cells give rise to differentiated tumor cells lacking tumor-regenerating capacity (8, 9).

Normal colon stem cells are exposed to toxins and drugs for an entire lifetime. To cope with this continuous challenge, stem cells must possess intrinsic resistance mechanisms that

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[1] The abbreviations used are: BIRC, baculoviral inhibitor of apoptosis protein repeat containing; ALDH1A1, aldehyde dehydrogenase 1A1; IAP, inhibitor of apoptosis protein; SMAC, second mitochondrial-derived activator of caspases.

protect their DNA from being mutated and that allow prolonged survival. Inheritance of these resistance mechanisms by cancer stem cells may protect them from the cytotoxic action of chemotherapeutic drugs. If cancer stem cells are indeed the major driving force behind tumor recurrence, novel strategies are required to target this subset of cancer cells. Indeed, several studies have shown that residual tumor tissue after chemotherapy is enriched for cancer stem cell-like cells (10, 11). However, the relationship between chemo-resistance and tumor-initiating potential and the mechanisms underlying cancer stem cell selective drug resistance are currently poorly understood (12–15).

Here we set out to address the relationship between colorectal cancer stem cells and drug resistance. To this end, we have generated cancer stem cell enriched human colonosphere cultures from colorectal liver metastases. In addition, we have generated colonosphere-derived stably differentiated progeny. These isogenic cell pairs were then used to identify proteome differences using mass spectrometry. Analysis of the data revealed that proteins governing cell survival are overrepresented in the cancer stem cell cultures. The most prominently overexpressed survival protein, BIRC6/BRUCE/Apollon, was identified as a key mediator of cancer stem cell resistance to cisplatin and oxaliplatin.

EXPERIMENTAL PROCEDURES

Colorectal Cancer Stem Cells and Differentiated Tumor Cell Cultures—Collection of tumor specimens, isolation and expansion of colorectal cancer stem cell and differentiated cell cultures was performed as described in Emmink *et al.* (16). Human colorectal tumor specimens were obtained from patients undergoing a liver resection for metastatic adenocarcinoma, in accordance with the ethical committee on human experimentation. Informed consent was obtained from all patients. All tumors were diagnosed as colorectal adenocarcinomas. Liver metastases were excised from segment VII (L145), segment IV (L146), and segment II-IV (L167). Differentiation status was not determined.

Isolation and Expansion of Colorectal Cancer Stem Cell Cultures—The obtained tissue fragments were washed extensively with phosphate-buffered saline and were mechanically dissociated using scalpels and vigorous trituration to yield small fragments (<1 mm^3) and single cells. Enzymatic digestion was performed using thermolysin 0.05% (Sigma, Type X) in Dulbecco's modified Eagle's medium/F12 containing 5 mM Hepes (Invitrogen, Carlsbad, CA) for 2 h at 37 °C. The suspension was then filtered through a 40-μm-pore size nylon cell strainer (BD Falcon) to separate the tissue fragments from the single cells. The single cell suspension was cultured in advanced Dulbecco's modified Eagle's medium/F12 (Invitrogen) supplemented with 0.6% glucose (BDH Lab. Supplies), 2 mM L-glutamine (Biowhittaker, Rockland, ME), 9.6 μg/ml putrescin (Sigma), 6.3 ng/ml progesterone (Sigma), 5.2 ng/ml sodium selenite (Sigma), 25 μg/ml insulin (Sigma), 100 μg/ml apotransferrin (Sigma), 5 mM hepes (Invitrogen), 0,005 μg/ml trace element A (Cellgro), 0.01 μg/ml trace element B (Cellgro), 0.01 μg/ml trace element C (Cellgro), 100 μM β-mercaptoethanol (Merck), 10 ml antibiotic-antimycotic (Invitrogen), 4 μg/ml gentamicine (Invitrogen), 0.002% lipid mixture (Sigma), 5 μg/ml glutathione (Roche), and 4 μg/ml Heparin (Sigma). Growth factors (20 ng/ml EGF (Invitrogen) and 10 ng/ml b-FGF (Abcam, Cambridge, UK)) were added to the cell culture medium freshly each week. All cell culture was carried out in nontissue culture treated flasks (BD Falcon) at 37 °C in a 5% CO_2 humidified incubator. *In vitro* differentiation was induced by culturing colon colonospheres for 3 weeks on collagen-coated dishes in Dulbecco's modified Eagle's medium/F12 (GIBCO) supplemented with 20% fetal bovine serum. Passage numbers of the clones used ($n = 3$) spheroid cultures and the accompanying differentiated tumor cell cultures were all below 10.

Cell lysis and SDS-PAGE—Paired colonospheres and differentiated tumor cell cultures were seeded in 10 cm^2 diameter tissue culture plates and cultured for 24 h in serum-free stem cell medium. Cells were subsequently washed twice with phosphate-buffered saline, centrifuged and washed with water to get rid of the excess salts. Lysis buffer (20 mM HEPES pH7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol) containing proteinase inhibitor was used to lyse cells. Equal amounts of protein (50 μg) were separated on NuPAGE Novex Bis-Tris Mini Gels (Invitrogen). Gels were stained with Coomassie brilliant blue G-250 (Pierce, Rockford, IL), washed and each lane was sliced into ten bands using a band pattern to guide the slicing. The gel slicing and in-gel digesting was performed in a laminar flow under keratin-free conditions.

In-gel Digestion—Before MS analysis, separated proteins were in-gel digested as described (17). Gel lanes corresponding to the different protein samples were sliced into ten bands. The bands were washed and dehydrated three times in 50 mM ammonium bicarbonate pH 7.9 + 50% acetonitrile. Subsequently, cysteine bonds were reduced with 10 mM dithiotreitol for 1 h at 56 °C and alkylated with 50 mM iodoacetamide for 45 min at room temperature in the dark. After two subsequent wash and dehydration cycles the bands were dried 10 min in a vacuum centrifuge and incubated overnight with 0.06 μg/μl trypsin at 25 °C. Peptides were extracted once in 1% formic acid and subsequently two times in 50% acetonitrile in 5% formic acid. The volume was reduced to 50 μl in a vacuum centrifuge prior to liquid chromatography tandem MS (LC-MS/MS) analysis (17).

NanoLC-MS/MS Analysis—Peptides were separated by an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings, Amsterdam, The Netherlands) equipped with a 20 cm $\times$ 75 μm ID fused silica column custom packed with 3 μm 120 Å ReproSil Pur C18 aqua (Dr Maisch GMBH, Ammerbuch-Entringen, Germany). After injection, peptides were trapped at 30 μl/min on a 5 mm $\times$ 300 μm ID Pepmap C18 cartridge (Dionex LC-Packings) at 2% buffer B (buffer A: 0.05% formic acid in MQ; buffer B: 80% acetonitrile + 0.05% formic acid in MQ) and separated at 300 nl/min in a 10–40% buffer B gradient in 60 min. Eluting peptides were ionized at 1.7 kV in a Nanomate Triversa Chip-based nanospray source using a Triversa LC coupler (Advion, Ithaca, NY). Intact peptide mass spectra and fragmentation spectra were acquired on a LTQ-FT hybrid mass spectrometer (Thermo Fisher, Bremen, Germany). Intact masses were measured at resolution 50.000 in the ICR cell using a target value of 1×10^6 charges. In parallel, after an FT prescan, the top 5 peptide signals (charge-states 2^+ and higher) were submitted to MS/MS in the linear ion trap (3 amu isolation width, 30 ms activation, 35% normalized activation energy, *Q* value of 0.25 and a threshold of 5000 counts). Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30s.

Label-free protein quantitation by spectral counting has emerged as a powerful alternative to labeling-based strategies, with each approach having its pros and cons. Notably, label-free analysis is less accurate than approaches in which proteins are labeled, in particular when small differences in protein levels across samples are quantified. However, with a good parallel workflow, label-free quantitation is simple, cheap, and allows for quantitation at a relative large dynamic range.

Database Searching, Statistics, and Ingenuity Pathway Analysis—MS/MS spectra were searched against the human International Protein Index database 3.31 (67,511 entries) using Sequest (version 27,

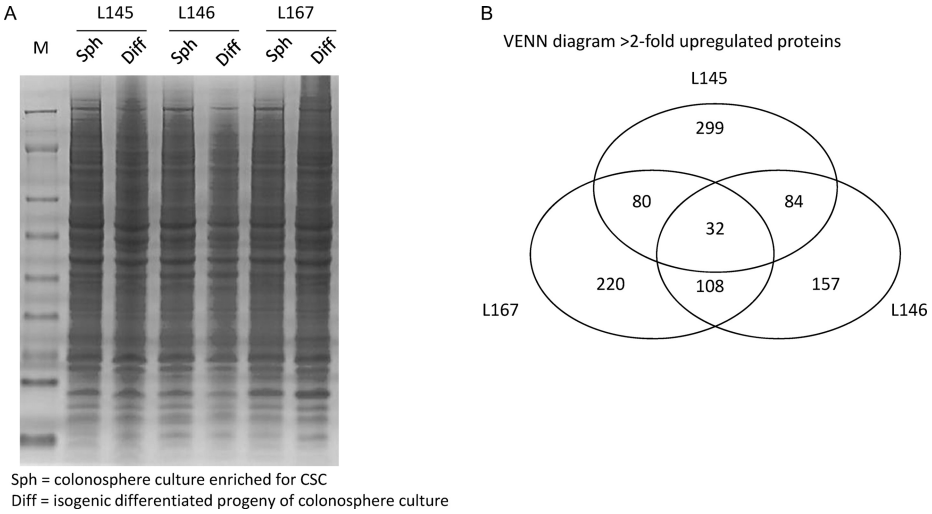

FIG. 1. **NanoLC-MS/MS/based analysis of proteome differences between colonospheres and isogenic differentiated tumor cells.** *A*, Coomassie-stained protein gradient gel loaded with protein samples from colonospheres (Sph) and differentiated tumor cells (Diff) of the indicated tumors. This gel was used for MS analysis. *B*, Venn diagram of all >twofold up-regulated proteins in colonospheres.

rev 12), which is part of the BioWorks 3.3 data analysis package (Thermo Fisher, San Jose, CA). MS/MS spectra were searched with a maximum allowed deviation of 10 ppm for the precursor mass and 1 amu for fragment masses. Methionine oxidation and cysteine carboxamidomethylation were allowed as variable modifications, two missed cleavages were allowed and the minimum number of tryptic termini was 1. After database searching the DTA and OUT files were imported into Scaffold 2.01.01 (Proteome software, Portland, OR). Scaffold was used to organize the gel-band data and to validate peptide identifications using the Peptide Prophet algorithm (18). Only peptide identifications with a probability >95% were retained. Subsequently, the ProteinProphet algorithm (19) was applied and protein identifications with a probability of >99% with two peptides or more in at least one of the samples were retained. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped. For each protein identified, the number of spectral counts (identified MS/MS spectra) in each sample (10 gel bands) was exported to Excel. For each sample the spectral counts per protein were normalized on the sum of the spectral counts for that sample. The list of differentially expressed proteins, including fold changes was imported in the online software package Ingenuity (Ingenuity IPA, version 7.6) and pathway and network analysis was performed with only direct relationships. Furthermore the list of differentially expressed proteins was imported in the online STRING database of known and predicted protein interactions. The interactions include direct (physical) and indirect (functional) associations between proteins.

Lentiviral Constructs and Transduction—The lentiviral short-hairpin RNA (shRNA) constructs targeting baculoviral inhibitor of apoptosis protein repeat containing 6 (BIRC6)[1] were obtained from the TRC-Mm1.0 library (Sigma Aldrich). The target set used for BIRC6 (NM_016252) included TRCN0000004157, TRCN0000004158, TRCN0000004159, TRCN0000004160, and TRCN0000004161. Of these constructs, transduction of cells with **58** and **59** produced the best knock down. As the control vector, we used the same vector containing a sequence targeting luciferase, TGACCAGGCATTCACAGAAAT.

Western Blotting and Antibodies—Lysates of colonospheres and their differentiated offspring were prepared in lysisbuffer (20 mM HEPES pH7.4, 1% Nonidet P-40, 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol). Equal amounts of protein were run out on NuPAGE Novex Tris-Acetate Mini Gel (Invitrogen) and were analyzed by Western blotting using antibodies directed against BIRC6 (Ab19609, Abcam) and β-Actin (AC-15, Novus Biologicals, Littleton, CO) was used.

Flow Cytometry and Cell Sorting—Dead cells were excluded using viability marker 7-aminoactinomycin D (7-AAD) (R&D, Detroit, MI) and cell doublets and clumps were excluded using doublet discrimination gating. Aldefluor®-positive cells were analyzed according to the manufacturer's protocol by using Aldefluor® and DEAB (STEMCELL Technologies). The cell sorting experiments were conducted with DAKO-Cytomation MoFlo High Speed Sorter.

Cisplatin and Oxaliplatin Sensitivity Assay—Control and BIRC6 knockdown colonospheres and differentiated tumor cells were cultured in the presence of oxaliplatin (Pharmachemie BV, Haarlem) or cisplatin (Pharmachemie BV, Haarlem) at the indicated concentrations for 5 days. Mitochondrial activity was evaluated using CellTiter 96® AQ_{ueous} Nonradioactive Cell Proliferation Assay (MTS) (Promega, Charbonnières, France). All absorbance values are expressed as percentages of vehicle-treated control wells.

RESULTS

Proteome Differences Between Colon Cancer Stem Cells and Their Differentiated Progeny—To compare the proteome of colon cancer stem cells to their differentiated progeny, we analyzed three isogenic pairs of colonospheres and differentiated tumor cells derived from freshly resected liver metastases. All colonosphere cultures were enriched for cancer stem cells based on their high clone- and tumor-forming potential (16).

The protein lysates of these cultures were fractionated on an SDS-PAGE gel (Fig. 1*A*), followed by in-gel tryptic digestion. Analysis of the extracted peptides was performed by Nano-LC-MS/MS, followed by database searching. In total, 3048 proteins were identified in all sets of cells together, with an average of 2269 proteins per isogenic couple (Table I). The number of proteins that was at least twofold up-regulated in the colonospheres of each isogenic pair varied from 377 to 491. Of these >twofold up-regulated proteins, 32 proteins were up-regulated in the colonospheres of all 3 isogenic pairs (Fig. 1*B*). The unsupervised heat map of global clustering shows that the similarity between isogenic pairs of colonosphere cells and differentiated tumor cells is greater than the similarity among the different colonospheres cultures

TABLE I

Proteome differences between colonospheres and isogenic differentiated cells. The numbers refer to all identified up-, down- and non-regulated proteins (top row), all up-regulated proteins (middle row), and all >twofold up-regulated proteins. The number of proteins in all three categories that were identified in 2/3 and in 3/3 pairs are shown in the last two columns

	L145	L146	L167	Overlap	
				2/3 pairs	3/3 pairs
All proteins	2242	2290	2376	1739	516
Total upregulated proteins	1078	1001	1093	676	229
>2 fold upregulated	491	377	437	275	32

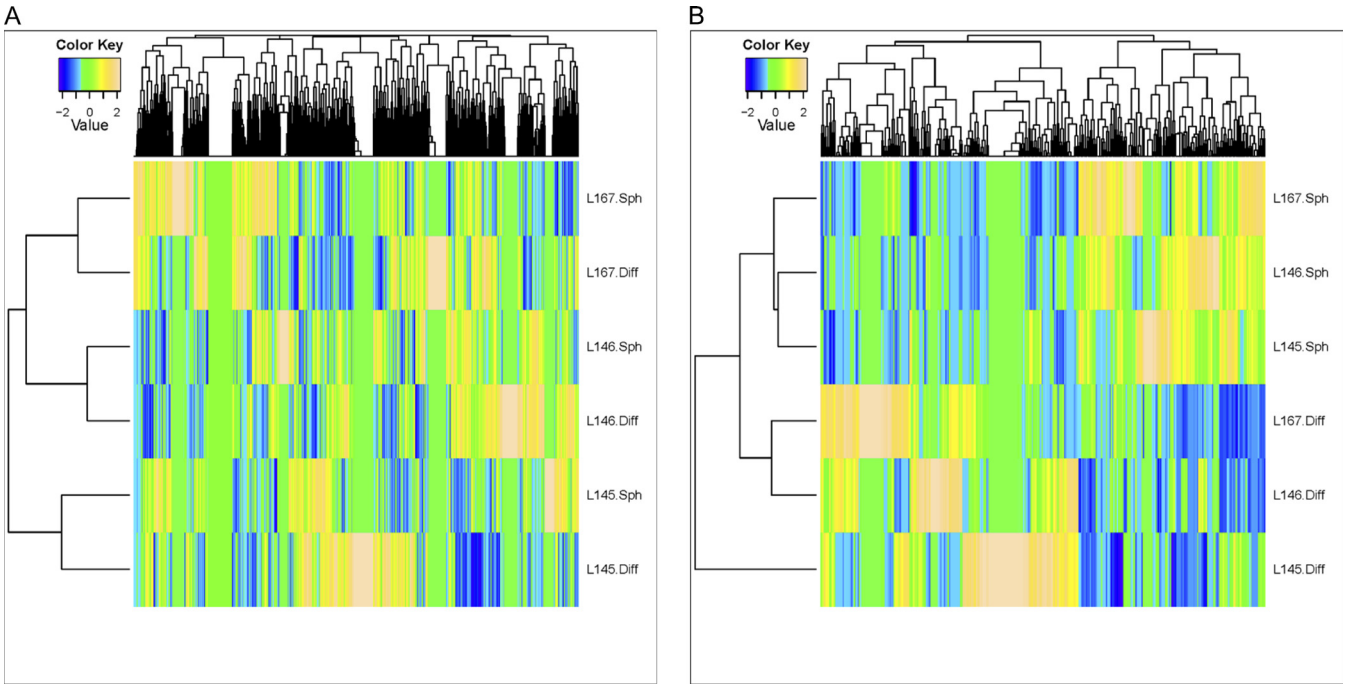

FIG. 2. **Separation of colonospheres from differentiated tumor cells by cluster analysis.** *A*, Heat map of unsupervised cluster analysis of all identified proteins. *B*, Heat map of supervised cluster analysis of all significantly up- and down-regulated proteins ($p < 0.05$ according to the Fisher exact test per pair.) The selected list of regulated proteins separates colonospheres from differentiated tumor cells.

(Fig. 2*A*). In contrast, supervised clustering including all significantly up- and down-regulated proteins shows that all colonosphere cultures and differentiated cultures now cluster together (Fig. 2*B*). This suggests that it may be possible to identify a cancer stem cell protein signature in colorectal cancer.

Proteins Associated with Survival are Up-regulated in Colon Cancer Stem Cells—Next, we divided the up-regulated proteins in three categories: (1) Top up-regulated, ≥ twofold up-regulated in three out of three isogenic pairs, (2) Subtop up-regulated, ≥1.5-fold up-regulated in three out of three isogenic pairs, and (3) Rest up-regulated, ≥1.5-fold up-regulated in two out of three isogenic pairs. A total amount of 119 proteins was found to be enriched in colonospheres (32 in category I; 22 in category II, and 65 in category III) (Table II, supplemental Table S1). Interestingly, among the top up-regulated proteins is ALDH1A1 (aldehyde dehydrogenase 1A1), which was recently identified by us and others as a bona fide colon (cancer) stem cell marker (11, 16, 20). The identification of ALDH1A1 demonstrates the validity of our proteomics approach to identify up-regulated factors in cancer stem cells.

Next, we used the Ingenuity Pathway Knowledge Base tool to identify biological functions and canonical pathways that distinguish colonospheres from differentiated cells. For this analysis all 119 colonosphere-enriched proteins (Table II, supplemental Table S1) were included. When classified according to function, proteins regulating "Cell Death " (20) and proteins regulating "Post-Translational Modification " (12) were most frequently identified. "Cell cycle control of chromosomal replication," "aryl hydrocarbon receptor signaling," "estrogen receptor signaling," and "mitochondrial dysfunction" were identified as the canonical pathways that are over-represented in colonosphere cultures (Table III).

Next, the STRING database was used to identify known and predicted protein interactions among the up-regulated proteins. STRING analysis of all up-regulated proteins showed five major clusters involved in redox regulation, transcription

TABLE II

All proteins up-regulated in colonospheres in comparison to differentiated tumor cells. The table shows all proteins that are up-regulated in colonospheres when compared to differentiated tumor cells according to the following criteria: (1) top up-regulated, ≥2-fold up-regulated in three out of three isogenic pairs (32) and (2) subtop up-regulated, ≥1.5-fold up-regulated in three out of three isogenic pairs (22). Sph = colonosphere culture enriched for cancer stem cells, Diff = isogenic differentiated progeny of colonosphere culture

Protein	Gene	Accession number	L145		L146		L167		Fold change	Location	Function
			Diff	Sph	Diff	Sph	Diff	Sph			
>2-fold up-regulated proteins in 3/3 combinations											
Glutathione peroxidase 2 (gastrointestinal)	GPX2	IPI00298176	0	13	0	23	0	12	∞	Cytoplasm	Enzyme
Glutathione peroxidase 1	GPX1	IPI00293975	0	10	0	8	0	12	∞	Cytoplasm	Enzyme
Ferritin, light polypeptide	FTL	IPI00852596	0	4	0	4	0	7	∞	Cytoplasm	Other
Neuroblastoma amplified sequence	NBAS	IPI00333913	0	5	0	6	0	3	∞	Unknown	Other
Baculoviral IAP repeat-containing 6	BIRC6	IPI00299635	0	4	0	6	0	3	∞	Cytoplasm	Enzyme
Microsomal glutathione S-transferase 2	MGST2	IPI00017767	0	3	0	4	0	3	∞	Cytoplasm	Enzyme
Glutathione peroxidase 4 (phospholipid hydroperoxidase)	GPX4	IPI00304814	0	2	0	5	0	3	∞	Cytoplasm	Enzyme
Eukaryotic translation initiation factor 3, subunit H	EIF3H	IPI00647650	0	3	0	3	0	3	∞	Cytoplasm	Translation regulator
CDGSH iron sulfur domain 3	CISD3	IPI00783359	0	2	0	4	0	3	∞	Unknown	Other
Endosulfine alpha	ENSA	IPI00220797	0	3	0	3	0	2	∞	Unknown	Transporter
Family with sequence similarity 98, member A	FAM98A	IPI00174442	0	2	0	2	0	3	∞	Unknown	Other
RAN binding protein 3	RANBP3	IPI00456728	0	2	0	2	0	3	∞	Nucleus	Other
Chromosome 11 open reading frame 31	C11ORF31	IPI00218054	0	2	0	2	0	3	∞	Nucleus	Other
Mitochondrial ribosomal protein S18B	MRPS18B	IPI00022316	0	2	0	3	0	2	∞	Cytoplasm	Other
RRS1 ribosome biogenesis regulator homolog (S. cerevisiae)	RRS1	IPI00014253	0	2	0	3	0	2	∞	Nucleus	Other
DEAH (Asp-Glu-Ala-His) box polypeptide 38	DHX38	IPI00294211	0	2	0	2	0	2	∞	Nucleus	Enzyme
C-terminal binding protein 1	CTBP1	IPI00012835	0	2	0	2	0	2	∞	Nucleus	Enzyme
SIN3 homolog A, transcription regulator (yeast)	SIN3A	IPI00170596	0	2	0	2	0	2	∞	Nucleus	Transcription regulator
Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	SDHB	IPI00294911	2	5	0	6	0	8	9.2	Cytoplasm	Enzyme
RNA binding motif protein 25	RBM25	IPI00004273	0	6	0	5	2	7	8.7	Nucleus	Other
E1A binding protein p400	EP400	IPI00064931	2	5	0	7	0	5	8.2	Nucleus	Other
Myosin, heavy chain 10, non-muscle	MYH10	IPI00397526	0	3	0	4	4	26	8.1	Cytoplasm	Other
Leucine rich repeat containing 16A	LRRC16A	IPI00014843	0	17	4	10	0	4	7.4	Unknown	Enzyme
CDC42 binding protein kinase beta (DMPK-like)	CDC42BPB	IPI00477763	2	6	0	4	0	3	6.2	Cytoplasm	Kinase
Eukaryotic translation initiation factor 3, subunit J	EIF3J	IPI00290461	2	6	0	2	0	3	5.2	Cytoplasm	Translation regulator
TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	TAF15	IPI00020194	0	3	0	2	2	5	4.9	Nucleus	Transcription regulator
PTK2 protein tyrosine kinase 2	PTK2	IPI00012885	0	3	0	3	2	4	4.8	Cytoplasm	Kinase
Protein arginine methyltransferase 5	PRMT5	IPI00441473	0	4	0	2	3	7	4.2	Cytoplasm	Enzyme
Activating signal cointegrator 1 complex subunit 3	ASCC3	IPI00430472	2	6	0	5	2	6	4.1	Nucleus	Enzyme
Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	IPI00218914	11	76	26	102	45	113	3.4	Cytoplasm	Enzyme
Mitochondrial ribosomal protein S27	MRPS27	IPI00022002	0	2	2	6	2	6	3.4	Cytoplasm	Other
Vacuolar protein sorting 13 homolog C (S. cerevisiae)	VPS13C	IPI00465428	9	19	5	13	5	13	2.3	Unknown	Other

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TABLE II—*continued*

Protein	Gene	Accession number	L145		L146		L167		Fold change	Location	Function
			Diff	Sph	Diff	Sph	Diff	Sph			
>1.5-fold up-regulated proteins in 3/3 combinations											
Adenylosuccinate lyase	ADSL	IPI00026904	0	5	3	5	0	5	4.8	Cytoplasm	Enzyme
Transmembrane protein 205	TMEM205	IPI00063130	0	5	0	3	3	5	4.2	Unknown	Other
Transformation/transcription domain-associated protein	TRRAP	IPI00069084	5	8	0	7	0	6	4.0	Nucleus	Transcription regulator
Nuclear receptor co-repressor 1	NCOR1	IPI00289344	0	3	0	4	3	5	3.9	Nucleus	Transcription regulator
ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	IPI00027481	0	7	0	11	9	17	3.8	Plasma Membrane	Transporter
CDGSH iron sulfur domain 1	CISD1	IPI00020510	0	3	0	8	6	10	3.4	Cytoplasm	Other
Methionine adenosyltransferase II, beta	MAT2B	IPI00002324	5	8	0	5	0	5	3.4	Cytoplasm	enzyme
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 11, 14.7kDa	NDUFA11	IPI00329301	2	5	0	6	4	6	2.7	Cytoplasm	Enzyme
Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	SDHA	IPI00305166	5	18	12	21	6	23	2.6	Cytoplasm	Enzyme
15 kDa selenoprotein	SEP15	IPI00030877	3	8	5	9	2	7	2.3	Cytoplasm	Enzyme
Aldehyde dehydrogenase 1 family, member B1	ALDH1B1	IPI00103467	8	17	15	46	14	22	2.2	Cytoplasm	Enzyme
Acetyl-CoA acyltransferase 1	ACAA1	IPI00012828	2	11	7	11	9	15	2.0	Cytoplasm	Enzyme
Eukaryotic translation initiation factor 5B	EIF5B	IPI00299254	6	12	6	11	3	8	2.0	Cytoplasm	Translation regulator
Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	IPI00011913	3	10	4	6	5	9	2.0	Nucleus	Other
UDP-glucose 6-dehydrogenase	UGDH	IPI00031420	4	22	30	45	19	37	1.9	Nucleus	Enzyme
Valyl-tRNA synthetase	VARS	IPI00000873	9	24	15	22	14	23	1.8	Cytoplasm	Enzyme
Tubulin tyrosine ligase-like family, member 12	TTLL12	IPI00029048	6	10	12	27	8	12	1.8	Unknown	Other
Interleukin enhancer binding factor 3, 90kDa	ILF3	IPI00298788	20	32	17	28	13	27	1.7	Nucleus	Transcription regulator
Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	CAD	IPI00301263	24	36	33	58	19	33	1.6	Cytoplasm	Enzyme
RAN binding protein 2	RANBP2	IPI00221325	28	45	22	35	28	42	1.5	Nucleus	Enzyme

TABLE III

Ingenuity analysis of colonosphere-enriched proteins. All 119 up-regulated proteins were analyzed by Ingenuity. The main Molecular and Cellular functions represented by this protein group are "Cell Death" (20) and "Post Translational Modifications" (12). The main canonical pathways represented by this protein group are also shown

Top functions	Associated molecules	Focus molecules
Molecular and cellular functions		
Cell death	ABCB1, ALDH1A1, ARMC10, BAX, **BIRC6**, CTBP1, EIF3H, EP400, GPX2, HSPA4, HTT, MCM2, MDC1, PRKDC, PTK2, RBM25, SDHA, SDHB, TIMM50, TRAP1	20
Post-translational modification	BAX, CTBP1, FTL, HSPA4, HTT, NEDD8, PRKDC, PRMT5, SEP15, ST13, TIMM50, TTN, ABCB1, ACO2, ADSL, AKR1C3, ALDH1A1, BAX, BDH2, GPX4, HSPA4, HTT, MAT2B, SDHA, TST, UGDH	12

Top functions	Associated molecules	Number of identified molecules	Number of molecules in pathway
Canonical pathways			
Cell cycle control of chromosomal replication	MCM2, MCM3, MCM5, MCM7	4	30
Aryl hydrocarbon receptor signaling	ALDH1A1, ALDH1B1, BAX, MCM7, MGST2, NEDD8	6	141
Estrogen receptor signaling	CTBP1, NCOR1, PRKDC, TAF15, TRRAP	5	134
Mitochondrial dysfunction	GPX4, NDUFA11, NDUFB11, SDHA, SDHB	5	133

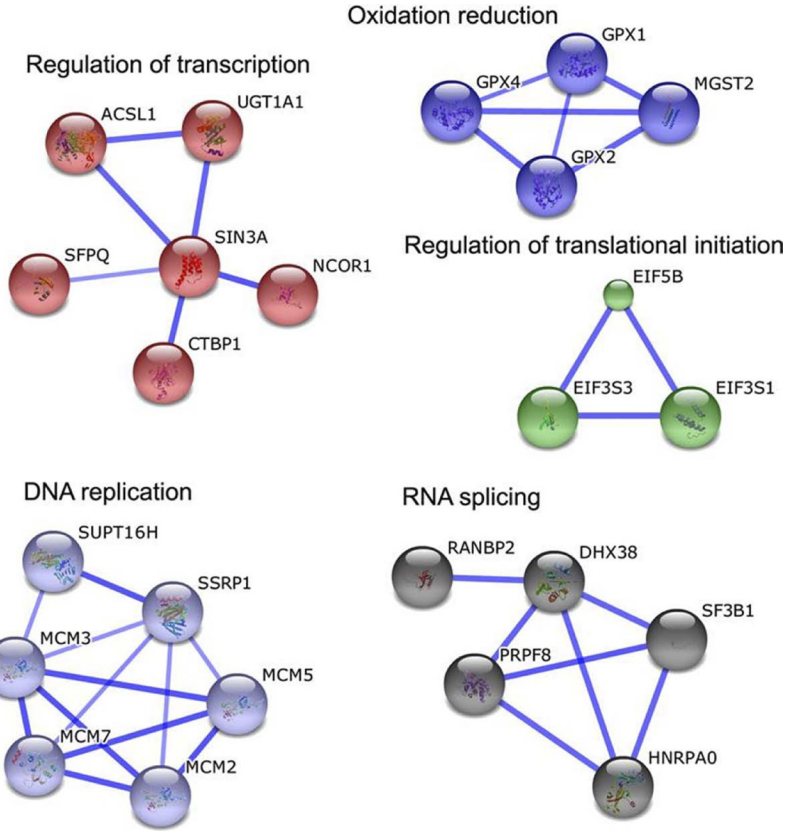

FIG. 3. **Colonopsphere-enriched proteins display functional interactions.** All colonosphere-enriched proteins (119) were analyzed against the STRING database for functional protein association networks. The strength of the associations is represented by line thickness. Networks with three or more protein interactions are shown. Required confidence (score) of protein association was 0.700 (high confidence).

control, RNA splicing, DNA replication, and regulation of translation initiation (Fig. 3).

Given our interest in drug resistance we further focused on proteins regulating cell death and survival (Table III). An extensive literature search revealed that the majority of overrepresented "Cell Death " regulators (15/20) have anti-apoptotic activity (Table IV). Of these, Baculoviral IAP repeat-containing 6 (BIRC6; also known as BRUCE or Apollon), an Inhibitor of Apoptosis Protein (IAP), is a key regulator of the intrinsic apoptosis pathway and has previously been implicated in drug resistance (21, 22).

BIRC6 Mediates Resistance of Colorectal Cancer Stem Cells to Platinum Compounds—Next, we tested whether

TABLE IV

Functional annotation of colonosphere-enriched "Cell Death" proteins. The 20 colonosphere-enriched cell death regulators that were identified by Ingenuity were classified according to their pro-and anti-apoptotic function, based on literature

Protein	Gene	Accession number	Fold change	Anti-apoptotic	Pre-apoptotic
Glutathione peroxidase 2 (gastrointestinal)	GPX2	IPI00298176	∞	+	
Baculoviral IAP repeat-containing 6	BIRC6	IPI00299635	∞	+	
Eukaryotic translation initiation factor 3, subunit H	EIF3H	IPI00647650	∞	+	
C-terminal binding protein 1	CTBP1	IPI00012835	∞	+	
Armadillo repeat containing 10	ARMC10	IPI00217968	∞	+	
Succinate dehydrogenase complex, subunit B, iron sulfur	SDHB	IPI00294911	9.2	+	+
RNA binding motif protein 25	RBM25	IPI00004273	8.7	+	+
E1A binding protein p400	EP400	IPI00064931	8.2	+	
PTK2 protein tyrosine kinase 2	PTK2	IPI00012885	4.8	+	
Aldehyde dehydrogenase 1 family, member A1	ALDH1A1	IPI00218914	3.4	+	
ATP-binding cassette, sub-family B (MDR), member 1	ABCB1	IPI00027481	3.8	+	
BCL2-associated X protein	BAX	IPI00071059	3.0		+
Succinate dehydrogenase complex, subunit A	SDHA	IPI00305166	2.6	+	
Huntingtin	HTT	IPI00002335	∞	+	+
Translocase of inner mitochondrial membrane 50	TIMM50	IPI00418497	3.1	+	
Mediator of DNA-damage checkpoint 1	MDC1	IPI00470805	2.9	+	+
Minichromosome maintenance complex component 2	MCM2	IPI00184330	1.7	+	
TNF receptor-associated protein 1	TRAP1	IPI00030275	1.6	+	
Protein kinase, DNA-activated, catalytic polypeptide	PRKDC	IPI00296337	1.4	+	
Heat shock 70kDa protein 4	HSPA4	IPI00002966	1.3	+	

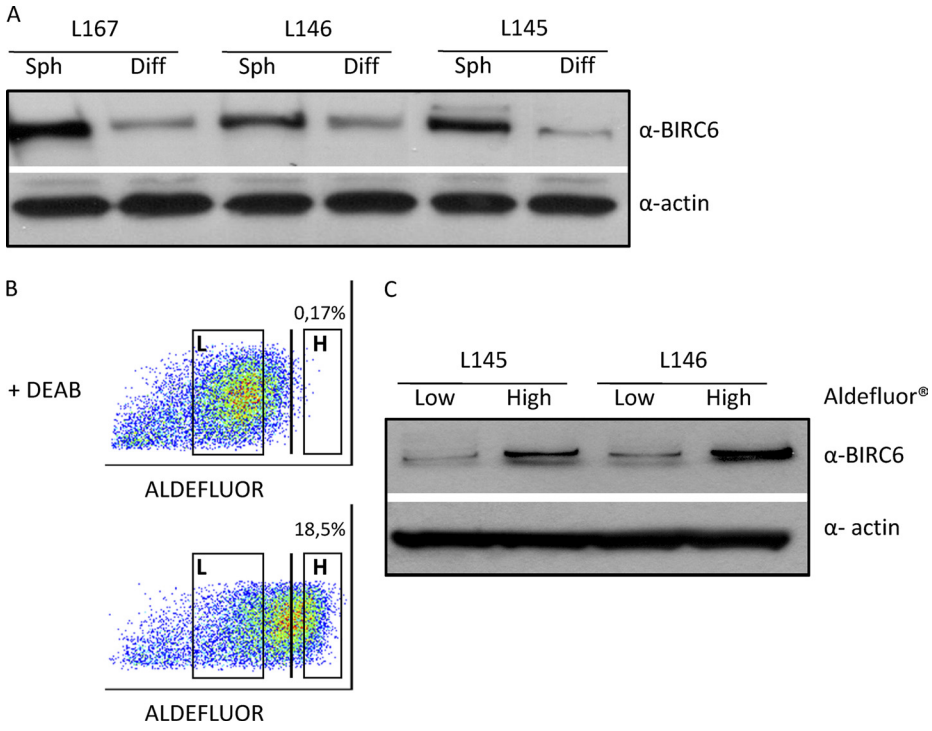

FIG. 4. **BIRC6 is expressed in the tumorigenic Aldefluor^{high} fraction of colonosphere cells.** *A*, Western blot analysis of BIRC6 levels in colonospheres and differentiated tumor cells in all pairs. *B*, Single cell cultures of L145 colonospheres were separated into Aldefluor®^{high} and Aldefluor®^{low} cell populations by FACS sorting. *C*, Western blot analysis of Aldefluor®^{high} and Aldefluor®^{low} cell populations for expression of BIRC6.

BIRC6 plays a role in mediating chemotherapy resistance in colorectal cancer stem cells. First, we analyzed BIRC6 expression in the three sets of isogenic cell pairs by Western blotting. In line with the proteomics data, BIRC6 was highly up-regulated in colonospheres when compared with differentiated cells (Fig. 4*A*). Western blot analysis of other top up-regulated proteins (GPX2, GPX1, and ALDH1) also confirmed the proteomics data (data not shown, and see (16)). Our previous results have shown that ALDH activity (as measured by the fluorescent substrate Aldefluor®) defines the tumorigenic and clonogenic cancer stem cell population within colonospheres (16). Therefore, we used FACS sorting to separate Aldefluor®^{high} from Aldefluor®^{low} cells (Fig. 4*B*) and analyzed BIRC6 expression in both tumor cell fractions. Fig. 4*C* shows that BIRC6 is highly expressed in tumorigenic Aldefluor®^{high} cells, but not in nontumorigenic Aldefluor®^{low} cells.

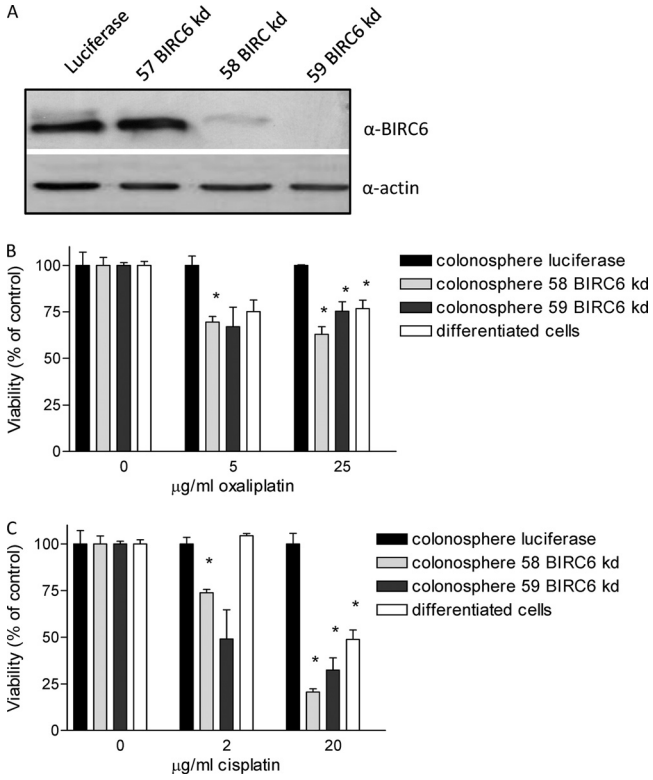

FIG. 5. **BIRC6 confers resistance against cisplatin and oxaliplatin.** *A*, Western blot analysis of BIRC6 expression in L145 colonospheres transduced with control (luciferase, 57) and BIRC6 knockdown vectors (58, 59). *B*, Control and BIRC6 knockdown L145 colonospheres were treated with oxaliplatin or cisplatin for 3 days using the indicated concentrations. Cell viability was then assessed by MTS assays for mitochondrial activity. Absorbance values (in triplicate) are expressed as percentage of vehicle-treated control wells. *Statistical significance (unpaired, 2-tailed *t* test: $p < 0.05$).

Next, we assessed the importance of BIRC6 in mediating colonosphere resistance to oxaliplatin and cisplatin, two frequently used chemotherapeutic drugs. To this end, expression of BIRC6 was suppressed in colonospheres by using a set of lentiviral RNA interference (RNAi) vectors. Two vectors (58 and 59) were found to suppress BIRC6 expression very efficiently (Fig. 5*A*).

Control and BIRC6 knockdown colonopsheres and differentiated tumor cells were treated with increasing concentrations of oxaliplatin or cisplatin and cell viability was measured by standard MTS assays. Knockdown of BIRC6 resulted in a significantly higher response of colonosphere cells to oxaliplatin and cisplatin (Fig. 5*B* and 5*C*). The sensitivity of BIRC6 knockdown cells to both drugs was comparable to that of the differentiated tumor cells. These results identify BIRC6 as an important mediator of resistance to oxaliplatin and cisplatin and suggest that high BIRC6 expression may selectively protect the cancer stem cell fraction against these drugs.

DISCUSSION

In the present study we have used a proteomics approach to identify potential regulators of the cancer stem cell phenotype in colorectal tumors. We identified known (ALDH1A1) and novel factors enriched in cancer stem cell cultures (colonospheres) when compared with stably differentiated tumor cells. Interestingly, STRING analysis revealed that distinct protein complexes involved in transcriptional repression, DNA replication, RNA splicing, translation initiation and redox control are significantly enriched in colonospheres when compared with differentiated tumor cells. Future work should reveal the function of these complexes in the maintenance of colorectal cancer stem cells.

Importantly, cancer stem cells were also characterized by high expression of a set of survival proteins, the most prominent of which was BIRC6. BIRC6 deletion is associated with sensitization to chemotherapy in *in vivo* and *in vitro* studies (21, 22). Furthermore, BIRC6 deletion promotes p53 stabilization and caspase 3 activation (23).

Our data show that specifically colorectal cancer stem cell cultures display increased resistance to oxaliplatin and to cisplatin and that BIRC6 is an important mediator of resistance. Previously, it was shown by gene expression profiling that BIRC1 and BIRC6 are up-regulated in colorectal tumors when compared with normal intestinal issue (24). Our results suggest that it is predominantly the cancer stem cell-fraction in colorectal tumors that expresses this survival protein.

BIRC6, also known as Apollon or Bruce, belongs to the family of IAP proteins. IAP's are major regulators of apoptosis due, at least in part, to their ability to inhibit caspase activation (25, 26). Human IAP family members include X-chromosome-linked IAP (XIAP, also known as BIRC4), cellular IAP 1 (c-IAP1 also known as BIRC2), c-IAP2 (also known as BIRC3), neuronal apoptosis inhibitory protein (also known as BIRC1), and survivin (also known BIRC5). IAP proteins contain one to three baculovirus IAP repeat (BIR) domains that are required for their anti-apoptotic activity (25). Our results are in line with previous studies showing that cancer stem cells express high levels of anti-apoptotic proteins and resist apoptotic stimuli (27, 28). Recently it was demonstrated that IL4-stimulated expression of survivin (BIRC5) protects colorectal cancer stem cells against apoptosis (10, 28, 29). The proteomics approach described here did not identify BIRC5 as a cancer stem cell-enriched protein. Possibly, different tumors resist apoptotic stimuli by increasing the expression of distinct IAP family members.

Because IAP's play an important role in tumor maintenance and therapy resistance they represent attractive targets for targeted therapy. Furthermore, IAP's are highly expressed in several cancer tissues (30). Several small molecule IAP inhibitors have been developed, including Smac-based peptides and Smac mimetics targeting a broad spectrum of IAP's (31, 32). Preclinical studies in mice carrying xenograft tumors have

shown promising antitumor efficacy in the treatment of malignant glioma, breast cancer, nonsmall cell lung cancer, and multiple myeloma. However, most of these preclinical studies have focused on BIRC4 and surviving (BIRC5), rather than on BIRC6 (33, 34). Several IAP inhibitors are being tested for their safety and anti-tumor efficacy in clinical trials either in combination with irradiation or with chemotherapy (35, 36). It is not yet established whether these compounds also target BIRC6. Second mitochondrial-derived activator of caspases (SMAC) mimetics bind to and inhibit the BIR domains in IAP's. Because SMAC also binds to BIRC6 (37), it is not unlikely that SMAC mimetics will inhibit BIRC6.

Based on the data presented here we propose that BIRC6 protects the cancer stem cell fraction of colorectal tumors against oxaliplatin and cisplatin. Targeting BIRC6 by SMAC mimetics or by novel specific BIRC6 inhibitors may therefore be effective in combination with platinum-based anticancer drugs. This may help eradicating the cancer stem cell fraction in colorectal tumors.

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This article contains supplemental Table S1.

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