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Original Article

Prominin-1 (CD133, AC133) and dipeptidyl-peptidase IV (CD26) are indicators of infinitive growth in colon cancer cells

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Abstract: Advanced colorectal cancer is characterized by uncontrolled growth and resistance against anti-cancer agents, including ErbB inhibitors. Recent data suggest that cancer stem cells (CSC) are particularly resistant. These cells may reside within a CD133+ fraction of the malignant cells. Using HCT116 cells we explored the role of CD133 and other CSC markers in drug resistance in colon cancer cells. CD133+ cells outnumbered CD133- cells over time in long-term culture. Both populations displayed the KRAS mutation 38G > A and an almost identical target profile, including EGFR/ErbB1, ErbB2, and ErbB4. Microarray analyses and flow cytometry identified CD26 as additional CSC marker co-expressed on CD133+ cells. However, knock-down of CD133 or CD26 did not affect short-term growth of HCT116 cells, and both cell-populations were equally resistant to various targeted drugs except irreversible ErbB inhibitors, which blocked growth and ERK1/2 phosphorylation in CD133- cells more efficiently than in CD133+ cells. Moreover, the MEK inhibitor AS703026 was found to overcome resistance against ErbB blockers in CD133+ cells. Together, CD133 and CD26 are markers of long-term growth and resistance to ErbB blockers in HCT116 cells, which may be mediated by constitutive ERK activity.

Keywords: Cancer stem cell, CD26, CD133, colon cancer, DPPIV, drug resistance, EGFR/ErbB, HCT116

Introduction

Colorectal cancer is a leading cause of morbidity and mortality in industrialized countries worldwide [1-4]. During the last decade, our knowledge about oncogene-dependent signaling and the mechanisms underlying progression of colorectal tumors has increased significantly, and has facilitated the development of novel anti-cancer agents [5-9]. These include conventional cytostatic drugs and molecular targeted drugs acting on various oncogenic kinases such as KIT, KDR, RAS, MEK or members of the EGF receptor (R) family [6-11]. Targeted drugs, including EGFR/ErbB blockers, have also been applied in combination with chemotherapy [7, 10, 12]. However, resistance against one or more drugs is still a challenge in the treatment of colon cancer patients, and the same holds true for other solid tumors. In colorectal cancer, the molecular mechanisms of resistance to anti-EGFR therapies are complex and are considered to be associated with mutations and hyperactivation of pro-oncogenic downstream effector molecules such as KRAS, BRAF or PIK3CA, or with inactivating mutations in tumor suppressor genes like PTEN [13]. Patients lacking mutations in pro-oncogenic genes have a higher probability to respond to EGFR-targeted therapy [13].

Table 1. Monoclonal antibodies (mAb) and reactivity of bulk, CD133+ and CD133- HCT116 colon cancer cells

mAb	Reactive Structure	CD	Source/ Isotype	Fluorochrome	Manufacturer	Reactivity with HCT116*		
						Bulk	CD133+**	CD133-**
L27	B1	20	m/IgG I	PE	BD-B	-	-	-
ML5	Nectadrin	24	m/IgG2a	FITC	BD-B	-	-	-
M-A261	DPP IV	26	m/IgG I	PE	BD-B	+++	++	+
581	HPCA1	34	m/IgG I	PE	BD-B	-	-	-
515	Pgp-1	44	m/IgG I	PE	BD-B	++++	++++	++++
HI30	LCA	45	m/IgG I	FITC	BD-B	-	-	-
HI186	Campath1	52	m/IgG2b	PE	BioL	-	-	-
487618	CEA	66e	m/IgG I	APC	R&D	++++	+++	+++
5EIO	Thy1	90	m/IgG I	FITC	BD-B	+	n.d.	n.d.
166707	Endoglin	105	m/IgG I	PE	R&D	+++	+++	++
LMM741	G-CSFR	114	m/IgG I	PE	BD-B	+	+	+
61708	M-CSFR	115	m/IgG I	PE	R&D	-	-	-
31916	GM-CSFRα	116	m/IgG1	PE	R&D	-	-	-
D2	KIT	117	m/IgG I	PE	BD-B	-	-	-
32703	IL-3Rα	123	m/IgG I	PE	R&D	-	-	-
AC133	AC133	133	m/IgG I	PE	Mil-BT	+++***	++++	-
BV10A4H2	FLT-3	135	m/IgG I	PE	BioL	+	-	-
105902	ALCAM	166	m/IgG I	PE	R&D	++++	++++	++++
MEM-260	IRp60	300a	m/IgG I	PE	Abcam	-	-	-
89106	KDR	309	m/IgG I	PE	R&D	++	-	+
HEA-125	EpCAM	326	m/IgG I	FITC	Mil-BT	++++	++++	++++
95106	MET	n.c.	m/IgG I	PE	R&D	++++	++++	++++
33255	IGF-IR	221	m/IgG I	PE	R&D	++++	++++	++++
2A2	LGR5	n.c.	m/IgG1	PE	Origene	+	+	+
EGFR-I	EGFR	n.c.	m/IgG2b	PE	BD-B	+++	+++	+++
191924	ErbB2	340	m/IgG2b	PE	R&D	+++	+++	+++
66223	ErbB3	n.c.	m/IgG1	PE	R&D	+++	+++	+++
182818	ErbB4	n.c.	m/IgG2a	PE	R&D	+	+	++

*Score reactivity: ++++, 75.01-100% of cells positive; +++, 50.01-75% of cells positive; ++, 25.01-50% of cells positive; +, 10.01-25% of cells positive; -, 0-10% of cells reactive. **Sorted cells. ***CD 133 was found to be expressed on a distinct subpopulation of bulk HCT116 cells. APC, allophycocyanin; BD-B, Becton Dickinson Biosciences; Biol, BioLegend; FITC, fluorescein isothiocyanate; HPCA 1, human progenitor cell antigen-1; IGF, insulin-like growth factor; IL- 3, interleukin-3; LCA, leukocyte common antigen; Mil-BT, Miltenyi Biotec; n.c., not yet clustered; PE, phycoerythrin.

Numerous studies have shown that most if not all neoplasms are composed of two different fractions of cells - a partially differentiated population with limited capacity to divide and a second cell population exhibiting the capacity of unlimited proliferation and self-renewal, the so-called cancer stem cells (CSC) [14-17]. The 'CSC-hypothesis' predicts that therapy is curative only when eliminating all CSC in a given neoplasm [14-17]. Recent data suggest that colon CSC reside within the CD133+ fraction of the clone [18-21]. The CD133 antigen, also known as prominin-1 or AC133, is a glycoprotein expressed on various mesenchymal cells without known specific function [22, 23]. However, recent data suggest that expression of CD133 on colon cancer cells is associated with drug resistance and with an increased metastatic potential [24-26]. The HCT116 cell line has been described as a useful model for studying the CD133+ CSC-phenotype in colon cancer cells [27-30]. In the present study, we used this cell line to study the role of CD133 in proliferation and survival of colon cancer cells

Table 2. PCR primers

Protein	Orientation	Primer Sequence	PCR Product Length
MEK1 (MAP2K1)	Forward	5' AACTCTCCGTACATCGTGGG 3'	
	Reverse	5' GGCGACATGTAGGACCTTGT 3'	332 bp
MEK2 (MAP2K2)	Forward	5' CGTACCTCCGAGAGAAGCAC 3'	
	Reverse	5' GGCAAAATCCACTTCTTCCA 3'	596 bp
CD133	Forward	5' TCAGGATTTTGCTGCTTGTG 3'	
	Reverse	5' GCAGTATCTAGAGCGGTGGC 3'	480 bp
β-actin	Forward	5' ATGGATGATGATATCGCCGCG 3'	
	Reverse	5' CTAGAAGCATTTGCGGTGGACGATGGAGGGGCC 3'	1020 bp

MAP2K1, mitogen-activated protein kinase 2 kinase 1; MAP2K2, mitogen-activated protein kinase 2 kinase 1; MEK, MAP/ERK kinase.

and their resistance against ErbB-targeting drugs.

Materials and methods

Reagents

The irreversible EGFR(ErbB1)/ErbB2 inhibitor pelitinib (EKB-569) was kindly provided by Wyeth (Cambridge, MA, USA). The MEK inhibitor AS703026 was a kind gift from Dr. J. Ogden and Dr. M. Wolf (Merck Serono, Darmstadt, Germany). The irreversible pan-ErbB inhibitors canertinib (CI-1033) and afatinib (BIBW2992), the reversible EGFR inhibitor erlotinib, the reversible pan-ErbB inhibitor lapatinib, the reversible EGFR-blocker gefitinib, the PDGFR/VEGFR/FGFR-blocker sunitinib, the multi-kinase inhibitor sorafenib, the Abl/Src/Kit-antagonist dasatinib, the Bcr-Abl targeting nilotinib, the PDGFR/Kit/Abl specific imatinib, the reversible pan-ErbB inhibitor BMS-599626, the pan-Aurora inhibitor VX-680, the Hsp90 inhibitor 17-AAG, and the HDAC inhibitor vorinostat were purchased from ChemieTek (Indianapolis, IN, USA). A specification of monoclonal antibodies (mAbs) used in our study is shown in **Table 1**. RNeasy Mini Kit and HotStarTaq Master Mix Kit were obtained from QIAGEN (Hilden, Germany), First Strand cDNA Synthesis Kit from Roche-Applied-Science (Mannheim, Germany), and RT-PCR primers (MEK1, MEK2, CD133, β-actin) from Eurofins MWG Operon (Ebersberg, Germany). ^{3}H-thymidine was purchased from Amersham (Buckinghamshire, UK) and the Vybrant MTT Cell Proliferation Assay Kit from Invitrogen (Carlsbad, CA, USA). Dulbecco's Modified Eagle Medium (DMEM), phenol red-free Iscove's MDM (IMDM), fetal calf serum (FCS), trypsin/EDTA, and L-glutamine were from Invitrogen (Carlsbad, CA, USA).

Culture of HCT116 cells

HCT116 human colon cancer cells were purchased from the German Resource Center for Biological Materials (Heidelberg, Germany). Cells were cultured in DMEM and 10% FCS and passaged using trypsin/EDTA. The identity of HCT116 was confirmed by flow cytometry and molecular investigations including short tandem repeat profiling [31], which was conducted at the German Resource Center for Biological Materials, and the presence of the KRAS 38G > A mutation was verified by DNA sequencing. Phenotyping and mutation analysis were repeated after multiple passages and after sorting into CD133+ and CD133- fractions.

Flow cytometry and cell sorting

HCT116 cells were stained with fluorochrome-conjugated mAbs directed against various cell surface antigens (**Table 1**). Antibody reactivity was determined by flow cytometry using a FACSCalibur (Becton Dickinson, San Diego, CA, USA) and FlowJo software (Tree Star, Ashland, OR, USA). Isotype-matched control antibodies were used in each experiment. CD133+ and CD133- HCT116 cells or CD26+ and CD26- HCT116 cells were purified by sorting on a FACSAria (BD Biosciences). The purity of sorted cells was up to 98%, and cell viability was > 90% in each case.

Reverse transcription PCR (RT-PCR)

PCR primers specific for MEK1, MEK2, AC133/CD133, and β-actin are given in **Table 2**. RT-PCR reactions were performed using First Strand cDNA Synthesis Kit and HotStarTaq Master Mix Kit as described [32].

Proliferation assays

HCT116 cells (bulk and fractions) were incubated with various concentrations (0.001-10 μM) of ErbB inhibitors or other drugs at 37°C for 48 or 72 hours. Cell survival was analyzed using the Vybrant MTT Cell Proliferation Assay Kit following the recommendation of the manufacturer (Invitrogen Molecular Probes) [33]. Synthesis of DNA was determined by measuring ^{3}H-thymidine uptake as reported [34]. In select experiments, combinations of pelitinib and AS703026 were applied (fixed ratio of drug concentrations) before measuring proliferation of HCT116 cells. Drug-interactions (additive versus synergistic) were determined by calculating combination index (CI) values using Calcusyn software (Calcusyn; Biosoft, Ferguson, MO). A CI value of 1 indicates an additive effect and CI values below 1 synergistic drug actions. In a separate set of experiments, DNA synthesis was determined by a BrdU colorimetric immunoassay according to the manufacturer's instructions (Roche-Applied-Science, Mannheim, Germany). All experiments were performed in triplicates.

Analysis of apoptosis and cell cycle progression

Unfractionated HCT116 cells and sorted fractions (CD133+ versus CD133- and CD26+ versus CD26-) were incubated with various concentrations of ErbB inhibitors (0.001-10 μM) for 48 or 72 hours and apoptosis was determined by staining externalized membrane phosphatidylserine with annexin V-FITC (BenderMedSystems, Vienna, Austria) or by labeling active caspase-3 with a PE-conjugated mAb (BD Biosciences). In addition, cell cycle distribution was determined using the DNA binding fluorochrome propidium iodide and labeled cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson).

RNA interference-mediated knockdown of CD133 and CD26

Cells ($24 \times 10^3/cm^2$) were transfected with siRNA using siLentFect™ Lipid Reagent (Bio-Rad Laboratories, Hercules, USA) following the manufacturer's protocol. Cells were incubated for 72 hours with 20 nM CD133 siRNA (sc-42820; Santa Cruz Biotechnology, Santa Cruz, CA) or 20 nM scramble control siRNA (sc-37007) at 37°C and 5% CO_2. For knockdown of CD26, a pLK0.1 clone containing an shRNA targeting human CD26 (5'-GACTGAAGTTATACTCCTTAA-3') was obtained from Open Biosystems (Huntsville, AL). Recombinant VSV-G pseudotyped lentiviruses were produced as described [35]. Cells were transduced in the presence of polybrene (7 μg/ml) and selected with puromycin (2 μg/ml) for 48 hours. Knockdown of CD133 and CD26 was confirmed by flow cytometry.

Western blot analysis

For cell signaling analyses, unfractionated or sorted (CD133+ and CD133-) HCT116 cells were starved in serum-free medium (24 hours) and then exposed to pelitinib (5 μM), canertinib (10 μM), afatinib (10 μM), or 0.1% DMSO as solvent control at 37°C for 6 hours. Cells were then challenged for 5 minutes with 100 ng/ml recombinant human EGF (Sigma, St. Louis, MO, USA) and 1 nM recombinant human heregulin 1 (HRG 1; Thermo Fisher Scientific, Fremont, CA, USA). Proteins (30 μg/lane) were then subjected to Western blotting as described [36] using antibodies against EGFR, phospho-EGFR(Tyr1068), AKT, phospho-AKT(Ser473), phospho-ERK1/2, S6, phospho-S6(Ser240/244) (Cell Signaling Technology, Danvers, MA, USA), ErbB2, phospho-ErbB2(Tyr1248), actin, (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and ERK1/2 (Upstate Biotechnology, Lake Placid, NY or Santa Cruz: sc-93). Secondary antibodies were peroxidase-tagged donkey-anti-rabbit (Promega, Madison, WI, USA), donkey-anti-goat IgG (Santa Cruz Biotechnology), goat anti-rabbit IgG (Cell Signaling Technology), or peroxidase-labeled donkey anti-goat IgG (Santa Cruz Biotechnology).

Gene chip experiments

To define mRNA expression patterns in CD133+ and CD133- HCT116 cells, DNA microarray analyses were performed using genome-wide human U133 2.0 plus GeneChips (Affymetrix, Santa Clara, CA) as described according to manufacturer's protocols (https://www.affymetrix.com). Robust Multichip Average signal extraction and normalization were done as reported (http://www.bioconductor.org/) [37]. Changes in mRNA expression levels were calculated as mRNA ratio between CD133+ and CD133- HCT116 cells. mRNA expression data are available at Gene Expression Omnibus:

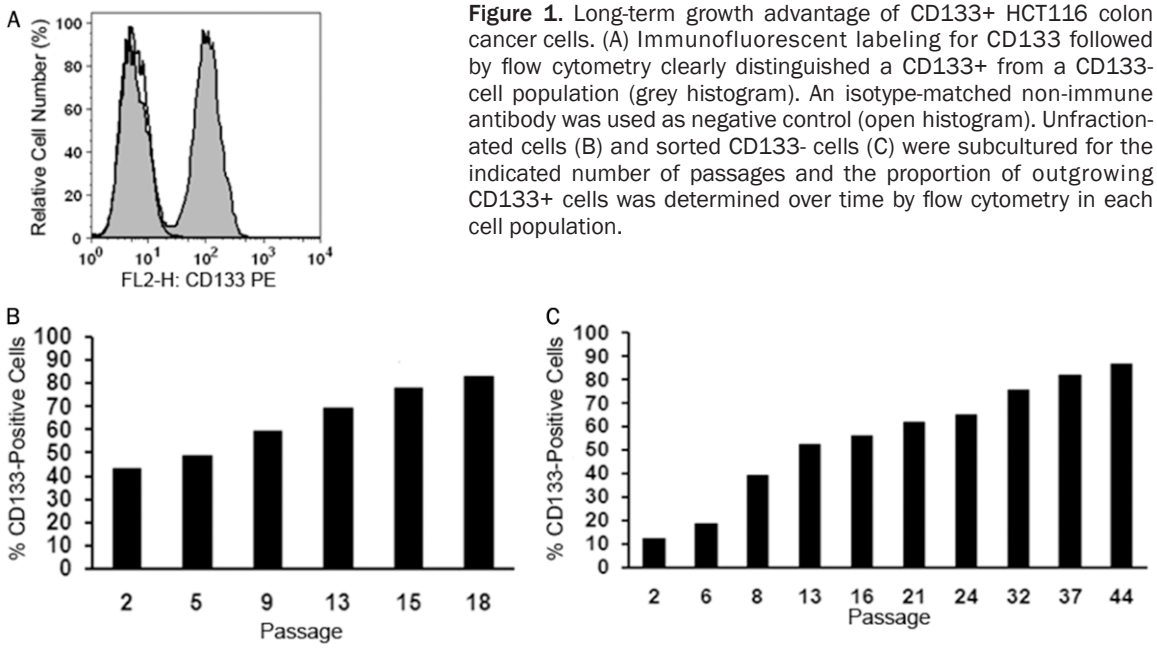

Figure 1. Long-term growth advantage of CD133+ HCT116 colon cancer cells. (A) Immunofluorescent labeling for CD133 followed by flow cytometry clearly distinguished a CD133+ from a CD133- cell population (grey histogram). An isotype-matched non-immune antibody was used as negative control (open histogram). Unfractionated cells (B) and sorted CD133- cells (C) were subcultured for the indicated number of passages and the proportion of outgrowing CD133+ cells was determined over time by flow cytometry in each cell population.

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33504S and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38049. Statistical analyses were performed with R-Bioconductor [38]. For annotation, R-packages hugene10st.db_1.0.2 and hugene10st-v1cdf_1.0.0 were used. For these analyses, we filtered genes with an expression level greater than log2 (100) in at least 2 of 6 samples and an inter-quartile range > 0.3. To assess differentially expressed genes we used moderated paired t-tests with empirical Bayes shrinkage of the standard errors (R-package: limma). To detect statistical significant pathways, gene set enrichment analysis was performed with R-package sigPathway. In pathway analyses, we focused on genes involved in cell growth and drug resistance.

Results

CD133+ HCT116 cells outnumber CD133- HCT116 cells in culture

As determined by flow cytometry, HCT116 cells are composed of a CD133+ and a CD133- cell population (**Figure 1A**). The differential expression of CD133 in sorted cell fractions was confirmed by RT-PCR and gene chip analysis. After serial passage, CD133+ cells were found to outnumber CD133- cells in long-term culture (**Figure 1B**). Even when sorted for CD133- cells, minute amounts of residual CD133+ cells (typically <5%) were outnumbering CD133- cells over time (**Figure 1C**). Surprisingly, in short-term culture, CD133+ and CD133- cells did not show differences in cell viability, proliferation rate or cell cycle distribution. Consequently, we wondered whether CD133+ cells may secrete an inhibitory factor that suppresses growth of CD133- cells. However, supernatants obtained from CD133+ cells did not affect the growth of CD133- cells (not shown).

Target protein expression in CD133+ and CD133- cells

Flow cytometry revealed that CD133+ and CD133- fractions (purity 90-98%) exhibit essentially the same cell surface membrane phenotype. Both cell subsets were found to express various tissue-specific and stem cell-related receptor antigens, including CD44, CD166 (ALCAM) and CD326 (EpCAM). In addition, both fractions stained positive for major drug targets, including EGFR (ErbB1), ErbB2, ErbB3, ErbB4, c-MET, and IGFR-1 (**Table 1**). Moreover, DNA sequencing identified mutant KRAS (38G > A) in both CD133+ and CD133- cells. Altogether, no obvious differences in target expression profiles were detected between CD133+ and CD133- HCT116 cells.

Comparative gene expression analysis of CD133+ and CD133- cells

We next screened for differentially expressed genes by global analysis of mRNA transcripts in CD133+ and CD133- HCT116 cells. Using DNA microarrays we found that CD133+ cells

Table 3. Major pathways upregulated in CD133+ relative to CD133- HCT116 cells

Gene Set Category	Pathway	Set Size*	Percent Up†	NTk q-value††	NEk q-value†††
GO:0006928	cell motility	25	40	0.0000	0.0000
GO:0051674	localization of cell	25	40	0.0000	0.0000
GO:0040011	locomotion	25	40	0.0000	0.0000
KEGG:04510	Focal adhesion	35	23	0.0000	0.0000
KEGG:04810	Regulation of actin cytoskeleton	28	32	0.0000	0.0000
SuperArray	Insulin Signaling Pathway	21	29	0.0000	0.0000
GO:0000902	cellular morphogenesis	39	46	0.0000	0.0000
GO:0030246	carbohydrate binding	29	38	0.0000	0.0000
GO:0008092	cytoskeletal protein binding	60	42	0.0000	0.0000
GO:0040007	growth	37	46	0.0000	0.0000
GO:0003779	actin binding	38	39	0.0000	0.0000
GO:0007167	enzyme linked receptor protein signaling pathway	22	50	0.0230	0.0000
GO:0001558	regulation of cell growth	24	50	0.0000	0.0000
GO:0040008	regulation of growth	27	52	0.0000	0.0000
SuperArray	Nitric Oxide	26	31	0.0230	0.0000
GO:0030695	GTPase regulator activity	39	41	0.0000	0.7791
SuperArray	EGF / PDGF Signaling Pathway	24	25	0.0000	0.7791
SuperArray	Hypoxia Signaling Pathway	24	38	0.0381	0.0000
GO:0008361	regulation of cell size	30	47	0.0000	0.7791
GO:0016049	cell growth	30	47	0.0000	0.7791
SuperArray	Breast Cancer / Estrogen Receptor Signaling	29	34	0.0741	0.0000
GO:0004857	enzyme inhibitor activity	25	48	0.0881	0.0000
GO:0007243	protein kinase cascade	52	40	0.0381	0.0000
GO:0015629	actin cytoskeleton	23	57	0.0488	0.0000
GO:0009966	regulation of signal transduction	46	43	0.0488	0.0000
SuperArray	G-Protein Coupled Receptors Signaling PathwayFinder	22	41	0.0881	0.0000
GO:0005578	extracellular matrix (sensu Metazoa)	23	22	0.0000	1.0000
GO:0031012	extracellular matrix	23	22	0.0000	1.0000
GO:0006066	alcohol metabolism	58	64	0.0000	1.0000
GO:0006820	anion transport	21	33	0.0938	0.0000
GO:0005996	monosaccharide metabolism	39	69	0.0256	1.0000
GO:0006006	glucose metabolism	28	71	0.0256	1.0000
GO:0015077	monovalent inorganic cation transporter activity	26	85	0.0256	1.0000
GO:0019318	hexose metabolism	38	68	0.0256	1.0000
GO:0031410	cytoplasmic vesicle	20	55	0.1017	0.0000
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	20	50	0.1649	0.0000
GO:0030529	ribonucleoprotein complex	138	79	0.0256	1.0000

Expression of mRNA levels in CD133+ cells compared to CD133- cells (methods and details are described in the text). *Set Size: Number of genes included in the pathway; †Percent Up: Percent upregulated genes in the pathway; ††NTk q-value: A multiplicity adjusted *p*-value <0.05 indicates that the genes in a gene set do not show the same pattern of associations with the group labels compared with the rest of the genes; †††NEk q-value: A multiplicity adjusted q-value <0.05 indicates that the gene set contains genes whose expression levels are associated with the group.

express markedly higher levels of various marker genes, including CD26, when comparing to CD133-cells (**Table 4A**). The differential expression of CD133 and CD26 was also detected at the protein level using flow cytometry (**Table 1**). Moreover, comparative pathway analyses on DNA microarrays revealed distinct expression of growth-regulatory, cell communication and motility pathways (**Tables 3**-**5**) as a function of the presence/absence of CD133, corroborating recent data published by Botchkina et al [39].

Irreversible ErbB inhibitors block the growth of CD133- but not of CD133+ cells

Despite the large spectrum of recognized targets (see Materials and methods for details),

Table 4. Top 10 upregulated and top 10 downregulated genes in CD133+ relative to CD133- HCT116 cells

A. Top 10 Upregulated Genes			
Gene Symbol	Gene Name	Log Fold Change	*P*-Value
PROM1	prominin 1	2.8	0.0106
EHF	ets homologous factor	2.3	0.0106
NRIP1	nuclear receptor interacting protein 1	2.2	0.0114
GPR110	G protein-coupled receptor 110	1.7	0.0188
IL18	interleukin 18 (interferon-gamma-inducing factor)	1.6	0.0114
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	1.4	0.0114
SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	1.4	0.0114
7A5	putative binding protein 7a5	1.4	0.0114
LOC390345	similar to ribosomal protein L10	1.3	0.0188
SNORA22	small nucleolar RNA, H/ACA box 22	1.3	0.0396
B. Top 10 Downregulated Genes			
Gene Symbol	Gene Name	Log Fold Change	*P*-Value
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	-1.7	0.0167
ODZ3	odz, odd Oz/ten-m homolog 3 (Drosophila)	-1.7	0.0106
PEG10	paternally expressed 10	-1.5	0.0114
MAP1B	microtubule-associated protein 1B	-1.4	0.0106
GLIS3	GLIS family zinc finger 3	-1.4	0.0188
CTGF	connective tissue growth factor	-1.3	0.0352
THBS1	thrombospondin 1	-1.3	0.0210
SEMA3C	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	-1.2	0.0377
RBM24	RNA binding motif protein 24	-1.2	0.0210
CDK6	cyclin-dependent kinase 6	-1.1	0.0114

Expression of mRNA levels in CD133+ cells compared to CD133- cells (methods and details are described in the text). Gene symbols and gene names (according to the Affymetrix hugene1.0-chip) are given as well as the log-fold change [log(mean group CD133+) minus log(mean group CD133-)] and the corresponding *p*-value of the t-test (adjusted for multiplicity using the method of Benjamini and Hochberg) of the top 10 up- and downregulated genes. A *p*-value <0.05 is indicating a significant difference between CD133+ and CD133- cells.

most of the drugs applied in this study failed to induce significant growth inhibition in unfractionated HCT116 cells, indicating pronounced drug resistance in this cell model. Nevertheless, inhibitors that bind to and interfere with ErbB receptor function in an irreversible manner, such as pelitinib, canertinib, and afatinib, effectively blocked cell growth when applied in concentrations ≥ 1 μM. The dose-dependent decline of cell numbers, evidenced by MTT assay (**Figure 2A**), correlated closely with diminished DNA synthesis determined by BrdU incorporation or ^{3}H-thymidine uptake (not shown). In contrast, flow cytometry analyses using annexin V or an antibody against activated caspase-3 failed to detect programmed cell death. These data suggest that irreversible ErbB blockers cause cell growth arrest rather than apoptosis in HCT116 cells. Remarkably, CD133- cells proved to be more sensitive to these ErbB inhibitors than CD133+ cells (**Figure 2B** and **2C**).

Effects of knockdown of CD133 or CD26 on cell growth and on the sensitivity against ErbB-inhibitory drugs

The obtained data suggested an association between CD133 expression and resistance against ErbB blockers. Surprisingly, siRNA-mediated knockdown of CD133 expression in CD133+ cells (**Figure 3A**) did not affect the growth rate of the cells (**Figure 3B**) nor did it decrease the resistance against pelitinib, canertinib or afatinib relative to control siRNA-transfected CD133+ cells (**Figure 3C**) suggesting that other proteins co-expressed with CD133 such as CD26 may confer inhibitor resistance in CD133+ cells. However, specific knockdown of CD26, which is typically co-expressed in CD133+ HCT116 cells (**Figure 4A**), failed to alter the growth rate (not shown) or the sensitivity of the cells against irreversible ErbB antagonists (**Figure 4B**) indicating that

Table 5. Comparative pathway analysis of expression of growth regulatory genes in CD133+ as opposed to CD133- HCT116 cells

Gene Symbol	Gene Name	Mean CD133+	Mean CD133-	StDev CD133+	StDev CD133-	*P*-Value
ACTL6A	actin-like 6A	8.2	8.1	0.3	0.4	0.6729
CAMK2D	calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	7.9	8.4	0.2	0.3	0.1264
CTGF	connective tissue growth factor	7.0	8.3	0.1	0.6	0.0622
IGFBP4	insulin-like growth factor binding protein 4	6.4	6.4	0.2	0.3	0.8422
IGFBP6	insulin-like growth factor binding protein 6	8.3	8.8	0.2	0.3	0.0894
CYR61	cysteine-rich, angiogenic inducer, 61	8.5	9.5	0.1	0.2	0.0059
QSOX1	quiescin Q6 sulfhydryl oxidase 1	8.3	8.2	0.5	0.4	0.7980
SHC1	SHC (Src homology 2 domain containing) transforming protein 1	8.7	9.0	0.1	0.1	0.0124
YEATS4	YEATS domain containing 4	8.2	7.9	0.3	0.4	0.4214
LTBP4	latent transforming growth factor beta binding protein 4	8.0	8.1	0.4	0.4	0.7153
BLZF1	basic leucine zipper nuclear factor 1 (JEM-1)	8.0	7.9	0.3	0.3	0.8172
RUVBL1	RuvB-like 1 (E. coli)	9.1	8.8	0.3	0.4	0.4854
SOCS1	suppressor of cytokine signaling 1	6.8	6.7	0.1	0.3	0.4293
SOCS2	suppressor of cytokine signaling 2	6.7	6.6	0.2	0.3	0.4856
ENOX2	ecto-NOX disulfide-thiol exchanger 2	6.5	6.3	0.3	0.3	0.5228
BRD8	bromodomain containing 8	7.2	7.5	0.2	0.4	0.2988
TMEM97	transmembrane protein 97	9.3	9.5	0.2	0.3	0.6351
CRIM1	cysteine rich transmembrane BMP regulator 1 (chordin-like)	8.5	9.3	0.2	0.4	0.0360
ING3	inhibitor of growth family, member 3	7.0	7.0	0.3	0.4	0.9326
SOCS4	suppressor of cytokine signaling 4	6.6	6.4	0.3	0.5	0.5587
BTG1	B-cell translocation gene 1, anti-proliferative	8.4	8.7	0.4	0.3	0.4571
PPP2CA	protein phosphatase 2 (formerly 2A), catalytic subunit, alpha isoform	9.9	9.9	0.3	0.3	0.8012
PPP2R1B	protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform	8.2	8.2	0.2	0.2	0.9676
RB1	retinoblastoma 1 (including osteosarcoma)	8.0	8.2	0.3	0.3	0.5369

Expression of mRNA levels in CD133+ cells compared to CD133- cells (methods and details are described in the text). Gene symbols and gene names (according to the Affymetrix human gene1.0 chip) are given as well as the mean values of the CD133+ and CD133- cells and the corresponding standard deviations (StDev). Furthermore, the *p*-values of the t-test to compare CD133+ to CD133- cells for each gene are shown. A *p*-value <0.05 indicates a significant difference between CD133+ and CD133- cells.

yet other still unidentified differentially activated genes must confer ErbB drug resistance in CD133+ cells. The efficacy of CD133 or CD26 knockdown was confirmed by flow cytometry (**Figures 3A** and **4A**) and Western blotting (not shown), respectively.

Effects of irreversible ErbB inhibitors on the phosphorylation of ErbB receptors and downstream effectors in CD133+ and CD133- cells

Western blot analyses revealed that pelitinib, canertinib and afatinib efficiently abrogated phosphorylation of EGFR (ErbB1), ErbB2, AKT and S6 in unfractionated as well as in CD133+ and CD133- cells. In contrast, phosphorylation of ERK1,2 was only blocked in the ErbB drug sensitive CD133- fraction, but not in the ErbB drug resistant CD133+ nor in the unfractionated cell population (**Figure 5**). Therefore, in the latter two less responsive cell populations, ErbB drug-dependent growth inhibition appears primarily mediated through silencing of the ErbB/RAS/PI3K/AKT/mTOR pathway, whereas in the more sensitive CD133- cells not only this pathway but also ErbB/RAS/MAPK signaling gets abrogated by the drugs, which may reinforce the antiproliferative response. Thus, the MAPK cascade may harbor crucial markers for the differential ErbB drug response of CD133- *versus* CD133+ cells.

The MEK inhibitor AS703026 induces cell growth arrest and overcomes ErbB inhibitor resistance

Since RAS downstream signaling via MAPK, in addition to PI3K, appears crucial for HCT116 cell growth we wondered whether co-silencing of ErbB and MAPK may be able to restore ErbB

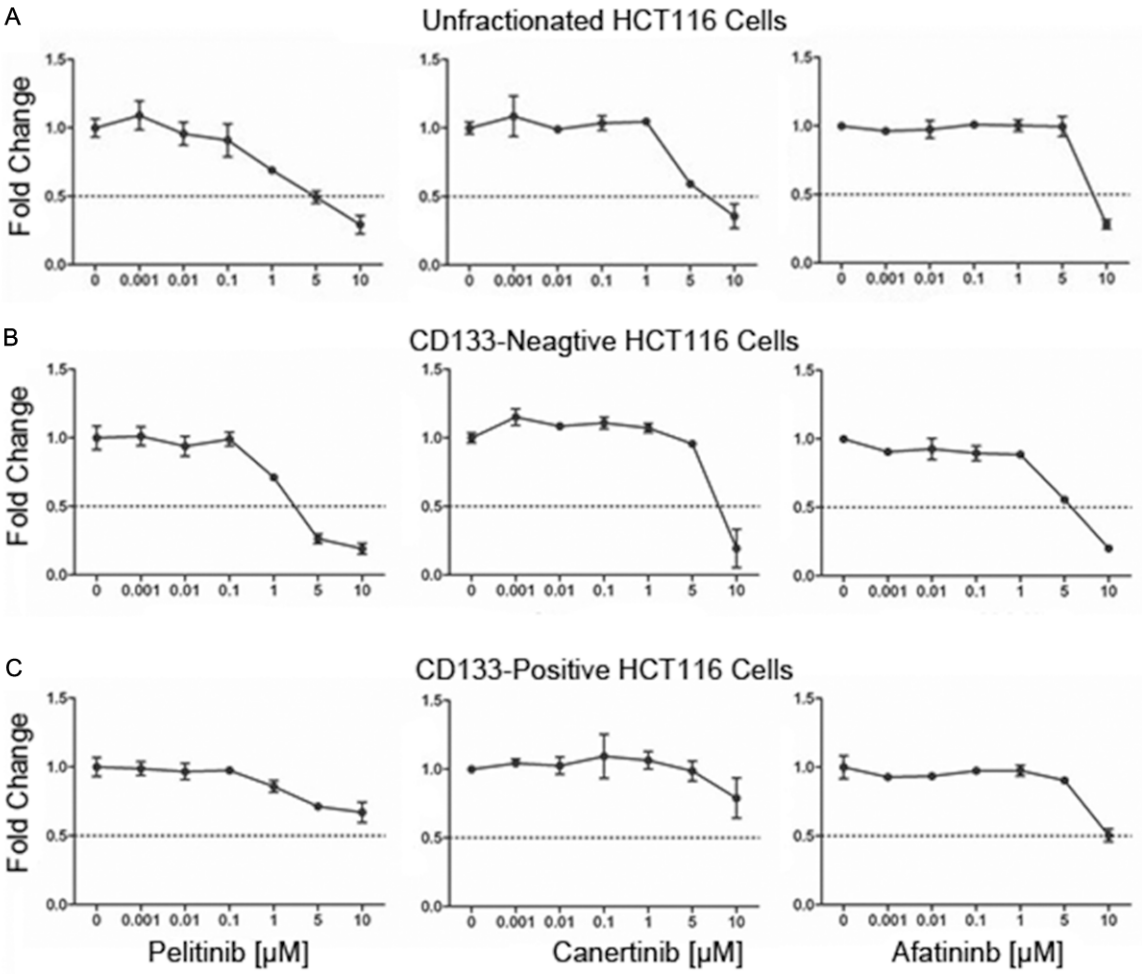

Figure 2. CD133+ HCT116 colon cancer cells are less sensitive to growth inhibition by the irreversible ErbB inhibitors pelitinib, canertinib, and afatinib as determined by MTT assay. Unfractionated (A), sorted CD133- (B) or CD133+ cells (C) were incubated for 72 hours with indicated concentrations of pelitinib, canertinib or afatinib. In vehicle control (0.1% DMSO), optical density, which is proportional to cell number, has been arbitrarily set at 1 and values from treated cultures have been related to control and are given as 'fold change'. Means ± SD, n = 3.

drug sensitivity in resistant CD133+ cells. Accordingly, the MEK inhibitor AS703026 was found to block growth of all HCT116 cell populations irrespective of expression of CD133 or CD26 (**Figure 6A**). Most importantly, however, this MEK inhibitor combined with an ErbB antagonist induced synergistic growth inhibition (**Figure 6B**). These data suggest that the MAPK pathway may harbor crucial markers for ErbB drug resistance in CD133+ HCT116 cells and that MEK-inhibition is a promising approach to overcome ErbB drug resistance in RAS-transformed colon cancer (stem) cells.

Discussion

Primary and acquired resistance against conventional and molecular targeted drugs is a major obstacle for successful treatment of colon cancer. Resistance of a subset of colorectal cancers to anti-EGFR therapy is associated with alterations in downstream effectors of the EGFR pathway including KRAS, BRAF, PIK3CA or PTEN [13]. However, not all alterations in RAS are necessarily associated with EGFR drug resistance. In metastatic colorectal cancer we recently identified a mutation in KRAS (p.G13D) that confers sensitivity to the EGFR blocking antibody cetuximab [40]. There is preclinical and clinical evidence suggesting that multiple tumor-specific features and molecular lesions contribute to drug resistance. Therefore, accessory lesions may act together with constitutively active RAS to yield a more resistant phenotype. It is thus of particular importance to identify additional mechanisms of drug resistance.

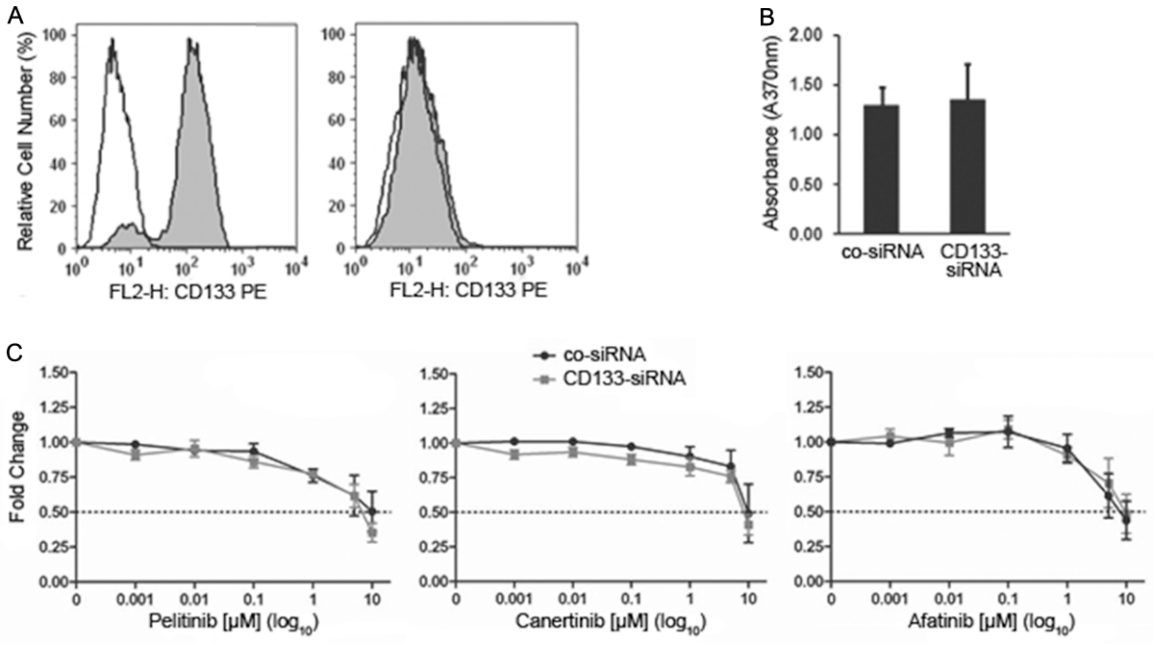

Figure 3. Knockdown of CD133 in CD133+ HCT116 colon cancer cells does not affect cell growth and ErbB drug resistance. (A) Flow cytometry analysis revealed that sorted CD133+ cells transfected with a non-targeting control siRNA retain high levels of CD133 (left panel), whereas a CD133-targeting siRNA causes complete knockdown of CD133 (right panel). (B) Growth of the sorted CD133+ cell population transfected either with non-targeting control siRNA (co-siRNA) or with CD133 siRNA was determined by BrdU colorimetric incorporation assay. (C) Sorted CD133+ cells transfected with non-targeting control siRNA (co-siRNA) or with CD133 siRNA were incubated for 72 hours with the indicated concentrations of the irreversible ErbB inhibitors pelitinib, canertinib or afatinib and then subjected to an MTT assay. In vehicle control (0.1% DMSO), optical density, which is proportional to cell number, has been arbitrarily set at 1 and values from treated cultures have been related to control and are given as 'fold change'. Means ± SD, n = 3.

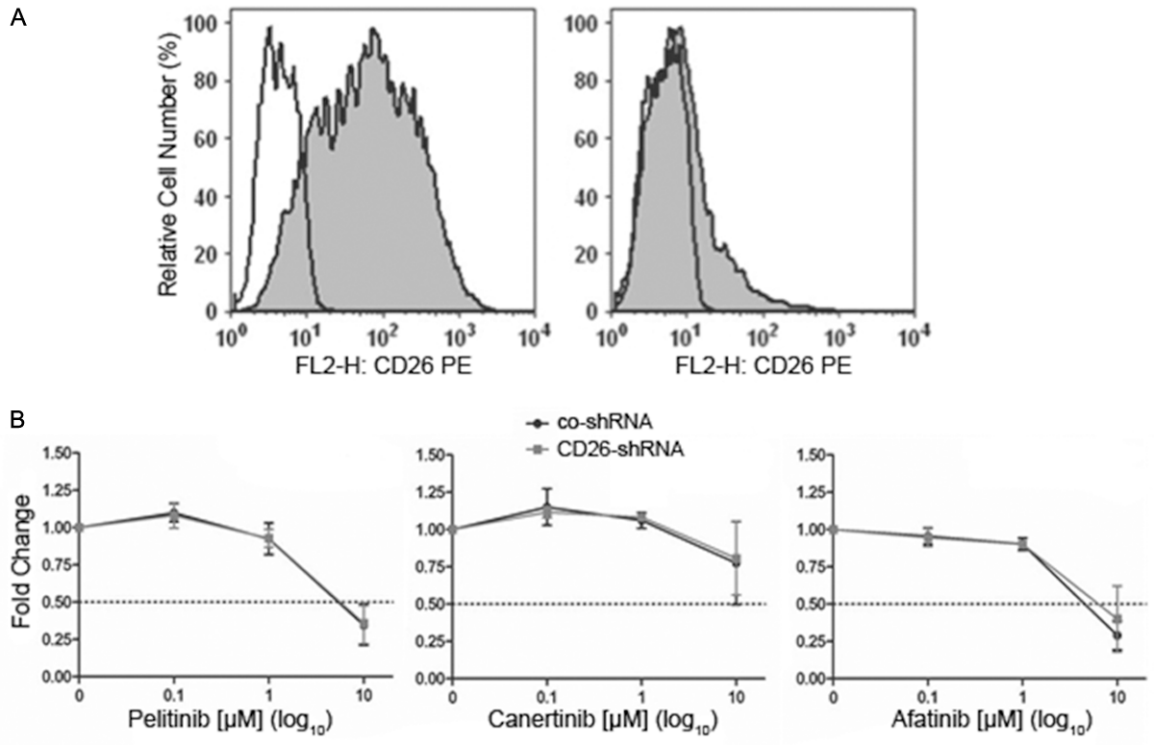

Figure 4. Knockdown of CD26 in CD133+ HCT116 colon cancer cells does not affect cell growth and ErbB drug resistance. (A) Flow cytometry analysis revealed that introduction of a non-targeting control shRNA into CD133+ cells does not lower the expression of CD26 (left panel), whereas a CD26-targeting shRNA causes strong downregulation of CD26 (right panel). (B) Sorted CD133+ cells transfected with non-targeting control shRNA (co-shRNA) or with CD26 shRNA were incubated for 72 hours with the indicated concentrations of the irreversible ErbB inhibitors pelitinib, canertinib or afatinib and then subjected to an MTT assay. In vehicle control (0.1% DMSO), optical density, which is proportional to cell number, has been arbitrarily set at 1 and values from treated cultures have been related to control and are given as 'fold change'. Means ± SD, n = 3.

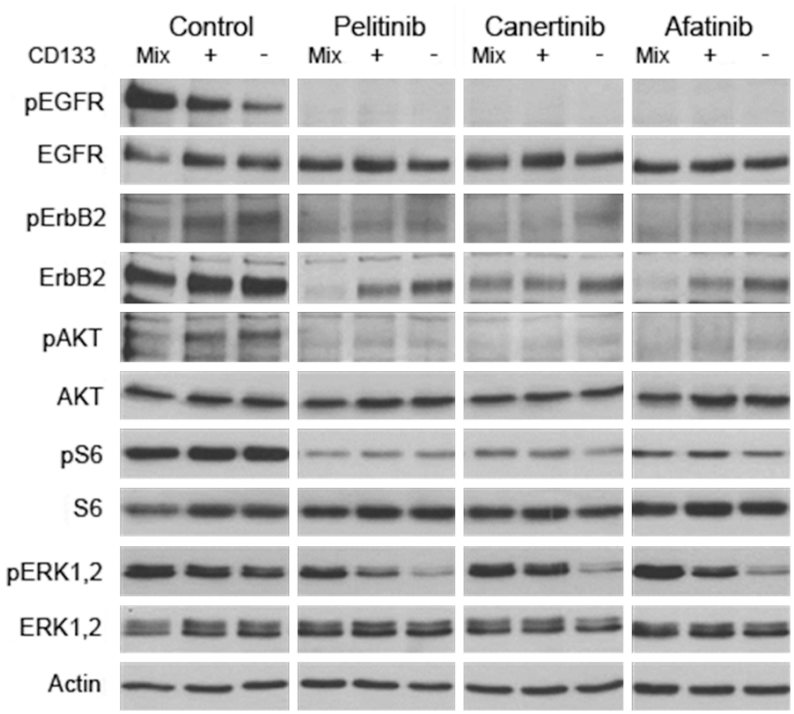

Figure 5. Effects of a 24-hours exposure of bulk HCT116 colon cancer cells (Mix) and of sorted CD133+ or CD133- cells to the irreversible ErbB inhibitors pelitinib, canertinib, or afatinib on the expression of phosphorylated (p) and total forms of EGFR, ErbB2, AKT, S6, and ERK1,2 as determined by Western blot analysis. Note that ErbB inhibitors lower pERK1/2 levels specifically in ErbB drug sensitive CD133- cells, but not in bulk or in CD133+ cells.

Colon cancer stem cells may reside within the CD133+ cell population [18-20]. In agreement with previous data we demonstrate that expression of CD133 (prominin-1) is associated with a long-term growth advantage of HCT116 cells relative to cells lacking this transmembrane glycoprotein [27-30]. In contrast, in short-term bioassays we were unable to define any gain in growth and survival of CD133+ versus CD133- cells. Both cell subsets invariably express hyperactivated mutant KRAS (38G > A) and are characterized by an almost identical repertoire of membrane proteins. Consequently, we hypothesized that small modifications in multiple gene sets and pathways may cause subtle functional differences in CD133+ cells that orchestrate to produce a robust long-term growth advantage in these cells. Accordingly, gene array analyses revealed several pathways related to cell growth and motility being upregulated in CD133+ HCT116 cells relative to CD133- cells. In contrast, no major differences in expression of drug resistance genes were found. Moreover, major drug targets including the EGFR/ErbB family (EGFR or ErbB1, ErbB2, ErbB3 and ErbB4) were expressed independently of CD133. Here we examined the anti-cancer efficacy of various reversible and irreversible ErbB blockers in cultures of HCT116 cells. While the reversible drugs erlotinib, gefitinib, lapatinib, and BMS599626 were not effective, irreversible ErbB blockers such as pelitinib, canertinib and afatinib dose-dependently reduced growth of HCT116 cells. Interestingly, CD133- cells were found to be more sensitive to growth inhibition by irreversible blockers than CD133+ and unfractionated HCT116 cells. Thus, it was tempting to speculate that CD133 is associated with resistance against irreversible ErbB drugs. Unexpectedly, however, genetic knockdown of CD133 failed to reestablish sensitivity in CD133+ cells. This data suggests that yet unidentified accessory factors that have been co-selected during enrichment of CD133+ cells may confer ErbB drug resistance. Accordingly, we observed that CD133+ cells invariably overexpress CD26, which has recently been proposed as a marker for metastatic and drug-resistant colorectal cancer cells [41].

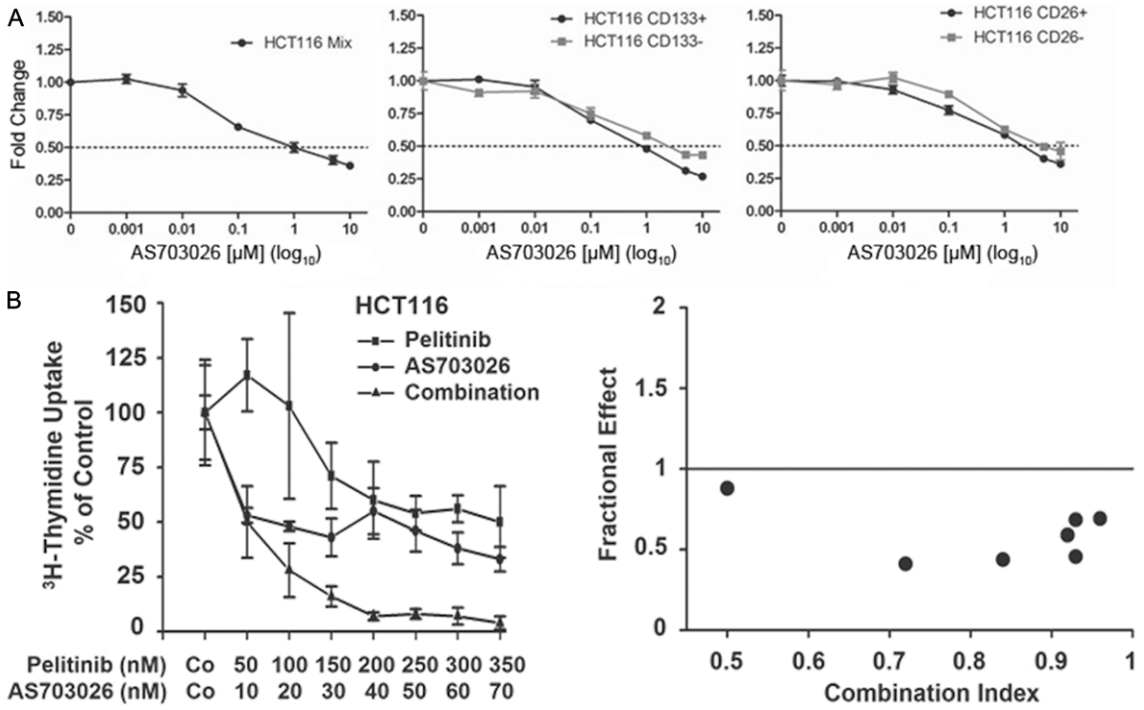

Figure 6. Effect of the MEK inhibitor AS703026 on the growth and the ErbB drug resistance of HCT116 colon cancer cells. (A) Unfractionated (left panel, Mix), sorted CD133+ and CD133- (middle panel), and sorted CD26+ and CD26- cells (right panel) were incubated for 72 hours with the indicated concentrations of AS703026 and then subjected to an MTT assay. In vehicle control (0.1% DMSO), optical density, which is proportional to cell number, has been arbitrarily set at 1 and values from treated cultures have been related to control and are given as 'fold change'. Means ± SD, n = 3. (B) Bulk cells were incubated with the indicated concentrations of pelitinib, AS703026 or a combination of both drugs held at a fixed concentration-ratio of 5:1 for 48 hours and DNA synthesis was measured by ^{3}H-thymidine uptake. Results are expressed as percent of control and represent the mean ± SD of triplicate determinations (left panel). Moreover, the combination index for exposure to pelitinib along with AS703026 is given (right panel). An index of <1 indicates synergistic drug interaction.

Unfortunately, however, knock-down of CD26 in CD133+ cells also failed to restore drug sensitivity arguing for yet other mechanisms of resistance. Moreover, phosphorylation of AKT and S6 was found to persist in all drug-treated HCT116 cell populations irrespective of CD133 expression as demonstrated by Western blot analysis. Notably, however, we observed specific downregulation of phosphorylated ERK1/2 in drug-sensitive CD133-, but not in resistant CD133+ or unfractionated cells. This indicates that constitutive MAPK hyperactivation obviously promotes ErbB drug resistance in CD133+ cells. These findings imply that subtle differences in the circuitry of ErbB, KRAS, RAF and PI3K, particularly at the bifurcation of RAS towards RAF or PI3K, do exist between CD133- and CD133+ cells and that this disparity contributes to relative resistance against ErbB kinase inhibitors. Specifically, in cells lacking CD133, RAS proteins are obviously less autonomous and dominant (i.e. more dependent on activation through upstream receptors) in activating downstream RAF, MEK and ERK1,2 than in cells expressing CD133. However, the definitive molecular link of CD133 with RAS has yet to be identified.

Since activation of ERK appears associated with ErbB drug resistant growth of HCT116 cells, we wondered whether abrogation of ERK activity may overcome resistance against ErbB blockers. Given as single drug, AS703026 - an inhibitor of the upstream kinase MEK - was found to block HCT116 cell growth even in ErbB inhibitor resistant CD133+ cells. Intriguingly, this compound was found to synergistically cooperate with irreversible ErbB antagonists in growth control and can overcome ErbB drug resistance in HCT116 cells. In conclusion, we present evidence demonstrating that expression of the cancer stem cell marker CD133

is associated with growth advantage and resistance against irreversible ErbB inhibitors in colon cancer (stem) cells, which can be overcome by concurrent blockade of MEK signaling.

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Disclosure of conflict of interest

ES and PV were supported by a research grant from Merck.

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