β-CATENIN in der kolorektalen Karzinogenese

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München 2010

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Dissertation

der Fakultät für Biologie

der Ludwig-Maximilian-Universität

München

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München, den 12. Juli 2010

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Tag der mündlichen Prüfung: 11.02.2011

MEINEN ELTERN

INHALTSVERZEICHNIS

ZUSAMMENFASSUNG

Dickdarmkrebs ist weltweit eine der häufigsten krankheitsbedingten Todesursachen. Daher ist die Aufklärung der zugrunde liegenden anatomischen und molekularen Mechanismen der kolorektalen Tumorentstehung und - Progression ein hochrelevanter Gegenstand der internationalen biomedizinischen Forschung. Während der vergangenen zwei Jahrzehnte führten zahlreiche Studien bereits zu vielen grundlegenden Erkenntnissen, so dass heute bereits allgemeine Modellvorstellungen über die Entwicklung dieses Tumortyps existieren und diskutiert werden. Es stellte sich heraus, dass die überwiegende Mehrzahl kolorektaler Läsionen auf der Dysregulation des hochkonservierten WNT Signalwegs beruht. Das hierdurch stabilisierte Protein β-CATENIN ist die treibende Kraft hinter der kolorektalen Karzinogenese. β-CATENIN nimmt in der Zelle zwei unterschiedliche wichtige Funktionen wahr: zum einen ist es ein Bestandteil der membranösen Zonula adhaerens und am Aufbau von Epithelverbänden beteiligt. In diesem Kontext fungiert β-CATENIN als Tumorsuppressor. Zum anderen kann β-CATENIN auch im Kern vorliegen, wo es zusammen mit TCF/LEF1 einen aktivierenden Transkriptionsfaktor darstellt. Die hierdurch regulierten Zielgene vermitteln die Aggressivität der Tumorzellen, so dass β-CATENIN in diesem Zusammenhang ein Onkoprotein darstellt. In kolorektalen Tumoren sind Zellen mit nukleärem β-CATENIN häufig an der Invasionsfront lokalisiert und zeigen Verlust der epithelialen Differenzierung bei gleichzeitigem Zugewinn mesenchymaler Attribute. Insofern stellt die Invasionsfront eine Zone epithelialer-mesenchymaler Transition (EMT) dar, ein Prozess der eng mit der Progression von Krebserkrankungen verknüpft zu sein scheint und auch von wichtiger Funktion in der Induktion des Tumorstammzellphänotyps ist. Diese Befunde bilden das Fundament für das Konzept der migrierenden Krebsstammzellen im Dickdarm, deren aggressive Eigenschaften sich im Wesentlichen auf die Aktivität nukleären β-CATENINs zurückführen lassen.

Im Mittelpunkt meiner Doktorarbeit standen somit Untersuchungen wissenschaftlicher Fragestellungen betreffend der Stabilisierung der WNT Aktivität in kolorektalen Tumoren und der Beteiligung von β-CATENIN an der Vermittlung des Tumorstammzellphänotyps. Ich konnte einen neuen denkbaren Mechanismus aufdecken, der in hochgradig mikrosatelliteninstabilen Tumoren zur Anreicherung von β-CATENIN führen könnte: in einem geringen Anteil dieser Tumore wies ich eine Frameshift-Mutation im *WTX* Gen nach, die vermutlich mit dem funktionellen Verlust

des Genprodukts assoziiert ist. Bei WTX handelt es sich um eine kürzlich entdeckte Komponente des β-CATENIN Degradationskomplexes. Demnach könnte dessen Aktivität aufgrund des Ausfalls von WTX beeinträchtigt sein, wodurch der Abbau von β-CATENIN inaktiviert wird. In einem weiteren Projekt beschäftigte ich mich ebenfalls mit der Regulation der WNT Aktivität und half zu zeigen, dass kolorektale Tumoren einen Verlust der Expression des β-CATENIN Zielgens und WNT Inhibitors DKK4 aufweisen, so dass der hierdurch aufgebaute negative *feedback loop* unterbrochen wird.

Der Fokus meiner Doktorarbeit lag allerdings auf Untersuchungen hinsichtlich des Tumorstammzellkonzepts. Es stellte sich heraus, dass der etablierte Tumorstammzellmarker CD133 zwar sehr wohl eine prognostische Bedeutung für die Tumorprogression hat, sein funktioneller Beitrag für die Aggressivität kolorektaler Tumore hingegen vermutlich eine untergeordnete Rolle spielt. Die Beteiligung von β-CATENIN an der Vermittlung von Tumorstammzelleigenschaften indes konnte in mehreren Projekten experimentell untermauert werden. Mir gelang die Identifizierung der neuen β-CATENIN Zielgene *p16*^{INK4A}, *TERT* und *FGF-2* und der Nachweis, dass diese tatsächlich auch in den vermeintlichen Tumorstammzellen der Invasionsfronten kolorektaler Tumore exprimiert werden. Die Tumorstammzellen zugeschriebene Eigenschaft geringer Proliferation wird im kolorektalen Karzinom demnach durch p16^{INK4A} vermittelt, dessen Expression auch mit einer signifikant schlechteren Überlebensprognose für die betroffenen Patienten korreliert ist. Als essentielle Komponente des Telomerase Enzymkomplexes ist TERT von hoher Wichtigkeit für das Tumorstammzellattribut der Langlebigkeit, so dass β-CATENIN auch hierfür direkt verantwortlich gemacht werden kann. Eine basale Herausforderung für Tumorstammzellen, die sich durch Migration auszeichnen, ist die Konfrontation mit sich permanent ändernden Umgebungsbedingungen und das Risiko des Verlusts der adäquaten Tumorstammzellnische. Durch die β-CATENIN vermittelte, autokrine Expression von FGF-2 könnten die Tumorstammzellen autark von dieser Nische werden, da ich nachweisen konnte, dass FGF-2 eine essentielle Rolle in der Aufrechterhaltung von Stammzelleigenschaften kolorektaler Tumorzellen innehat.

Zusammenfassend liegt der Hauptbeitrag meiner Doktorarbeit in der Untermauerung der fundamentalen Bedeutung stabilisierten β-CATENINs für die kolorektale Tumorentstehung und - Progression. In Unterstützung des Konzepts der migrierenden Krebsstammzellen konnte ich bestätigen, dass nukleäres β-CATENIN funktionell an der Vermittlung von Tumorstammzelleigenschaften im kolorektalen Karzinom beteiligt ist.

1 EINLEITUNG

1.1 Krebs

Nach Erkrankungen des Herz-Kreislaufsystems stellen bösartige (maligne) Tumore weltweit die zweithäufigste Todesursache dar. So schätzte man für das Jahr 2007 das Auftreten von mehr als sieben Millionen Todesfällen als direkte Folge von Krebsleiden, wobei jährlich in etwa zwölf Millionen neue Krebspatienten hinzukommen. Demnach beträgt das individuelle Lebenszeit-Risiko an Krebs zu erkranken über 11% und an einem Tumor zu sterben annähernd 6%. Männer sind dabei häufiger betroffen als Frauen und die Inzidenzen in der westlichen Welt sind deutlich höher als in Schwellen- und Entwicklungsländern (Garcia et al., 2007). Letzteres liegt vor allem daran, dass die Menschen in den Industrieländern teils sehr viel älter werden und der Großteil aller Tumore erst ab einem Lebensalter von etwa 60-70 Jahren auftritt. Der derzeit stattfindende demografische Wandel wird folglich unweigerlich eine weitere Zunahme an Tumorerkrankungen mit sich bringen. Zusätzliche erhebliche Einflüsse auf das Auftreten von Krebserkrankungen haben außerdem genetische Prädispositionen, Umweltbelastung (z. B. Strahlung) sowie Lebensgewohnheiten (z. B. Ernährung und Tabakkonsum). Im Jahr 2004 entfielen in etwa 436.000 aller Neuerkrankungen auf Deutschland, wo im selben Jahr über 200.000 Menschen ihrer Krankheit erlagen (Batzler et al., 2008). Aufgrund dieser sehr großen Todeszahlen ergibt sich, dass seitens der Medizin, der Forschung und der Industrie enorme Anstrengungen unternommen werden, um die Aussichten für betroffene Patienten künftig verbessern zu können. Hierzu zählen neben der Grundlagenforschung auch neue, patienten- und tumorindividuelle Diagnose- und Therapieverfahren (Theranostik), aber insbesondere auch Präventionsprogramme und regelmäßige Vorsorge-Untersuchungen zur Früherkennung maligner Veränderungen (Neoplasien) bestimmter Zell- und Gewebetypen. Für Tumorarten, bei denen dies bereits häufig durchgeführt wird (z. B. Gebärmutterhals, Brust, Prostata), sind zum Teil schon erste Erfolge erzielt worden. So schätzt man, dass sich die Zahl der neu diagnostizierten Krebsfälle durch Prävention und Früherkennung um bis zu mehr als 50% senken lassen wird (Garcia et al., 2007). Dies gilt insbesondere auch für das kolorektale Karzinom (KRK).

1.2 Das kolorektale Karzinom (KRK)

1.2.1 Epidemiologie

Im Darm sind besonders das Kolon und das Rektum häufig von gutartigen (benignen) und malignen epithelialen Tumoren, den Adenomen und den Karzinomen betroffen. Durch diagnostische Verfahren wie der Darmspiegelung (Koloskopie) könnten auch kleinere Veränderungen der Darmschleimhaut (Mucosa) frühzeitig erkannt und die Inzidenz des KRK mittels rechtzeitiger Entfernung derartiger Tumorfrühstadien stark verringert werden. Dementsprechend gehört in Deutschland seit dem Oktober 2002 die Koloskopie ab dem 55. Lebensjahr zur angebotenen Darmkrebsvorsorge, deren Kosten auch von den gesetzlichen Krankenkassen übernommen werden. Dennoch war das KRK im Jahr 2004 die zweithäufigste Krebsneuerkrankung in Deutschland (16,9%) und ebenfalls die zweithäufigste krebsbedingte Todesursache (Abbildung 1) mit fast 28.000 verstorbenen Patienten (13,4%) (Batzler et al., 2008). Ähnlich ist die prozentuale Verteilung weltweiter Fälle des KRK, wo man für 2007 mit etwa 1,2 Millionen Neuerkrankungen und knapp der Hälfte an Verstorbenen rechnete (Garcia et al., 2007). In vielen Fällen erfolgte hier die Diagnose eines KRK zu spät, so dass die gängigen Therapieformen nicht mehr vom gewünschten Erfolg gekrönt waren.

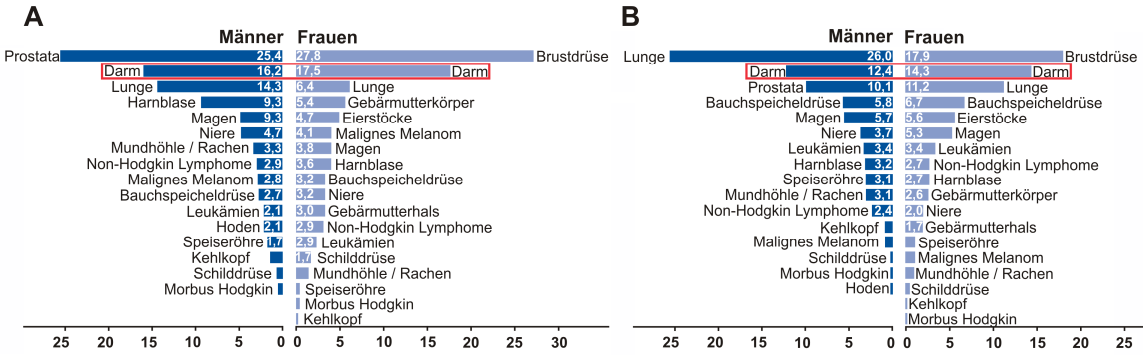

Abbildung 1: Prozentualer Anteil ausgewählter Tumorlokalisationen an Krebs-Neuerkrankungen und an allen Krebssterbefällen in Deutschland 2004

Darmkrebs gehört zu den häufigsten (A) Tumorerkrankungen mit schlechtem Verlauf (B) (rote Kästen). Angegeben sind die Inzidenzen in %; (*Batzler et al, 2008)*.

1.2.2 Diagnose, Therapie und Prognose

Die Diagnose kolorektaler Läsionen wird nach operativer Entnahme maßgeblich von Pathologen erstellt, die das Tumormaterial makroskopisch, mikroskopisch sowie molekularbiologisch analysieren und gemäß gängiger Kriterien, z. B. der Weltgesundheitsorganisation (WHO) oder der Union Internationale Contre le Cancer (UICC), klassifizieren. Die UICC empfiehlt hierbei eine Klassifikation nach dem TNM-System. Diesem liegen die Infiltrationstiefe des Primärtumors bezüglich der Basalmembran, der Mucosa (Schleimhaut) und der Sub-Mucosa (T0, T1, T2, T3, T4) sowie der metastatische Befall regionaler Lymphknoten (N0, N1) und das (überwiegend in der Leber vorkommende) Auftreten von Fernmetastasen (M0, M1) zugrunde. Von einem invasiven Karzinom und demnach einer malignen Erkrankung spricht man generell ab dem T-Stadium 1. In diesem haben die Tumorzellen nämlich die Basalmembran als Verankerung epithelialer Zellen durchbrochen und sind in benachbarte Gewebe eingedrungen. Passenderweise folgen die jeweiligen Therapieformen und die Überlebensprognosen für die Patienten u. a. den anatomischen Eindringtiefen ihrer jeweiligen Tumore. Therapieansätze können zusätzlich zu chirurgischen Maßnahmen auch die Gabe von Chemotherapeutika (5-Fluoro-Uracil (5-FU), Irinotecan, Oxaliplatin) beinhalten sowie den Einsatz bestimmter Antikörper (z. B. Erbitux®, Vectibix®), die spezifisch gegen Zell-Oberflächen-Moleküle wie den Epidermal Growth Factor (EGFR) gerichtet sind. Als Folge der Antikörperbindung kann u. a. deren Aktivität und somit verstärkende Wirkung auf die Tumorzellen gezielt blockiert werden (*targeted therapy*). Der Erfolg der gewählten Therapieform lässt sich v. a. anhand der 5-Jahres-Überlebensrate bestimmen, die für das KRK in Deutschland kumuliert bei etwa 60% liegt (Batzler et al., 2008). Von entscheidender Wichtigkeit für eine derart positive Prognose ist hierbei die Diagnosestellung eines KRK vor dem Auftreten von Fernmetastasen (UICC Stadium IV). Diese führen zu einer Senkung der 5-Jahres-Überlebenswahrscheinlichkeit auf 5% und sind in der Regel auch direkt für den Tod der betroffenen Patienten verantwortlich (de la Chapelle, 2004). Doch auch Tumore, die entsprechend früh entdeckt und therapiert wurden, können nach einiger Zeit als sog. Rezidiv erneut wiederauftauchen und haben dann bei Diagnosestellung oft bereits Fernmetastasen entwickelt, so dass die 5-Jahres-Überlebenswahrscheinlichkeit der Patienten deutlich geringer ausfallen wird. Hierfür

verantwortlich könnten die sogenannten Mikrometastasen sein, bei denen von erhöhten Resistenzen gegen Therapeutika ausgegangen wird und die bei chirurgischen Eingriffen und pathologischer Klassifikation übersehen worden waren. Für die Diagnose- und Prognose-Erstellung wären molekulare Hinweise, die bereits anhand des Primärtumors einerseits Auskunft über den späteren Krankheitsverlauf und andererseits über eventuelles Therapieansprechen (individuelle Prädiktion) geben, somit von großer Wichtigkeit. Ein wesentliches Ziel der Theranostik ist es daher, derartige Hinweise als definierte Biomarker zu etablieren und diese in den klinischen Alltag einfließen zu lassen.

1.2.3 Die Adenom-Karzinom-Sequenz und das KRK als Modelltumor

Das KRK ist für das Auffinden derartiger Marker ein sehr geeigneter Tumor-Typ. Hier ist nämlich die schrittweise Entwicklung einer malignen Erkrankung von frühesten hyperplastischen Vorläufer-Läsionen wie den aberranten Kryptenfoci (AKF) über gutartige Adenome (Polypen) und örtlich begrenzte Karzinome (Karzinom in situ) bis hin zu einem vollinvasiven und metastasierenden Karzinom histopathologisch außerordentlich gut definier- und nachvollziehbar. Dieser Tumorigeneseprozess wird daher auch als Adenom-Karzinom Sequenz bezeichnet (Muto et al., 1975), die sich vom Adenom bis hin zur Metastase wahrscheinlich über einen Zeitraum von etwa 20 Jahren erstreckt (Jones et al., 2008). Wie jeder andere Krebs auch entwickelt sich das KRK aus einem Zusammenspiel von genetischen und epigenetischen Veränderungen der Tumorzellen sowie der Interaktion mit den sie umgebenen Zellen, Organstrukturen und Komponenten der extrazellulären Matrix. Interessanterweise weisen zumindest Änderungen in der genetischen Komponente der Tumorentstehung einige Parallelen zur Adenom-Karzinom Sequenz des KRK auf. So ordneten Vogelstein und Mitarbeiter den einzelnen histologisch abgrenzbaren Stufen kolorektaler Tumore eine Reihe von Mutationen in Tumorsuppressorgenen und Proto-Onkogenen zu, die sich in der Großzahl dieser Läsionen finden und entwickelten daraus das *multistep carcinogenesis model* (Abbildung 2A) (Fearon and Vogelstein, 1990).

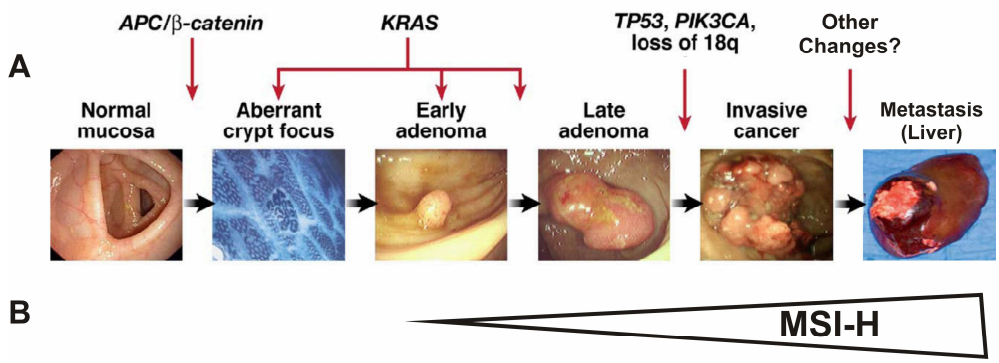

Abbildung 2: Das Multistep Carcinogenesis Modell der kolorektalen Karzinogenese

(A) Darmkrebsentstehung aufgrund schrittweise auftretender genetischer Alterationen oder (B) als Folge hochgradiger Mikrosatelliteninstabilität. Für Details siehe Text; *modifiziert nach (Pino and Chung, 2010).*

Ausgehend davon, dass zur Aktivierung von Onkogenen (*gain of function*) ein, und zur Inaktivierung von Tumorsuppressorgenen (*loss of function*) zwei unabhängige genetische Mutationen (*hits*) notwendig sind, bedarf es diesem Modell nach mindestens sieben genetischer Alterationen in der Entwicklung eines KRK: in der Mehrzahl aller KRK (ca. 85%) wird der neoplastische Prozess durch Verlust des auf Chromosom 5q gelegenen Tumorsuppressorgens *APC* (*ADENOMATOUS POLYPOSIS COLI*) initiiert. Dieses wird aufgrund dieser Schlüsselstellung auch als *gatekeeper* der kolorektalen Karzinogenese bezeichnet (Kinzler and Vogelstein, 1996). APC hat als eine Komponente des Spindelapparates u. a. eine grundlegende Rolle bei der Chromosomen Segregation inne (Kaplan et al., 2001). Bei funktionellem Ausfall des Proteins zeichnet sich diese Gruppe von Tumoren folglich häufig durch generell hohe chromosomale Mutationsraten aus und wird daher auch als CIN (*chromosomal instability*) KRK klassifiziert. Dem Verlust des *APC* folgen im Weiteren Mutationen im Proto-Onkogen *KRAS* sowie in Tumorsuppressorgenen, welche sich auf den Chromosomen 17p (*TP53*) und 18q (*DCC*, *SMAD4*) befinden. Diese über defektes APC initiierte Tumorentstehung findet sich auch in einem erblichen Syndrom des KRK wieder, wo Familienmitglieder bereits mit einer mutierten Variante (Allel) des *APC* Gens geboren werden: die familiäre adenomatöse Polyposis (FAP). FAP-Patienten entwickeln im Gegensatz zu Patienten mit sehr spät auftretenden sporadischen Neoplasien bereits bis zur Pubertät hunderte von Polypen im Kolon und im Rektum und zeigen mit annähernd 100% Penetranz das Auftreten von Adenokarzinomen in frühen Lebensjahren (de la Chapelle, 2004) (Abbildung 3).

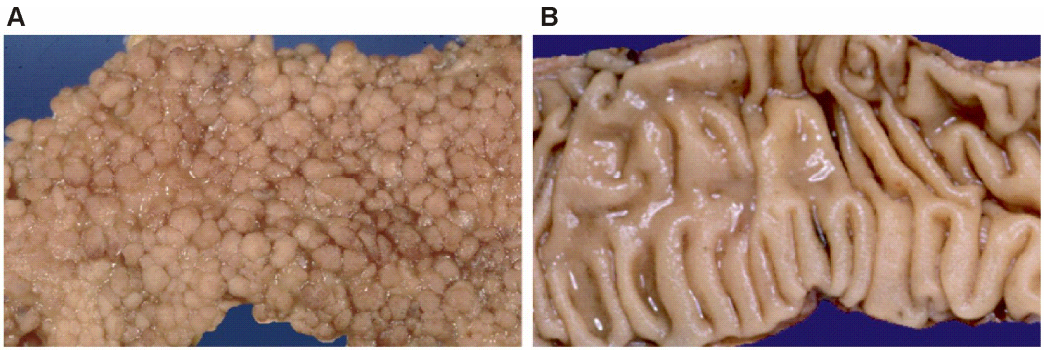

Abbildung 3: Familiäre Adenomatöse Polyposis (FAP)

(A) Hunderte von Polypen (Adenome) im Dickdarm eines FAP-Patienten; (B) Normaler Dickdarm; *(Weinberg, 2007)*.

Das über die Keimbahn vererbte mutierte *APC* Gen stellt somit den ersten *hit* nach Knudsons *Two-Hit*-Hypothese dar (Knudson, 1993), während eine zweite, somatische Mutation (Punktmutation oder *loss of heterozigosity,* LOH) zum Ausfall des verbleibenden gesunden *APC* Gens bis zum jungen Erwachsenenalter führt (Albuquerque et al., 2002). Allerdings konnte in Analysen der Mutationsfrequenzen der Gene *APC*, *KRAS* und *TP53* in humanen Fällen von KRK eine Koinzidenz aller drei Mutationen von lediglich 6,6% nachgewiesen werden (Smith et al., 2002). Dies lässt somit Zweifel an der allgemeinen Gültigkeit des Vogelstein-Modells aufkommen und eröffnet Spielraum für andere Modelle der kolorektalen Tumorprogression. Diese Zweifel gelten insbesondere für eine Gruppe von Dickdarmtumoren, die sich primär durch eine epigenetisch bedingte Karzinogenese auszeichnet statt durch Mutationen: den sporadischen hochgradig mikrosatelliteninstabilen (MSI-H) Tumoren. Dieser Form des KRK entsprechen stadienabhängig etwa 6-10% aller Fälle (Ogino and Goel, 2008). Im Gegensatz zu Tumoren des CIN Typs sind sporadische MSI-H KRK nicht durch früh auftretende *APC* Mutationen, sondern meist (ca. 75%) durch *gain of function* im *BRAF* Gen charakterisiert (Ogino and Goel, 2008), was häufig mit dem *CpG island methylator phenotype* (CIMP) assoziiert ist (Weisenberger et al., 2006). Aufgrund dieses Phänotyps kommt es im Folgenden zum Verlust der Expression von *MLH1* (*MUT-L homologue 1*) durch Methylierung im Promotor/Exon1 Bereich dieses Gens. Da es sich bei MLH1 um eine essentielle Komponente des Mismatch Reparatur Systems (MMR) handelt, kommt es als Konsequenz der Methylierung zu Instabilitäten in Mikrosatelliten, die – wenn sie sich in genkodierenden DNA-

Abschnitten von Tumorsuppressorgenen befinden – weiter zur malignen Transformation beitragen können (Abbildung 2B). Auch vom MSI-H Typ des KRK gibt es eine familiäre Modellerkrankung: das hereditäre nicht polypöse kolorektale Karzinom (HNPCC), wo die Mikrosatelliteninstabilität als Folge von Mutationen in verschiedenen Komponenten des MMR auftritt (de la Chapelle, 2004). Trotz der Unterschiede in den Karzinogenesewegen von Tumoren des CIN oder des MSI-H Typs lassen sich letztendlich auch molekular annähernd alle Fälle von Dickdarmkrebs durch ein gemeinsames Attribut zusammenfassen: die Stabilisierung des Onkoproteins β-CATENIN.

1.3 β-CATENIN und der WNT Signalweg

1.3.1 Die duale Funktion von β-CATENIN

β-CATENIN übernimmt in kolorektalen Epithelzellen zwei sehr unterschiedliche Aufgaben, die auch funktionell direkt mit verschiedenen zellulären Phänotypen korreliert sind (Harris and Peifer, 2005) (Abbildung 4).

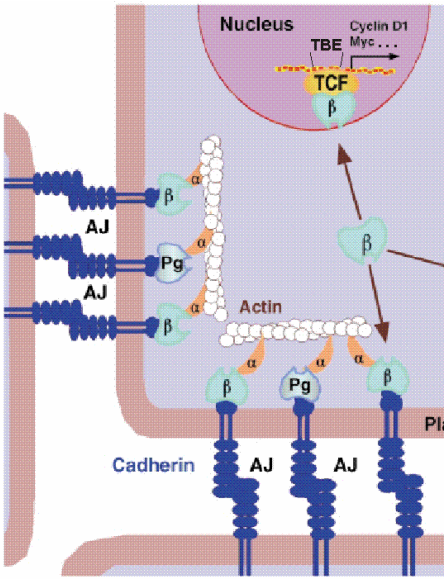

Abbildung 4: Die duale Funktion von β-CATENIN in Zelladhäsion und transkriptioneller Aktivierung

β-CATENIN (β) ist zum einen am Aufbau der Zonula Adhaerens/Adherens Junctions (AJ) beteiligt, zum anderen kann es auch im Nukleus lokalisiert sein, wo es zusammen mit TCF einen aktiven Transkriptionsfaktor darstellt. Für Details siehe Text; *modifiziert nach* (*Conacci-Sorrell et al., 2002*).

Zum einen ist β-CATENIN maßgeblich an der Konstruktion der Zonula adhaerens (*adherence junctions*) beteiligt und ist somit eine essentielle Komponente für den Aufbau und die Aufrechterhaltung des epithelialen Phänotyps (Brabletz et al., 2001; Hirohashi, 1998). Die Zell-Zell Kontakte der Zonula adhaerens werden durch homophile Interaktionen der extrazellulären Domänen des Kalzium-abhängigen Transmembranproteins E-CADHERIN vermittelt und führen unter anderem zum Erhalt einer geordneten Gewebe-Architektur wie auch der Zellpolarität. Auf zytoplasmatischer Seite interagiert E-CADHERIN mit β-CATENIN und PLACOGLOBIN (γ-CATENIN), welche dann ihrerseits via α-CATENIN den Kontakt zum β-AKTIN-Zytoskelett herstellen (Brembeck et al., 2006; Conacci-Sorrell et al., 2002).

Im Gegensatz zu dieser membranständigen Lokalisation kann β-CATENIN auch im Zellkern (Nukleus) vorliegen, wo es seine zweite wichtige physiologische Aufgabe wahrnimmt: hier interagiert β-CATENIN mit HMG (*high mobility group*) DNA Bindungsfaktoren der TCF/LEF Familie (T-CELL FACTOR/LYMPHOID ENHANCER FACTOR) (Behrens et al., 1996; Molenaar et al., 1996) und stimuliert die transkriptionelle Aktivierung von Zielgenen, welche DNA-Bindemotive für diesen Proteinkomplex in ihren Promotor/Enhancer Regionen aufweisen. Diese TCF Bindeelemente (TBEs) sind durch das zentral gelegene Sequenzelement A/TTCAAAG (Hatzis et al., 2008) definiert. Die Expressionsstärke der Zielgene, welche unter anderem Proliferation und Dedifferenzierung vermitteln, wird ferner durch die Aktivität von *chromatin remodelling* Komplexen, insbesondere Histon Deacetylasen (HDACs) und Histon Acetyltransferasen (HATs) feinreguliert. Diese interagieren entweder direkt mit β-CATENIN beziehungsweise TCF/LEF oder werden via weiterer Brückenfaktoren an die TBEs rekrutiert und zeigen hier inhibierende (GRO/TLE (GROUCHO/TRANSDUCTION-LIKE ENHANCER OF SPLIT), CTBP (C-TERMINAL BINDING PROTEIN)) oder stimulierende (SWI/SNF, p300/CBP (C-AMP RESPONSE ELEMENT BINDING PROTEIN)) Wirkung auf die Genaktivierung (Barker et al., 2001; Cavallo et al., 1998; Hecht et al., 2000; Le et al., 2008; Roose et al., 1998; Valenta et al., 2003).

1.3.2 Der kanonische WNT Signalweg

Physiologisch wird die subzelluläre Lokalisation von β-CATENIN über die Aktivität des evolutionär hochkonservierten kanonischen WNT Signalweg reguliert, dessen zentrales Effektormolekül es ist (Bienz and Clevers, 2000) (Abbildung 5).

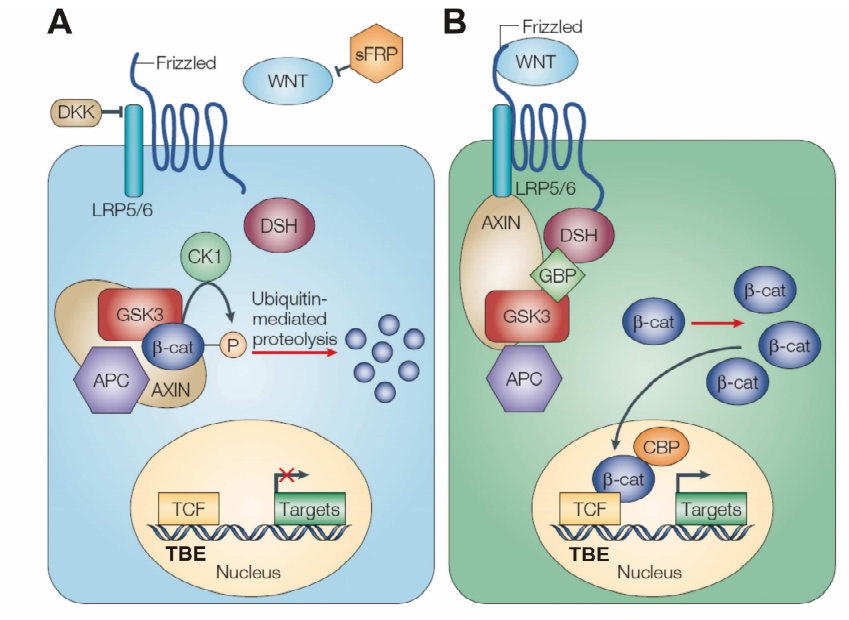

Abbildung 5: Der kanonische WNT Signalweg

(A) In Abwesenheit von WNT Liganden wird β-CATENIN durch den Degradationskomplex gebunden und für den Abbau im Proteasom markiert. (B) Nach Bindung von WNT an der Zelloberfläche dissoziiert der Komplex, β-CATENIN wird stabilisiert und im Cytoplasma/Nukleus angereichert; Zielgene können aktiviert werden. Details siehe Text; *modifiziert nach (Moon et al., 2004)*.

Bei Sättigung der membran-assoziierten β-CATENIN-Bindungsstellen wird im inaktiven Zustand des Signalwegs überschüssiges β-CATENIN durch den sogenannten Degradationskomplex gebunden und für den Abbau vorbereitet. Dieser zytoplasmatisch vorliegende Multiprotein-Komplex besteht zumindest aus den Molekülen GLYCOGEN SYNTHASE KINASE 3β (GSK3β), AXIN/CONDUCTIN und APC, wobei AXIN das Gerüstprotein dieses Komplexes darstellt. Nachfolgend wird β-CATENIN durch die CASEIN KINASE Iα (CKIα) an Serin 45 phosphoryliert, was wiederum das Signal für weitere N-terminale Serin-/Threonin-Phosphorylierungen durch die GSK3β ist (Gregorieff and Clevers, 2005). So markiert wird β-CATENIN durch einen E3-Ubiquitin Ligase SCF-Komplex (SKP/CULLIN/F-BOX)

polyubiquitiniert und dem zytoplasmatischen 26 S Proteasom Komplex zur Degradation zugeführt (Aberle et al., 1997; Bienz and Clevers, 2000). Physiologisch inhibiert wird dieser Prozess durch die Bindung von WNT Liganden an die sieben-Transmembran-Rezeptoren der FRZ (FRIZZLED) Familie und ihre Korezeptoren LRP 5/6 (LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN). Der genaue Ablauf der von den Rezeptoren ausgehenden Signaltransduktion, welche zur Hemmung des Degradationskomplexes führt, ist zwar noch nicht genau verstanden, aber man vermutet, dass die membranöse Translokation des Gerüstproteins AXIN hierfür eine maßgebliche Ursache darstellt. Vermittelt wird diese Translokation wahrscheinlich über das durch FRZ aktivierte Protein DSH (DISHEVELLED) und LRP 5/6 (Clevers, 2006). Als Folge der WNT Aktivierung kann β-CATENIN nicht mehr abgebaut werden, akkumuliert in der Zelle und gelangt auf noch nicht genau verstandenem Weg in den Nukleus, wo es seine transaktivierende Wirkung entfaltet. Doch auch in Anwesenheit von WNTs kann die Aktivität des Signalwegs gehemmt werden. Dies geschieht, indem die Liganden bereits extrazellulär durch ebenfalls sekretierte Proteine der SFRP-Familie (SECRETED FRIZZLED RELATED PROTEINS) gebunden werden, oder ihre Interaktion mit LRP 5/6 durch die Aktivität der Familie der DKK (DICKKOPF) Proteine gehemmt wird (Clevers, 2006).

1.3.3 Die Rolle des kanonischen WNT Signalwegs für die intestinale Kryptenarchitektur

Das adulte Intestinum setzt sich zusammen aus Dünndarm, Dickdarm (Kolon) und Enddarm (Rektum). Anatomisch sind diese Abschnitte recht ähnlich aufgebaut und charakterisiert durch eine enorme Anzahl von Einstülpungen. Diese sogenannten Darmkrypten dienen der Oberflächenvergrößerung und somit der effizienteren Aufnahme von Nährstoffen und/oder Wasser aus der Nahrung. Im Dünndarm erfüllen den gleichen Zweck zusätzlich noch die sogenannten Villi, die von den Krypten ausgehend ins Darmlumen hineinragen (Abbildung 6).

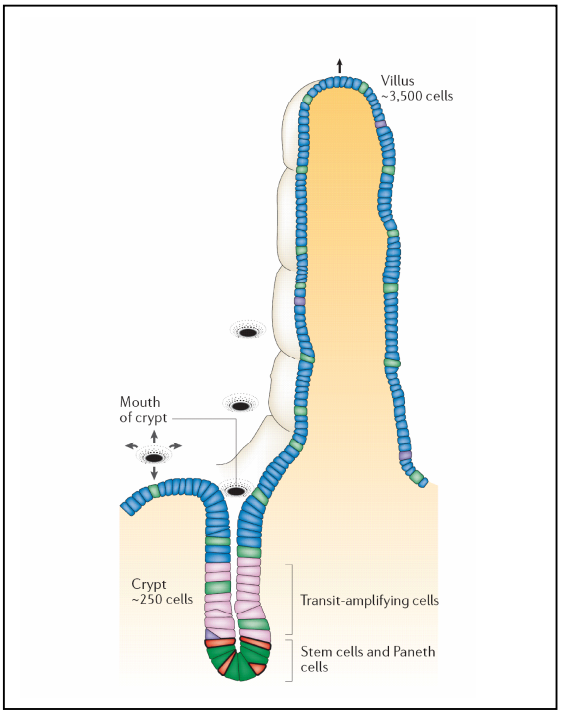

Abbildung 6: Die Krypten-/Villus-Architektur des Darmes

Der Darm besteht aus vielen Krypten, die der Oberflächenvergrößerung dienen. Im Dünndarm dienen demselben Zweck zusätzlich noch die Villi. Reguliert wird die Bildung dieser Architektur von basal in der Krypte lokalisierten Stammzellen. Für Details siehe Text; *(Crosnier et al., 2006).*

Das Darmepithel, welches lediglich aus einer Zellschicht besteht, zeichnet sich durch eine hohe Rate konstanter Selbsterneuerung aus. So dauert der Lebenszyklus einer neugebildeten Zelle in der Kryptenbasis von einer Phase hoher Proliferation (*transiently amplifying unit cells*) über den Differenzierungszustand mit Sekretions- und/oder Absorptionsaufgaben bis hin zum programmierten Zelltod, der Apoptose, und dem Abschilfern der Zelle am Krypten- oder Villusapex (Anoikis) lediglich 3-6 Tage (Gregorieff and Clevers, 2005). Somit werden allein ca. 10^{11} Zellen des menschlichen Dünndarms täglich über den Stuhl mit ausgeschieden (Potten and Loeffler, 1990). Ableiten lassen sich alle diese Zellen von gemeinsamen, sich selbst erneuernden, multipotenten adulten Stammzellen, die sich basal in den Krypten befinden (Potten et al., 2009; van der Flier and Clevers, 2009). Die treibende Kraft hinter dieser enormen Erneuerungsrate ist der WNT Signalweg (Abbildung 7A).

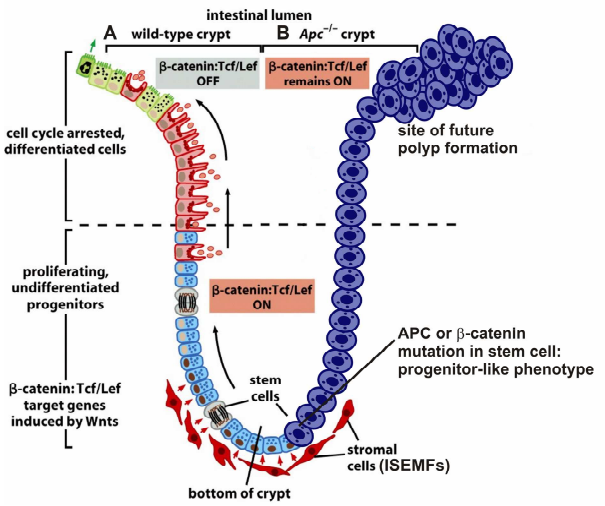

Abbildung 7: β-CATENIN in der Biologie der Dickdarmkrypte

Schematische Darstellung einer Dickdarmkrypte. Im Normalzustand (A) ist der WNT Signalweg lediglich im unteren Drittel der Krypte aktiv (ON). Hier sind die Zellen gekennzeichnet durch Dedifferenzierung und Proliferation. An der Basis der Krypten sind die Stammzellen lokalisiert. Nach apikal nimmt die Aktivität des Signalwegs ab (OFF), die Zellen stoppen den Zellzyklus und differenzieren um ihrer physiologischen Aktivität nachzukommen, bis sie an der Spitze der Krypte ins Darmlumen abschilfern (Anoikis). Bei Defekten im WNT Signalweg (B) werden die Zellen unabhängig von dieser Regulation, proliferieren weiter und behalten dabei ihren Vorläufer-Phänotypen. Es kommt zur Adenombildung; *modifiziert nach (Weinberg, 2007).*

So liegt nukleäres β-CATENIN in wenigen Zellen an der Kryptenbasis vor, wodurch die dort liegenden Zellen durch hohe Zellteilung und Dedifferenzierung charakterisiert sind (van de Wetering et al., 2002). Außerdem findet sich nukleäres β-CATENIN im Mausmodell auch in den intestinalen Stammzellen, die gleichzeitig exklusiv durch das β-CATENIN Zielgen *Lgr5* (*Leucine-rich-repeat-containing G-protein coupled receptor 5*) charakterisiert und somit nachweisbar sind (Barker et al., 2007). So könnte der WNT Signalweg hier auch eine essentielle Funktion für den Aufbau der intestinalen Stammzellnische inne haben. In apikaler gelegenen Zellen jenseits der *transiently amplifying unit* befindet sich β-CATENIN hingegen an der Zellmembran. Dementsprechend sind die Zellen durch hohe epitheliale Differenzierung und fehlende Proliferation gekennzeichnet (van de Wetering et al., 2002). Die Rolle des WNT Signalwegs für die Aufrechterhaltung der intestinalen Homöostase wurde auch in *in vitro* Kulturen intestinalen Gewebes bestätigt (Ootani et al., 2009) und eindrucksvoll in Experimenten mit transgenen Mäusen bewiesen, die entweder darmspezifisch den WNT Inhibitor Dkk1 exprimierten oder einen genomischen

Knockout von *β-catenin* oder *Tcf4* (*Tcf4*$^{-/-}$) aufwiesen (Fevr et al., 2007; Korinek et al., 1998; Pinto et al., 2003). In allen Modellen kam es zum Verlust der proliferativen Zellfraktion sowie des Stammzell-Kompartiments, was letztlich den Kollaps der Kryptenarchitektur zur Folge hatte. Über die physiologische WNT Quelle in der Kryptenbasis herrscht noch Unklarheit. Es wird aber vermutet, dass intestinale subepitheliale Myofibroblasten (ISEMFs) hierbei eine entscheidende Rolle spielen (Gregorieff and Clevers, 2005). Da diese in apikaleren Bereichen nicht vorhanden sind, könnte sich die hier fehlende nukleäre Lokalisation von β-CATENIN somit durch einen abnehmenden WNT Gradienten ausgehend von den ISEMFs erklären lassen. Ergänzend zu diesem rein passiven Effekt gibt es auch Untersuchungen die nahelegen, dass dem WNT Signalweg in luminalen Kryptenregionen aktiv entgegengewirkt wird, z. B. durch den HEDGEHOG (HH) Signalweg (Akiyoshi et al., 2006; van den Brink et al., 2004).

1.3.4 Die Bedeutung von β-CATENIN im kolorektalen Karzinom

In kolorektalen Tumorzellen erfolgt die Stabilisierung von β-CATENIN unabhängig von Ligandenbindung durch Mutationen in Komponenten des WNT Signalwegs (Abbildung 7B). In CIN Tumoren erfolgt dies überwiegend durch *loss of function* von APC, welches hier auch oft die erste Mutation in der malignen Entartung darstellt. Wie erwähnt, zeichnen sich MSI-H KRK initial zwar durch andere Mechanismen der Karzinogenese aus, benötigen zur vollen Transformation häufig jedoch ebenfalls die Aktivität stabilisierten β-CATENINs. In dieser Gruppe von Tumoren wird dieser Zustand nicht ausschließlich über APC Mutationen erreicht (Huang et al., 1996; Shitoh et al., 2001), sondern auch Mutationen im *AXIN2* Gen (Liu et al., 2000) oder in *β-CATENIN* (Polakis, 2000) selbst wurden hier beschrieben. Darüber hinaus sind in diesem speziellen Tumortyp auch noch weitere Mechanismen der β-CATENIN Stabilisierung denkbar. Interessanterweise wird der bivalente Charakter von β-CATENIN bezüglich seiner subzellulären Lokalisation trotz Akkumulation in allen Tumorzellen auch in vielen gut bis mäßig differenzierten KRK wiedergespiegelt (Abbildung 8). Diese Plastizität wird auch als das β-CATENIN Paradoxon bezeichnet (Fodde and Brabletz, 2007).

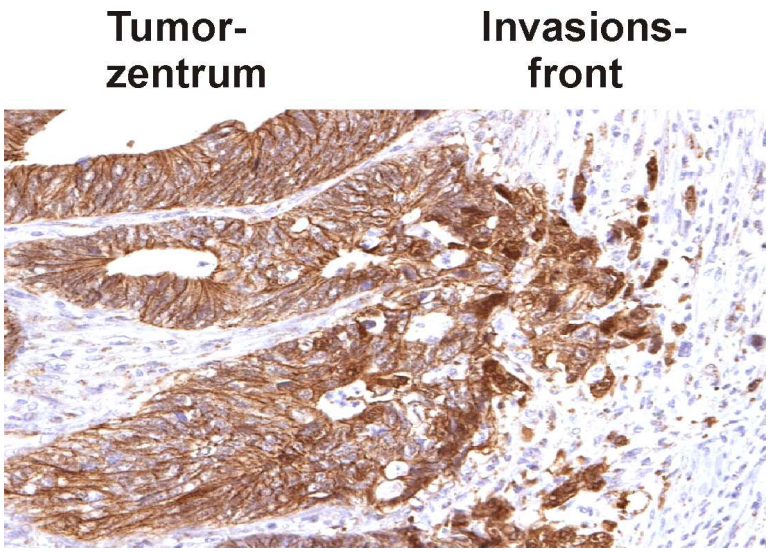

Abbildung 8: EMT und nukleäre Akkumulation von β-CATENIN an der Invasionsfront kolorektaler Tumore

β-CATENIN Immunhistochemie (braune Färbung) eines KRK. Im Tumorzentrum sind die Zellen epithelial differenziert und zeigen membranöses β-CATENIN, während die Zellen der Invasionsfront nukleäres β-CATENIN aufweisen und durch eine epitheliale-mesenchymale Transition (EMT) charakterisiert sind; *aus (Brabletz et al., 2001).*

So findet sich nukleäres β-CATENIN in diesen Tumoren vornehmlich in wenigen, teils einzeln, teils in kleinen Clustern vorliegenden Tumorzellen (sog. *buds*) der Invasionsfront wieder, welche den Übergangsbereich zwischen neoplastischen und normalen Gewebeanteilen darstellt (Brabletz et al., 1998; Brabletz et al., 2001). Zellen zentraler Tumoranteile enthalten hingegen wenig bis kein nukleäres β-CATENIN, wobei zwischen Invasionsfront und zentralen Arealen eine graduelle Abnahme nukleären β-CATENINs zu beobachten ist. Dieses Verteilungsmuster von β-CATENIN findet sich ebenfalls in den Metastasen kolorektaler Karzinome wieder. Diese Beobachtung spricht daher für eine hohe Plastizität und regulatorische Komponenten, die zusätzlich zur Stabilisierung von β-CATENIN als Folge von Mutationen dessen nukleäre Translokation manipulieren können. Der regulatorische Anteil in der Progression eines KRK kann demnach als Erweiterung des linearen Modells der Karzinogenese nach Vogelstein aufgefasst werden. Die entsprechenden Signale hierfür könnten von der sich während des Tumorwachstums verändernden zellulären Umgebung (*environment*) stammen und zum Beispiel die über Oberflächenrezeptoren vermittelte Aktivierung weiterer Signalwege (*pathways*)

beinhalten (Barker and Clevers, 2001; Vermeulen et al., 2010). Denkbar ist hier in etwa die Beteiligung von verschiedensten *pathways*, welche die Aktivität der AKT Kinase erhöhen. Diese ist nämlich wiederum bekannt dafür die nukleäre Translokation und die transkriptionelle Aktivierung von β-CATENIN durch bestimmte Phosphorylierungen entscheidend beeinflussen zu können (Fang et al., 2007). Aber auch zusätzliche Stimulation durch den WNT Signalweg selbst könnte hier von Bedeutung sein. In Zellkultur konnte nämlich gezeigt werden, dass auch kolorektale Tumorzellen, die bereits Mutationen in β-CATENIN besitzen, zur vollen Aktivierung weiterhin auf diese Liganden angewiesen sind (Bafico et al., 2004). Im Gegensatz zu Zellen aus zentralen Arealen ist auffallend, dass die Zellen der Invasionsfront durch fehlendes membranständiges β-CATENIN ihren epithelialen Phänotyp aufgegeben haben und in der Lage sind zu dissoziieren (*budding*) sowie mesenchymal zu dedifferenzieren. So zeigen besonders vereinzelte Zellen den Verlust von E-CADHERIN bei gleichzeitiger Expression der mesenchymalen Markermoleküle FIBRONEKTIN und VIMENTIN (Brabletz et al., 2001). Von daher zeichnet sich die Invasionsfront als Zone einer epithelialen-mesenchymalen Transition (EMT) aus (Abbildung 8), einen Prozess den man auch *in vitro* mit kultivierten kolorektalen Tumorzellen simulieren kann (Brabletz et al., 2001; Conacci-Sorrell et al., 2003). Derartige Tumorzellen mit mesenchymalem Charaker sind durch hohe Migrations- und Invasionsfähigkeit gekennzeichnet (Spaderna et al., 2008). Da diese Prozesse von grundlegender Relevanz für die Progression zu einem metastatischen Zustand und somit in der Regel für den Tod der Patienten sind, weist dies auch auf eine elementare Bedeutung der EMT im Verlauf der Tumorigenese hin (Thiery, 2002). Unterstützt wird dies durch Daten, die belegen, dass das Ausmaß des *buddings* mit stark vermindertem Patientenüberleben korreliert ist (Hase et al., 1993; Ueno et al., 2002). Aufgrund der Tatsache, dass die jeweiligen Metastasen ebenfalls durch epitheliale und mesenchymale Regionen gekennzeichnet sind, wird allerdings vermutet, dass es sich an der Invasionsfront lediglich um eine transiente EMT handelt, die durch den Umkehrprozess der mesenchymal-epithelialen Transition (MET) in der Metastase wieder revertiert wird. Bedingt durch die hohe Korrelation von nukleärem β-CATENIN und EMT an der Invasionsfront kolorektaler Karzinome ergibt sich somit die Frage nach dem Zielgen-Programm von β-CATENIN und ob dieses auch kausal für die EMT und die Progression der Tumore eine Rolle spielt.

1.3.5 Zielgene von β-CATENIN

Tumore sind durch einige ganz besondere funktionelle Eigenschaften gekennzeichnet, die sie von gesunden Geweben unterscheiden und die als Mindestanforderungen von Krebszellen erfüllt sein müssen, um eine voll maligne Krankheit ausbilden zu können: den *hallmarks of cancer* (Hanahan and Weinberg, 2000). Eine ganze Reihe von mittlerweile identifizierten β-CATENIN Zielgenen sind interessanterweise an der Regulierung einzelner dieser Hallmarks funktionell beteiligt (Brabletz et al., 2009).

Proliferation (*hallmark: self-sufficiency in growth signals*)

Ein wesentliches Charakteristikum von Tumorzellen ist ihre extrem hohe Teilungsrate, die letztlich notwendig ist um überhaupt große Tumore entstehen zu lassen. *CYCLIN D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999) und das Proto-Onkogen *cMYC* (He et al., 1998) sind zwei proliferationsinduzierende β-CATENIN Zielgene, was die zentrale Rolle von β-CATENIN in der kolorektalen Karzinogenese unterstreicht. Besonders interessant ist hierbei, dass cMYC über die Hemmung der p53-abhängigen Zelltod-Maschinerie gleichzeitig auch noch die Erfüllung des Hallmarks der **Apoptose-Resistenz** erklären kann. Experimentell bestätigt sind in diesem Zusammenhang bereits die β-CATENIN Zielgene *SURVIVIN* (Zhang et al., 2001a) und das anti-apoptotisch wirkende BCL-W (Lapham et al., 2009). Die besondere Rolle von cMYC in der kolorektalen Karzinogenese wird eindrucksvoll in einer Arbeit hervorgehoben, die in transgenen Mäusen alle phänotypischen Effekte einer inaktivierenden *Apc* Mutation nur durch gleichzeitigen knockout von c*Myc* komplett revertieren konnte (Sansom et al., 2007). Paradoxerweise ist jedoch die Invasionsfront humaner kolorektaler Tumore trotz Zellen mit nukleärem β-CATENIN sowie cMYC- (Brabletz et al., 2000) und CYCLIN D1-Expression (Jung et al., 2001) eine Zone geringer Proliferation, was sich durch die simultane Expression des Zellzyklusinhibitors $p16^{INK4A}$ (Jung et al., 2001) erklären lassen könnte. Biologisch ließe sich dieser negative Einfluss auf die Proliferation dadurch erklären, dass eine hohe Zellteilungsrate oft im Widerspruch steht zu anderen funktionellen Prozessen wie der Migration oder der Invasion.

Invasion und Metastasierung (*hallmark: tissue invasion and metastasis*)

Für die Fähigkeit zur Invasion müssen Zellen auch Migrationsfähigkeit aufweisen können. Hierzu sind besonders mesenchymale Zellen gut in der Lage. β-CATENIN reguliert passenderweise unter anderem die Expression des EMT-Induktors TENASCIN C (Beiter et al., 2005) aber auch die Expression von E-CADHERIN Repressoren wie SLUG (Conacci-Sorrell et al., 2003) oder die mesenchymalen Markerproteine VIMENTIN und FIBRONEKTIN (Gilles et al., 2003; Gradl et al., 1999). Somit ist β-CATENIN direkt für die Induktion von EMT verantwortlich (Brembeck et al., 2004). Um in benachbarte Gewebe invadieren zu können, müssen mesenchymale Zellen zunächst die sie umgebende extrazelluläre Matrix überwinden. Dies wird ermöglicht durch das β-CATENIN Zielgen *MMP7* (*MATRIX METALLOPROTEINASE7*) (Brabletz et al., 1999), welches eine extrazellulär wirkende Metalloproteinase ist, die extrazelluläre Matrix und extrazelluläre Oberflächenmoleküle proteolytisch abbaut oder modifiziert. Weitere β-CATENIN Zielgene, die direkt die Invasionsfähigkeit kolorektaler Tumorzellen modulieren, sind außerdem *UPA* (*UROKINASE PLASMINOGEN AKTIVATOR*) (Hiendlmeyer et al., 2004) oder *LAMININ-5 γ2* (Hlubek et al., 2001). Schließlich ist β-CATENIN auch an der Metastasierung kolorektaler Tumoren selbst beteiligt, indem es die Expression des prometastatischen Zielgens *S100A4* (Stein et al., 2006) hochreguliert, welches in humanen KRK mit hoher Aggressivität und schlechter Prognose korreliert.

Angiogenese (*hallmark: sustained angiogenesis*)

Da die Diffusionsgrenze für Sauerstoff im menschlichen Gewebe bei etwa 200 µm liegt, ist eine entsprechend dichte Vaskularisierung zur Sicherstellung der Blut- und somit Sauerstoffzufuhr eine essentielle Voraussetzung für das Überleben von Tumoren. Ein für die Neo-Angiogenese und die Stabilisierung reifer Blutgefäße essentieller Faktor ist VEGF (VASCULAR ENDOTHELIAL GROWTH FACTOR), der ebenfalls durch β-CATENIN transkriptionell aktiviert wird (Easwaran et al., 2003; Zhang et al., 2001b).

Das Hallmark des **unbegrenzten replikativen Potentials** (*limitless replicative potential*) ist eine weitere essentielle funktionelle Eigenschaft, die Zellen während ihrer malignen Transformation erlangen müssen. Normalerweise ist nämlich die

Anzahl möglicher Zellteilungen durch die sich während jedes Zellzyklus verkürzenden Telomere auf ungefähr 50 limitiert. Um dieses sogenannte *Hayflick limit* (Hayflick, 1965) überwinden zu können, exprimieren Tumorzellen oft das Enzym TERT (TELOMERASE REVERSE TRANSCRIPTASE). Durch dessen Aktivität werden die Enden der Chromosomen auch nach vielen Zellgenerationen noch stabilisiert und dadurch das Überleben der Zellen erst ermöglicht. Interessanterweise findet sich Expression von TERT in kolorektalen Karzinomen ebenfalls in Bereichen der Invasionsfront (Kolquist et al., 1998), so dass β-CATENIN auch als geeigneter Kandidat für die Vermittlung der daraus folgenden Immortalisierung in Betracht gezogen werden kann.

Zusammenfassend lässt sich somit sagen, dass β-CATENIN zumindest an der Realisierung von bis zu fünf von sechs *hallmarks of cancer* direkt beteiligt ist, was seine herausragende Rolle in der kolorektalen Tumorprogression eindrucksvoll unterstreicht.

Darüber hinaus gibt es auch noch einige Zielgene von β-CATENIN, die nicht direkt einem der Hallmarks zugeordnet werden können, deren Rolle für die kolorektale Karzinogenese jedoch trotzdem interessante Fragestellungen aufwirft. So wird zum Beispiel der negative WNT Regulator DKK4 ebenfalls transkriptionell durch β-CATENIN aktiviert (Pendas-Franco et al., 2008), was zumindest bei intaktem WNT Signalweg eine hemmende Wirkung auf dessen Aktivität bewirkt. Welche funktionelle Auswirkung dieser negative *feedback loop* in der kolorektalen Karzinogenese spielt, ist jedoch noch nicht genau bekannt.

Die lange Liste von β-CATENIN Zielgenen ist in jüngerer Zeit außerdem noch durch einige Kandidaten wie *Lgr5* erweitert worden, die mittlerweile zur Charakterisierung der intestinalen Stammzellen verwendet werden und somit auch einen Bezug zur wichtigen Rolle des WNT Signalwegs in der Embryogenese herstellen. Seit einigen Jahren wird in diesem Zusammenhang intensiv an der Hypothese gearbeitet, dass sich Tumore, ähnlich wie normale Gewebe, ebenfalls von der Existenz solcher multipotenten Zellen ableiten lassen: den sogenannten tumorinitiierenden Zellen oder auch Tumorstammzellen/Krebsstammzellen (KSZ) (Clarke et al., 2006), deren Eigenschaften nunmehr selbst zu einem neuen *hallmark of cancer* geworden sind. Diese Zellen machen zwar nur einen sehr geringen

Bestandteil der Gesamt-Tumormasse aus, sind allerdings die für die Tumorentstehung und -progression relevanten Zellen, während die Nicht-Tumorstammzellen hierzu nicht in der Lage sind. Im KRK werden für Tumorstammzellen charakteristische Markermoleküle wie CD44, CD166 und CD133 vermutlich ebenfalls durch β-CATENIN reguliert (Hatzis et al., 2008; Katoh and Katoh, 2007; Wielenga et al., 1999). Dies legt den Schluss nahe, dass der deregulierte WNT Signalweg im KRK zusätzlich zu den etablierten *hallmarks of cancer* auch maßgeblich an der Vermittlung des Tumorstammzellphänotyps beteiligt ist (Reya and Clevers, 2005). Dies konnte jüngst in einer Publikation experimentell gezeigt werden (Vermeulen et al., 2010).

1.4 Das Krebsstammzell-Konzept

1.4.1 EMT und stemness: das Konzept der migrierenden Krebsstammzellen

Aufgrund der Beobachtung dass „Tumorstammzelligkeit“ (*stemness*) tatsächlich durch nukleäres β-CATENIN vermittelt wird, ergibt sich im Zusammenhang mit der beschriebenen Vermittlung der EMT und ihrer funktionellen Besonderheiten eine höchst interessante Hypothese: hiernach könnte es sich bei den Zellen der Invasionsfront eines KRK um migrierende Krebsstammzellen (mKSZ) handeln. Diese wären demnach nicht nur zur Wanderung in der Lage, sondern könnten durch ihre Multipotenz auch zur Kolonialisierung entfernter Organe und somit zur Bildung von Metastasen verantwortlich sein (Brabletz et al., 2005b) (Abbildung 9). Diese Hypothese wird von Arbeiten unterstützt die zeigen, dass in Mamma- und Pankreas-Krebszellen EMT und ein stammzellartiger Phänotyp molekular direkt miteinander verknüpft sind (Mani et al., 2008; Wellner et al., 2009). Bezogen auf das KRK könnte dies bedeuten, dass Mutationen im WNT Signalweg durch die Induktion von Proliferation zwar zu hyperplastischen Läsionen wie Adenomen führen können, dass aber erst die zusätzliche Induktion von EMT und somit Tumorstammzellen zum voll aggressiven Dickdarmtumor beiträgt.

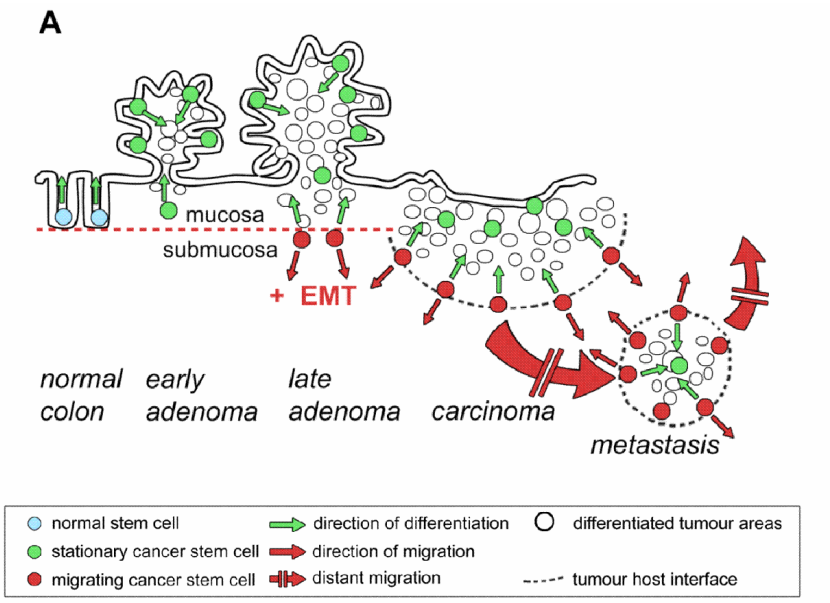

Abbildung 9: Das Konzept der migrierenden Krebsstammzellen

Im KRK zeigen Tumorzellen der Invasionsfront (*tumour host interface*) EMT bei nukleärer Expression von β-CATENIN. Bei diesen Zellen könnte es sich um migrierende Krebsstammzellen (mKSZ) handeln, die für die Progression und die Metastasierung der Tumore verantwortlich sind; *(Brabletz et al., 2009).*

Entsprechend findet man in KRK auch Expression von β-CATENIN Zielgenen, die der Idee eines Zwei-Phasen-Models folgen. Erst mit Eintritt in Phase zwei, die durch Tumorzell-Dissimination und Metastasierung gekennzeichnet ist, wird das Zielgenprogramm ergänzt von Mediatoren der Migration, der Invasion und der EMT (Brabletz et al., 2005b). Das Umschalten nach Phase zwei wird im KRK hierbei durch die Manipulation der β-CATENIN Aktivität erreicht, welche wiederum maßgeblich durch das sich verändernde *environment* beeinflusst wird. Für die mKSZ könnte dies bedeuten, dass das *environment* nicht nur ihre *stemness* aufrechterhält (Stammzellnische) (Bissell and Labarge, 2005), sondern diese durch entsprechende Signale, wie z. B. von HGF (HEPATOCYTE GROWTH FACTOR), dessen Rezeptor c-MET wiederum selber ein β-CATENIN Zielgen ist, überhaupt erst induziert wird (Boon et al., 2002; Vermeulen et al., 2010). Unterstützt wird die Wirkung von β-CATENIN dabei vermutlich von ZEB1 (ZINC FINGER E-BOX-BINDING HOMEOBOX1), einem Transkriptionsfaktor der Zinkfingerfamilie. ZEB1 ist ein Induktor von EMT, der ebenfalls an der Invasionsfront kolorektaler Tumore exprimiert wird (Spaderna et al., 2008) und zumindest in pankreatischen Tumoren an der

Vermittlung von *stemness* beteiligt ist (Wellner et al., 2009). *In vitro* zeichnen sich derartige durch EMT induzierte Stammzellen durch eine stark erhöhte Resistenz gegen Chemotherapeutika aus (Gupta et al., 2009), eine Eigenschaft die in Patienten vor allem den Mikrometastasen zugeschrieben wird. Aufgrund dieser Tatsachen ergibt sich die sehr interessante Hypothese, dass es sich bei den vermeintlichen koKSZ der Invasionsfront von KRK um die Mikrometastasen handelt, die für das Auftreten von Rezidiven und Metastasen nach erfolgreicher Operation des Primärtumors verantwortlich sind.

1.4.2 Kolorektale Krebsstammzellen

Tatsächlich zeigen auch primäre, das heißt direkt aus frisch operativ entfernten Tumoren gewonnene kolorektale Krebsstammzellen (koKSZ), eine erhöhte Resistenz gegen Therapeutika wie Oxaliplatin oder 5-FU (Dylla et al., 2008; Todaro et al., 2007). Es wird vermutet, dass sich solche Tumorstammzellen direkt von ihren normalen organspezifischen Gegenstücken ableiten, da nur diese lange genug überleben um überhaupt die für die maligne Transformation nötigen Mutationen zu akkumulieren. In eleganten Experimenten mit transgenen Mäusen konnte so gezeigt werden, dass sich koKSZ direkt auf normale intestinale Stammzellen zurückführen lassen (Barker et al., 2009; Zhu et al., 2009). Für die Resistenz von Tumorstammzellen gegen cytotoxische Substanzen kommen mehrere Mechanismen als Erklärung in Frage. Zum einen teilen sich Stammzellen definitionsgemäß sehr selten, gerade aber sich schnell teilende Zellen stellen für diese Medikamente besonders gute therapeutische Ziele dar. Zum Zweiten zeichnen sich Stammzellen und Tumorstammzellen durch funktionelle Eigenschaften aus, die es ihnen erlauben die durch die Therapien zerstörte DNA hocheffizient wieder zu reparieren (Bao et al., 2006) oder aber aufgenommene schädliche Stoffe mit hoher Effizienz aktiv wieder auszuschleusen. Interessanterweise ist ein Molekül das eine derartige *drug pump* darstellt, selbst auch ein Zielgen des WNT Signalwegs (*MDR1*, *MULTI DRUG RESISTANCE 1*) (Yamada et al., 2000). Dies sind somit weitere Befunde, die das Konzept der koKSZ in Abhängigkeit von nukleärem β-CATENIN weiter unterstützen. Darüber hinaus scheint in koKSZ die Expression von IL4 (INTERLEUKIN 4) ein

weiterer Mechanismus gegen therapiebedingte Apoptose zu sein und dadurch den betreffenden Zellen erhöhte Resistenzen vermitteln zu können (Todaro et al., 2007).

Zur Isolation von koKSZ dienen spezifische Oberflächenmoleküle, über die sich die Zellen zum Beispiel in einer FACS- (*fluorescent activated cell sorting*) Analyse anreichern lassen. Im KRK gelang die Isolation von Zellpopulationen, die sich durch entsprechende Eigenschaften auszeichnen (ca. 2,5% - 5,4% aller Tumorzellen), mit Hilfe des Oberflächenmarkers CD133 (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) oder einer Kombination aus CD44 und CD166 (Dalerba et al., 2007) sowie der enzymatischen Aktivität der Aldehyd Dehydrogenase 1 (ALDH1) im sogenannten Aldefluor-Assay (Huang et al., 2009). Entsprechend des Konzepts der koKSZ korreliert passenderweise auch die Expression des Tumorstammzellmarkers CD133 in Kombination mit nukleärem β-CATENIN in Primärtumoren des humanen KRK mit einem signifikant schlechteren Überleben der Patienten (Horst et al., 2009a). Allerdings scheint hierbei der funktionelle Beitrag von CD133 für die Progression vermutlich eher von geringer Bedeutung zu sein, gerade im Vergleich zu CD44 (Du et al., 2008). Solche funktionelle Eigenschaften von koKSZ beinhalten u. a. die Fähigkeit zur Selbsterneuerung durch asymmetrische Teilung, die Fähigkeit Nachkommen zu bilden, die in sämtliche für das Organ zutreffende Zelltypen differenzieren können (Vermeulen et al., 2008), sowie als Goldstandard die Fähigkeit zur Tumorinitiation nach subkutaner Injektion in immundefiziente Wirtsmäuse (Xenografts) in geringer Zellzahl. Weiterhin konnten koKSZ auch als die für die Metastasierung relevanten Zellen identifiziert werden (Pang et al., 2010). Um mit diesen Zellen *in vitro* Analysen durchführen zu können, sind ganz besondere Zellkulturbedingungen nötig, da die Zellen ihrer natürlichen Nische beraubt wurden und sie folglich ihren Stammzellcharakter verlieren könnten und differenzieren würden. Die geeigneten *in vitro* Bedingungen umfassen die nicht-adhärente Propagierung als sogenannte Sphäroide (Kolosphären) in Gegenwart hoher Konzentrationen der Wachstumsfaktoren EGF (EPIDERMAL GROWTH FACTOR) und FGF-2 (FIBROBLAST GROWTH FACTOR 2) (Ricci-Vitiani et al., 2007). Diese scheinen demnach in der Lage zu sein, die Tumorstammzellnische der *in vivo* Situation simulieren zu können. Interessanterweise können auch aus etablierten kultivierten kolorektalen Tumorzelllinien derartige Kolosphären mit vielen Eigenschaften von Tumorstammzellen generiert werden (Dallas et al., 2009; Fillmore

and Kuperwasser, 2008; Yeung et al., 2010). Dies legt den Schluss nahe, dass Tumorstammzellen mit ihren funktionellen Charakteristika auch unter artifiziellen *in vitro* Bedingungen einen kleinen Prozentsatz aller Zellen ausmachen, worauf sich die Fähigkeit der Kultivierung von Tumorzelllinien überhaupt erst zurückführen ließe. Durch diese Erkenntnis lassen sich somit Fragestellungen im Kontext von Tumorstammzellen auch in etablierten Tumorzelllinien untersuchen und es muss nicht zwangsläufig auf schwer zu gewinnendes und aufwendig zu kultivierendes primäres Material zurückgegriffen werden. Möglich ist die Isolation dieser Zellen ebenfalls mit Hilfe der beschriebenen Markermoleküle (Yeung et al., 2010) oder durch die funktionelle Eigenschaft von KSZ fluoreszierende Farbstoffe, wie z. B. Hoechst 33342, aktiv auszuschleusen, was in FACS Analysen die charakteristische Zellfraktion der Side Population (SP) zur Folge hat (Sussman et al., 2007).

2 FRAGESTELLUNGEN

Zusammenfassend kann festgestellt werden, dass das von Vogelstein vorgeschlagene Modell der linearen Tumorentstehung viel zum Verständnis der kolorektalen Karzinogenese beigetragen hat (Kinzler and Vogelstein, 1996), es jedoch angesichts neuerer Daten insbesondere bezüglich der EMT und des Konzepts der Tumorstammzellen in einen weitaus komplexeren Kontext einzuordnen ist. Da β-CATENIN in diesem Zusammenhang eine zentrale Rolle einnimmt und an der Vermittlung vieler tumorrelevanter Prozesse beteiligt ist, sollte im Verlauf meiner Promotion die Bedeutung des deregulierten WNT Signaltransduktionsweges für die Karzinogenese im Gastrointestinaltrakt mit besonderem Fokus auf die *stemness* weitergehend untersucht werden. Insbesondere wurden in meiner kumulativen Dissertation folgende Fragestellungen bearbeitet:

1. Welche Mechanismen führen in humanen KRK zur Stabilisierung von β-CATENIN, insbesondere in MSI-H KRK?
2. Welche Auswirkung hat der β-CATENIN induzierte negative *feedback loop* durch DKK4 in humanen KRK und wie wird dieser unterbrochen?
3. Welche prognostische und funktionelle Rolle spielen koKSZ Marker wie CD133 in der Progression humaner KRK?
4. Welche neuen β-CATENIN Zielgene sind funktionell an der Aufrechterhaltung von Tumorstammzell-Attributen wie Langlebigkeit, geringer Teilungsrate, Tumor-Initiation und Therapieresistenz beteiligt? Sind β-CATENIN Zielgene selber auch funktionell am Aufbau der Tumorstammzellnische beteiligt?

3 ERGEBNISSE

3.1 In einigen hochgradig mikrosatelliteninstabilen (MSI-H) kolorektalen Tumoren finden sich Mutationen im WTX – Gen

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RESEARCH ARTICLE Open Access

Mutations in the *WTX* - gene are found in some high-grade microsatellite instable (MSI-H) colorectal cancers

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Abstract

Background: Genetically, colorectal cancers (CRCs) can be subdivided into tumors with chromosomal instability (CIN) or microsatellite instability (MSI). In both types of CRCs genes that are involved in the degradation of β-CATENIN are frequently mutated. Whereas in CIN CRCs *APC* (Adenomatous Polyposis Coli) is affected in most cases, high grade MSI (MSI-H) CRCs frequently display mutations in various genes, like the *APC*-, *AXIN2*- or *CTNNBI* (β-CATENIN) gene itself. Recently in Wilms tumors, *WTX* (Wilms tumor gene on the X-chromosome) was discovered as another gene involved in the destruction of β-CATENIN. As the *WTX*-gene harbors a short T_6-microsatellite in its N-terminal coding region, we hypothesized that frameshift-mutations might occur in MSI-H CRCs in the *WTX* gene, thus additionally contributing to the stabilization of β-CATENIN in human CRCs.

Methods: DNA was extracted from 632 formalin-fixed, paraffin-embedded metastatic CRCs (UICCIV) and analyzed for MSI-H by investigating the stability of the highly sensitive microsatellite markers BAT25 and BAT26 applying fluorescence capillary electrophoresis (FCE). Then, in the MSI-H cases, well described mutational hot spot regions from the *APC*-, *AXIN2*- and *CTNNBI* genes were analyzed for genomic alterations by didesoxy-sequencing while the *WTX* T_6-microsatellite was analyzed by fragment analysis. Additionally, the PCR products of T_5-repeats were subcloned and mutations were validated using didesoxy-sequencing. Furthermore, the *KRAS* and the *BRAF* proto-oncogenes were analyzed for the most common activating mutations applying pyro-sequencing. mRNA expression of *WTX* from MSI-H and MSS cases and a panel of colorectal cancer cell lines was investigated using reverse transcription (RT-) PCR and FCE.

Results: In our cohort of 632 metastatic CRCs (UICCIV) we identified 41 MSI-H cases (6.5%). Two of the 41 MSI-H cases (4.8%) displayed a frameshift mutation in the T_6-repeat resulting in a T_5 sequence. Only one case, a male patient, expressed the mutated *WTX* gene while being wild type for all other investigated genes.

Conclusion: Mutations in the *WTX*-gene might compromise the function of the β-CATENIN destruction complex in only a small fraction of MSI-H CRCs thus contributing to the process of carcinogenesis.

Background

Genetically, colorectal cancers (CRCs) might be subdivided into two groups. One group is characterized by chromosomal instability (CIN) and follows the classical multistep carcinogenesis model where mutations result in the activation of proto-oncogenes (gain of function) or the inhibition of tumor suppressor genes (loss of function) by this driving the process of colorectal carcinogenesis [1]. The other group is characterized by high grade instability of microsatellites (MSI-H) and can be subdivided into sporadic and heritable forms and accounts for approximately 15% of all CRCs. The majority of sporadic MSI-H CRCs is characterized by loss of expression of the *MLH1* (MUT-L homologue 1) gene, a component of the mismatch repair (MMR) system due to methylation of its promoter/exon 1 region. These MSI-H CRCs belong to the CIMP (CpG island methylator phenotype) and are highly associated with mutations in the *BRAF* proto-oncogene (up to 75%) [2]. In contrast, the heritable forms of MSI-H CRCs, known as

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hereditary non polyposis colorectal cancers (HNPCC), harbor mutations in genes of the MMR-system, like *MLH1*, *MSH2* (MUT-S homologue 2), *MSH6* or *PMS2* (post mitotic segregation 2). Thus, in MSI-H tumors, the function of the MMR-system is lost [3]. This in turn leads to frameshift mutations in microsatellites, which might contribute to the malignant transformation of tumor cells when located in the coding sequences of tumor suppressor genes like the *TGFBR2* (TGF-β receptor type 2) [4]. This type of mutation and the associated occurrence of neoantigenic structures might explain why sporadic MSI-H CRCs have a better prognosis than microsatellite stable (MSS) CRCs [5].

Interestingly, the stabilization of β-CATENIN, which is the executor of the canonical WNT-signaling pathway, is affected in both, MSI-H and MSS CRCs. In the WT situation β-CATENIN is earmarked for degradation by a multi-protein complex assembled of at least APC, AXIN2, PP2A (pyro-phosphatase 2 A) and GSK3B (glycogen synthase kinase 3β). In MSS CRCs, the stabilization of β-CATENIN is mostly achieved by mutations in the tumor suppressor gene *APC*, which is considered to be the gatekeeper of colorectal carcinogenesis [1]. In contrast in MSI-H CRCs, the stabilization of β-CATENIN seems to be a later event and is achieved by loss of function in *APC* [6-8] in only 14-56%, while in 24% mutations are found in the *AXIN2*- [8] or in up to 43% in the *CTNNBI*-gene itself, depending on if investigating sporadic or heritable cases of CRC [6,8-11]. Expectedly in MSI-H CRCs, the loss of function mutations in the *APC*- and *AXIN2*-tumor suppressor genes partly results from frameshift mutations, thus highlighting the causative role of the MMR system in MSI-H CRCs.

Recently in Wilms tumors, *WTX* (Wilms Tumor gene on the X-Chromosome) was discovered as another component of the β-CATENIN degradation complex where it directly interacts with β-CATENIN and APC [12,13]. Moreover, Wilms tumors are often characterized by a deregulated WNT-signaling pathway which can be attributed to mutations in the *WTX*-gene in 7 to 30% of all cases [14,15]. Due to its location on the X-chromosome, *WTX* resembles a one hit tumor suppressor which has therefore a higher penetrance in men than in women.

Because of its role in the degradation of β-CATENIN and the presence of a short six basepair long T-repeat (T_6) in the N-terminus encoding part of the *WTX*-gene, we hypothesized that *WTX* might also contribute to the stabilization of β-CATENIN in MSI-H CRCs. Therefore, we investigated if frameshift mutations are present in the T_6-repeat of the *WTX*-gene in the MSI-H fraction of a collection of metastatic CRCs (UICCIV).

Methods

Clinical samples

632 cases of formalin fixed, paraffin-embedded (FFPE) tissue of metastatic CRCs (UICCIV) were selected for the investigation. The usage of these cases for scientific reasons was approved by the local ethics committee of the Medical Faculty of the Ludwig-Maximilians-Universität München.

DNA and RNA isolation

After removal of paraffin wax using graded xylene- and alcohol dilutions following routine protocols, tumor cells were manually dissected from the slides. Subsequently, DNA and RNA were extracted using QIAamp DNA Micro kits or RNeasy FFPE kits (Qiagen, Hilden, Germany), respectively following the instruction manuals.

Cell lines, Cell culture and RNA isolation

Cultivated colorectal tumor cell lines RKO, LoVo, SW480, Caco2, DLD-1, HCT15, HCT116, LS174T and HT29 were purchased from the ATCC (LGL Promochem GmbH, Wesel, Germany) and maintained in DMEM (Biochrom, Berlin, Germany) containing 7.5% (v/v) fetal bovine serum (Biochrom, Berlin, Gemany). RNA was isolated using RNeasy Mini kits (Qiagen, Hilden, Germany) according to the user's instructions.

PCR, reverse transcription, RT-PCR, fluorescence capillary electrophoresis (FCE), didesoxy- and pyro-sequencing

For the determination of MSI-H polymerase chain reactions (PCRs) specific for the monomorphic mononucleotide microsatellites *BAT25* and *BAT26* were done using 1 μl DNA as the template in the presence of 1.5 mM $MgCl_2$, 200 μM dNTPs (Fermentas, St. Leon, Germany), 400 nM of each of *BAT25* or *BAT26* specific primer pairs (Additional file 1, Table S1), respectively together with 1 U HotStarTaq Polymerase (Qiagen, Hilden, Germany) following the user's manual. Male DNA (Promega, Mannheim, Germany) was used as the template for positive- and water for negative controls. PCRs specific for the mutation hot spots of the *APC*- (exon 15) [7], *AXIN2*- (exon 8) [8] and *CTNNBI*- (exon 3) [16,17] genes as well as the T_6-microsatellite of the *WTX*-gene were done using the same protocol together with gene-specific primers (Additional file 1, Table S1). 1 μl of the final PCR products was used for subsequent sequencing using BigDye Terminator v1.1 kits (Applied Biosystems, Darmstadt, Germany) together with appropriate primers (500 nM, Additional file 1, Table S1). Reactions were purified using DyeEx v2.0 kits (Qiagen, Hilden, Germany) following the handbook. 4 μl purified

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Table 1 Overview of patient age, gender and mutations found in MSI-H CRCs

Patient ID	Age	Gender	*WTX*	*APC Exon 15*	*AXIN2 Exon 8*	*CTNNB1 Exon 3*	*KRAS Codon 12/13*	*BRAF Codon 600*
1	65	m	T_5	WT	WT	WT	WT	WT
2	n.a.	f	T_5	WT	WT	WT	WT	c.1799T>A
3	52	m	T_6	WT	WT	WT	WT	WT
4	74	m	T_6	WT	1 bp del (G) codon 665	WT	WT	n.a.
5	68	f	T_6	WT	WT	WT	c.35G>A	WT
6	28	m	T_6	2 bp del (AG) codon 1462	WT	WT	WT	WT
7	72	f	T_6	WT	WT	WT	WT	c.1799T>A
8	65	f	T_6	n.a.	n.a.	n.a.	WT	WT
9	63	f	T_6	c.2312A>G p.E770G	WT	WT	WT	c.1799T>A
10	n.a.	m	T_6	c.2393A>G p.G797D	WT	WT	WT	c.1799T>A
11	77	f	T_6	WT	WT	WT	WT	WT
12	65	f	T_6	WT	WT	WT	WT	c.1799T>A
13	75	m	T_6	1 bp ins (A) codon 1554	WT	WT	WT	c.1799T>A
14	69	f	T_6	WT	c.2062C>T (mut/mut) p.P687L	c.64G>A p.V22I	WT	WT
15	72	f	T_6	WT	WT	WT	WT	c.1799T>A
16	46	m	T_6	WT	c.2073C>T p.691Q>STOP	WT	WT	WT
17	45	f	T_6	1 bp ins (A) codon 1554	WT	WT	WT	c.1799T>A
18	42	m	T_6	WT	WT	WT	WT	WT
19	n.a.	m	T_6	WT	WT	WT	WT	WT
20	76	m	T_6	WT	WT	WT	WT	c.1799T>A
21	76	m	T_6	WT	WT	WT	WT	WT
22	78	f	T_6	WT	c.2037C>T p.H679Y and c.2071C>T p.691Q>STOP	WT	WT	WT
23	56	f	T_6	WT	WT	c.134C>T p.S45F	WT	c.1799T>A
24	33	m	T_6	WT	WT	WT	WT	WT
25	73	f	T_6	WT	WT	WT	WT	c.1799T>A
26	65	f	T_6	WT	c.2103T>C p.S701P	WT	WT	c.1799T>A
27	77	f	T_6	WT	WT	WT	WT	WT
28	69	m	T_6	WT	WT	WT	WT	WT
29	43	m	T_6	WT	WT	WT	WT	WT
30	64	f	T_6	WT	WT	WT	WT	WT
31	43	m	T_6	WT	WT	WT	WT	WT
32	43	m	T_6	WT	WT	WT	WT	WT
33	54	m	T_6	WT	WT	WT	WT	WT
34	66	f	T_6	WT	c.2062C>T p.P687L	WT	WT	WT
35	40	f	T_6	WT	WT	WT	WT	WT
36	60	m	T_6	n.a.	n.a.	n.a.	n.a.	WT

Table 1 Overview of patient age, gender and mutations found in MSI-H CRCs *(Continued)*

37	63	f	T_6	WT	WT	WT	c.35G>A	WT
38	73	m	T_6	WT	WT	WT	WT	WT
39	72	f	T_6	WT	c.2077A>G p.H692R	WT	WT	WT
40	65	f	T_6	n.a.	n.a.	n.a.	WT	n.a.
41	52	m	T_6	c.2626C>T p.876R>STOP and 2 bp del (AG) codon 1462	WT	WT	WT	WT

PCR product were mixed together with 16 μl highly-deionized formamide (HiDi, Applied Biosystems, Darmstadt, Germany), heated for 2 minutes at 90°C, cooled down immediately on ice and loaded onto the genetic analyzer 3130 (Applied Biosystems, Darmstadt, Germany). Results were finally analyzed with the help of the Sequencing Analysis v5.2- (Applied Biosystems, Darmstadt, Germany) and Geneious-software (Biomatters Ltd., Australia).

For sequencing the fragments generated from the WTX-gene, PCRs were repeated using the same but unlabelled primers (Additional file 1, Table S1) as for the screening for mutations. Resulting PCR products were subcloned using the CloneJet PCR cloning kit (Fermentas, St. Leon, Germany) according to the user's manual and finally sequenced as described above.

For reverse transcription, 1 μg of RNA was converted into cDNA using QuantiTect Reverse Transcription kits (Qiagen, Hilden, Germany) in the presence (+) or absence (-) of reverse transcriptase (RT) following the user's manual. For subsequent *WTX* specific PCRs 1 μl of a fiftyfold diluted cDNA solution was used as the template but else applying the same conditions as described above. RT-PCR specific for the housekeeping gene *ACTB* (β-ACTIN, [GenBank: NM_001101]) served as a control using β-ACTIN specific primers (Additional file 1, Table S1). Products from RT-PCR were separated on 2% 0.5 × TBE agarose gels containing 100 ng/ml ethidium bromide and visualized under UV-light.

For the analysis microsatellite-stability PCR products were separated by capillary electrophoresis using an ABI 3130 sequencing analyzer by mixing 1 μl of the PCR products with 18.5 μl highly-deionized formamide (HiDi) and 0.5 μl of the GeneScan-500 LIZ size standard (both Applied Biosystems, Darmstadt, Germany), heated for 2 minutes at 90°C, cooled down immediately on ice, and subsequently loaded onto the genetic analyzer 3130. Finally, results were analyzed with the help of the GeneMapper v4.0 software (Applied Biosystems, Darmstadt, Germany).

Mutations in codons 12 and 13 of the *KRAS*- and codon 600 of the *BRAF*-gene were done as described [18,19] using Pyromark-Gold reagents (Qiagen, Hilden Germany) together with the appropriate primers (Additional file 1, Table S1) following the user's instructions.

Results

First of all, MSI-H CRCs were identified from our collection of 632 cases of metastatic CRCs (UICCIV) by investigating the stability of the highly sensitive and specific monomorphic mononucleotide markers *BAT25* and *BAT26* [20,21] using PCR based fluorescence capillary electrophoresis (FCE). We detected instability of both microsatellite markers in 41 out of 632 cases (6.5%) compared to control DNA (Figure 1A, tumor ID #1, #2 (both MSI-H), #42 (MSS) and Table 1). These 41 cases were taken for all further investigations as we aimed to concentrate on *WTX* frameshift mutations only in MSI-H CRCs. Second, these cases were analyzed for mutations in the *BRAF*- and *KRAS*- proto-oncogenes (Table 1) applying pyro-sequencing. We found two out of 40 evaluable cases (5%) with activating mutations in *KRAS* codons12 and 13 and 12 out of 39 cases (30.8%) with an activating mutation in codon 600 of the *BRAF* gene (p.V600E). Third, *WTX* frameshift mutations were found in 2 out of the 41 investigated MSI-H CRCs (4.9%) as shown by FCE and resulted in a T_5-repeat (Figure 1B, tumor ID #1, #2), while no such mutations were present in MSS CRCs (Figure 1B, ID #42). These findings were further confirmed by subcloning the truncated *WTX* PCR products and subsequent didesoxy-sequencing. Both approaches also revealed signals from the wild type T_6-repeat in both cases (not shown for direct sequencing), which probably stem from contaminations with normal tissue, as well as signals derived from the second, intact X-chromosome, the latter of course only in case of female patients. The detected frameshift leads to a stop codon (TGA) at position 621 of the coding sequence of the *WTX*-gene, which is of 3.408 bp in length (GenBank: NM_152424). When expressed, such mutated *WTX* alleles will inevitably result in a truncated protein of 207 instead of wild typic 1.135 amino acids. Such a protein will have no functional properties with respect to the degradation of β-CATENIN, since the APC and

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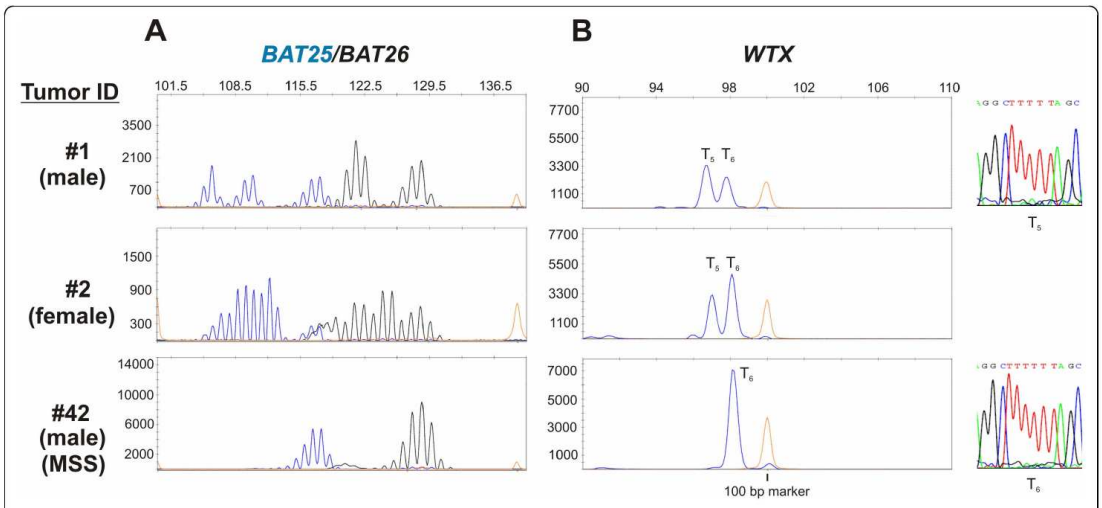

Figure 1 MSI-H colorectal tumors display frameshift mutations in the T_6-microsatellite of the *WTX*-gene. (A) Genomic DNA from 632 patients with metastatic CRC (UICCIV) was analyzed for the presence of MSI-H with the help of the two monomorphic microsatellite markers *BAT25* and *BAT26*. Shown are representative results from two CRCs with MSI-H (ID #1, #2) and one CRC with MSS (ID #42). (B) Two tumors of the 41 MSI-H cases of CRCs harbored frameshift mutations in the coding sequence of the T_6-repeat of the *WTX*-gene resulting in the occurrence of a T_5-repeat (ID #1 and #2) compared to only wild type T_6-repeats in tumors with MSS CRCs (ID #3). Left: shown by FCE, right: shown by direct sequencing.

β-CATENIN interaction domains are located C-terminally of amino acid 207, namely positions 307-789 and in the C-terminus, respectively [12,13]. Tumor ID #2 was additionally characterized by a mutation in the *BRAF* gene (Table 1). Fourth, we wanted to increase the evidence that the *WTX* mutations might have a functional relevance for the stabilization of β-CATENIN in the context of the other members of the WNT signaling pathway *APC*, *AXIN2* and *CTNNB1*, which have been described to be mutated in MSI-H CRCs. Therefore, we investigated the described mutational hot spot regions of these three genes in the 41 MSI-H CRCs [7,8,16,17] by direct didesoxy-sequencing (Table 1). Mutations in the APC gene were detected in 6 out of 38 evaluable cases (15.8%), in the *AXIN2*-gene in 7 of 38 (18.4%) and in the *CTNNBI*-gene in 2 of 38 (5.3%) cases. As far as to our knowledge, some of the detected mutations have not been described previously and thus their functional consequences remain to be determined. Importantly, however, we could not find any additional mutations in the two tumors displaying the *WTX* T_5 frameshift. Fifth, we analyzed the expression of *WTX* mRNAs in the tumors of the patients with *WTX* frameshift mutations in order to assess whether these might be of functional relevance for the stabilization of β-CATENIN. Due to the clonal origin of cancers and the fact that only one X-chromosome is transcriptionally active in women whereas men carry only a single X-chromosome, mutations in X-chromosomal encoded genes like the *WTX*-gene will have a direct functional consequence known as haplo-insufficiency when being expressed. Thus, we checked, first of all, if WTX is commonly expressed in CRCs and especially in the patients carrying T_5-repeats in their WTX-genes. The tumors of both patients expressed *WTX* mRNA (Figure 2A, tumor ID #1 and #2), as did MSI-H tumors from patients with wild type *WTX* genes (Additional file 2, Figure S1 A patient ID #4 - #6) or tumors from MSS CRCs (Figure 2A, tumor ID #42 and Additional file 2, Figure S1 A, tumor ID #43 and #44), as well as a panel of cultivated colorectal tumor cell lines (Additional file 2, Figure S1 B). Next, we investigated the length of the T_6- microsatellite in the *WTX*-mRNA. We found that only one of the two tumors (ID #1, a male patient) harbored the T_5-frameshift mutation on the transcript level (Figure 2B, tumor ID #1) and thus inevitably also on the protein level. However, this latter fact could not be verified immunohistochemically due to lack of specific antibodies distinguishing between wild type and mutated WTX protein. The T_5-repeat containing tumor of the female patient (ID #2) expressed wild-type *WTX*-mRNA, indicating that the mutated gene was located on the inactivated X-chromosome.

Discussion

MSI-H CRCs constitute approximately 15% of all CRCs [3] and are generally considered to be

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http://www.biomedcentral.com/1471-2407/10/413

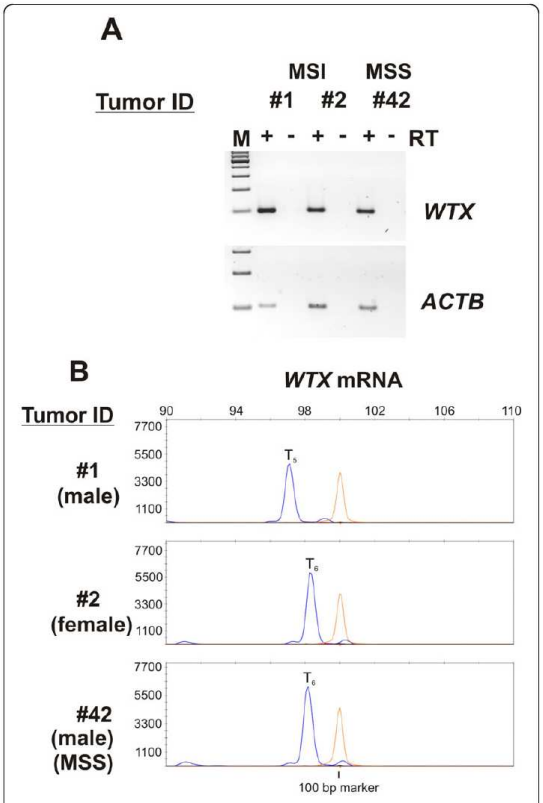

Figure 2 Frameshift mutations in the *WTX*-gene are expressed on the mRNA level. (A) CRCs (ID #1, #2 and #42) expressed *WTX* mRNA. (B) One of the two CRCs with MSI-H and a genomic frameshift mutation in the *WTX*-gene displayed expression of the mutated allele on the level of mRNA (ID #1) whereas the other tumor (ID #2) as well as MSS-CRCs (ID #42) harbored wild type mRNA indicated by the presence of only T_6-repeats.

characterized by a better prognosis than tumors of the MSS type [5]. This is reflected by the fact that only 7.9% of all MSI-H CRCs show progression to a metastatic state (UICCIV), while 27.9% of MSS cases succeed in forming distant metastases [22]. Thus, the proportion of MSI-H CRCs among tumors in UICCIV has been found to be less compared with the mean value of 15% in all stages (UICCI - UICCIV) [23] which is in support with 41 MSI-H cases found in 632 UICCIV cases of CRCs (6.5%). These results were obtained using the two mononucleotide markers *BAT25* and *BAT26* instead of the five marker set recommended by the Bethesda guidelines from the National Cancer Institute [20] because these two markers were shown to be of almost the same diagnostic value as all five NCI markers in combination [21]. Furthermore, due to their monomorphic character, usage of *BAT25* and *BAT26* makes a comparison of normal tissue dispensable.

The difference in clinical behavior of colorectal MSI-H and MSS tumors is also reflected by differences in the mechanisms of genetic instability. MSS CRCs usually mutate the *APC* tumor suppressor gene by gross deletions in the genome known as loss of heterozygosity (LOH) or point mutations [24]. As this occurs as the entry mutation in the process of colorectal carcinogenesis *APC* is here known as the gatekeeper [1]. In contrast, MSI-H CRCs seem to evolve via a different pathway as mutations in the *BRAF* or less often *KRAS* proto-oncogene occur early [3]. Our findings show that in UICCIV less tumors carry *BRAF* gene mutations (28% compared with up to 75% in MSI-H tumors of all stages [2]) indicating that in MSI-H tumors *BRAF* mutations might be somehow protective for progression into UICCIV. Alternatively, the amount of heritable HNPCC tumors might be higher in our collection which do not show mutations in the *BRAF* gene [2]. Due to changes in the cellular methylation system in sporadic MSI-H tumors the expression of several tumor suppressor genes is turned off due to methylation of its promoter/exon1. This also seems to be the cause for the loss of the expression of the *MLH1* (*MUT-L homologue*) gene. As a consequence, instability of microsatellites occurs which affects in a second wave of mutations components of the WNT-signaling pathway, like the *APC*-, or the *AXIN2*-gene and, as shown here in a small group of cases, additionally in the *WTX*-gene. Since we concentrated on defects leading to the stabilization of β-CATENIN in MSI-H CRCs, a collection of UICCIV tumors was used because it was expected that later states should contain most if not all mutations leading to the stabilization of β-CATENIN. One study analyzed in a group of 45 MSI-H CRCs the occurrence of mutations in the three genes encoding *AXIN2* (11/45 - 24.4%), *APC* (4/28 - 14.3%) and *CTNNBI* (5/45 - 11.1%) [8], thus assigning approximately 50% of MSI-H CRCs with defects in components of the WNT signaling pathway. Assuming that mutations in the WNT-signaling pathway might also be important in MSI-H tumors, it suggests that additional mechanisms might contribute to the stabilization of β-CATENIN in this tumor entity. The situation turns out to be even more complex as heritable and sporadic forms of tumors display partly great differences in their mutational spectrum as e.g. shown for the *CTNNB1* gene where sporadic tumors do not display mutation [11] compared to 43% in heritable HNPCC cases [10]. Analyzing our mixed collection of UICCIV MSI-H CRCs, we found 15 out 38 evaluable cases (39.5%) with mutations in the established components of the WNT signaling pathway. Alternatively, this might be a stage specific effect as it was shown that

MSI-H tumors are found less frequently in the UICCIV group than MSS tumors which is not the case in the other stages (UICCI - UICCIII) thus indicating a special biology of MSI-H tumors in UICCIV [22]. Here, we add frameshift mutations in the recently identified *WTX*-gene, a component of the β-CATENIN degradation complex [13], as an additional mechanism which were found at a frequency of 2/41 among MSI-H cases (4.9%). This frameshift occurred in a 6 thymidine repeat that is located in the N-terminal coding region giving rise to an unfunctional gene product when being transcribed as shown here and translated due to the absence of the APC- and β-CATENIN interaction domains [12,13]. Moreover, we detected several monorepeats of five basepairs in length distributed over the *WTX* open reading frame which we did not investigate for instability because they are to small to be targets for microsatellite frameshifts [25].

In general a rate of two out of 41 is very low but it is misleading to conclude from mutation rates on the importance of genes as even intronic regions display mutation rates of 54.2% which are known to be only passenger mutations [26]. Moreover, even high mutation frequencies of 39% in the *TCF4* gene (T-cell transcription factor 4) [27] which has an important role in WNT-signaling are not warranting that the mutations might have a physiologic effect [28]. Thus, the finding that 1 patient (2.5%) expressed the genetic alteration on the mRNA level may be an indicator that alterations in the *WTX*-gene might have a functional role for the stabilization of β-CATENIN and have in this case the status of a driver mutation. This accounts especially in the context of absent other mutations in the WNT pathway (Table 1) and when considering that this patient with tumor ID #1 was a 65 years old man with probably a sporadic tumor and that *WTX* is located on the X-chromosome. Moreover, our mutation frequency is consistent with another study, which detected only a single mutation in 47 CRCs (2.1%), irrespective of the MSI status [29]. While the functional consequene of this described point mutation is unknown, this and our work at least indicate that mutations in *WTX* might be involved in colorectal carcinogenesis in a small proportion of both, MSI-H as well as MSS tumors. Interestingly, our result that the tumor of a female patient displayed the mutation in the *WTX*-gene on the transcriptionally inactive X-chromosome is in support with findings from Wilms tumors where mutations of the *WTX*-gene were also found on the inactivated X-chromosome in women [14]. Obviously, larger cohorts are needed for definitively answering the open questions about the frequency of *WTX*-gene mutations and the prevalence of mutations on the inactivated X-chromosome in female patients.

Conclusion

Taking together, we demonstrate that mutations in the *WTX*-gene might play a role in the process of MSI-H colorectal carcinogenesis in a small subgroup of these tumors as has been modeled for Wilms Tumors before [30]. This implies that *WTX*-gene mutations should be reconsidered in future studies dealing with MSI-H CRCs, especially in the context of the other known mutations leading to stabilization of β-CATENIN. Importantly, a functional role of these mutations for the process of colorectal carcinogenesis has to be investigated in further works in the future.

Additional material

Additional file 1: Table S1 Oligonucleotides used in this study.

Additional file 2: Figure S1 CRCs as well as colorectal tumor cell lines express *WTX* mRNA. (A) All cases of CRCs tested expressed *WTX* mRNA irrespective of being MSI-H (ID #1, #2 and #4 to #6) or MSS (ID #42 to #44). (B) A panel of cultivated CRC cell lines also expressed *WTX* mRNA. PCR specific for the housekeeping gene *ACTB* (β-ACTIN) served as a control.

Acknowledgements

We thank Jutta Hügel-Tegge, Gabi Charell, Margit Mattei, and Nicole Perera for their help in preparing and providing genomic DNA for the cases under investigation. This work was supported by the Wilhelm-Sander Stiftung, Germany (grant No 2004-111.2, to AJ and TK).

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Authors' contributions

SKS coordinated the study, designed and partly optimized the analytical tools. He generated and analyzed the data. Moreover, he wrote the draft of the manuscript. SP generated and analyzed data in part, MP developed and optimized part of the mutation detection system of the APC gene as part of his MD work, SO was involved in the initial planning of the study, TK approved the study and AJ designed and coordinated the study. He was involved in the analysis of the data and brought the manuscript into its final form. All authors read and approved to the final form of the manuscript.

Author's information

This project is part of the PhD thesis of SKS.

Competing interests

The authors declare that they have no competing interests.

Received: 22 December 2009 Accepted: 9 August 2010
Published: 9 August 2010

References

1. Kinzler KW, Vogelstein B: **Lessons from hereditary colorectal cancer.** *Cell* 1996, **87**:159-170.
2. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, *et al*: **CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer.** *Nat Genet* 2006, **38**:787-793.

3. Ogino S, Goel A: **Molecular classification and correlates in colorectal cancer.** *J Mol Diagn* 2008, **10**:13-27.
4. Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan RS, Zborowska E, Kinzler KW, Vogelstein B, *et al*: **Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability.** *Science* 1995, **268**:1336-1338.
5. Popat S, Hubner R, Houlston RS: **Systematic review of microsatellite instability and colorectal cancer prognosis.** *J Clin Oncol* 2005, **23**:609-618.
6. Shitoh K, Furukawa T, Kojima M, Konishi F, Miyaki M, Tsukamoto T, Nagai H: **Frequent activation of the beta-catenin-Tcf signaling pathway in nonfamilial colorectal carcinomas with microsatellite instability.** *Genes Chromosomes Cancer* 2001, **30**:32-37.
7. Huang J, Papadopoulos N, McKinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JK, Markowitz SD, Morin P, *et al*: **APC mutations in colorectal tumors with mismatch repair deficiency.** *Proc Natl Acad Sci USA* 1996, **93**:9049-9054.
8. Liu W, Dong X, Mai M, Seelan RS, Taniguchi K, Krishnadath KK, Halling KC, Cunningham JM, Boardman LA, Qian C, *et al*: **Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling.** *Nat Genet* 2000, **26**:146-147.
9. Polakis P: **Wnt signaling and cancer.** *Genes Dev* 2000, **14**:1837-1851.
10. Miyaki M, Iijima T, Kimura J, Yasuno M, Mori T, Hayashi Y, Koike M, Shitara N, Iwama T, Kuroki T: **Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis colorectal cancer.** *Cancer Res* 1999, **59**:4506-4509.
11. Salahshor S, Kressner U, Pahlman L, Glimelius B, Lindmark G, Lindblom A: **Colorectal cancer with and without microsatellite instability involves different genes.** *Genes Chromosomes Cancer* 1999, **26**:247-252.
12. Grohmann A, Tanneberger K, Alzner A, Schneikert J, Behrens J: **AMER1 regulates the distribution of the tumor suppressor APC between microtubules and the plasma membrane.** *J Cell Sci* 2007, **120**:3738-3747.
13. Major MB, Camp ND, Berndt JD, Yi X, Goldenberg SJ, Hubbert C, Biechele TL, Gingras AC, Zheng N, Maccoss MJ, *et al*: **Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling.** *Science* 2007, **316**:1043-1046.
14. Perotti D, Gamba B, Sardella M, Spreafico F, Terenziani M, Collini P, Pession A, Nantron M, Fossati-Bellani F, Radice P: **Functional inactivation of the WTX gene is not a frequent event in Wilms' tumors.** *Oncogene* 2008, **27**:4625-4632.
15. Rivera MN, Kim WJ, Wells J, Driscoll DR, Brannigan BW, Han M, Kim JC, Feinberg AP, Gerald WL, Vargas SO, *et al*: **An X chromosome gene, WTX, is commonly inactivated in Wilms tumor.** *Science* 2007, **315**:642-645.
16. Mirabelli-Primdahl L, Gryfe R, Kim H, Millar A, Luceri C, Dale D, Holowaty E, Bapat B, Gallinger S, Redston M: **Beta-catenin mutations are specific for colorectal carcinomas with microsatellite instability but occur in endometrial carcinomas irrespective of mutator pathway.** *Cancer Res* 1999, **59**:3346-3351.
17. Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW: **Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC.** *Science* 1997, **275**:1787-1790.
18. Ogino S, Kawasaki T, Brahmandam M, Yan L, Cantor M, Namgyal C, Mino-Kenudson M, Lauwers GY, Loda M, Fuchs CS: **Sensitive sequencing method for KRAS mutation detection by Pyrosequencing.** *J Mol Diagn* 2005, **7**:413-421.
19. Poehlmann A, Kuester D, Meyer F, Lippert H, Roessner A, Schneider-Stock R: **K-ras mutation detection in colorectal cancer using the Pyrosequencing technique.** *Pathol Res Pract* 2007, **203**:489-497.
20. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: **A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer.** *Cancer Res* 1998, **58**:5248-5257.
21. Deschoolmeester V, Baay M, Wuyts W, Van Marck E, Van Damme N, Vermeulen P, Lukaszuk K, Lardon F, Vermorken JB: **Detection of microsatellite instability in colorectal cancer using an alternative multiplex assay of quasi-monomorphic mononucleotide markers.** *J Mol Diagn* 2008, **10**:154-159.
22. Malesci A, Laghi L, Bianchi P, Delconte G, Randolph A, Torri V, Carnaghi C, Doci R, Rosati R, Montorsi M, *et al*: **Reduced likelihood of metastases in patients with microsatellite-unstable colorectal cancer.** *Clin Cancer Res* 2007, **13**:3831-3839.
23. Fallik D, Borrini F, Boige V, Viguier J, Jacob S, Miquel C, Sabourin JC, Ducreux M, Praz F: **Microsatellite instability is a predictive factor of the tumor response to irinotecan in patients with advanced colorectal cancer.** *Cancer Res* 2003, **63**:5738-5744.
24. Albuquerque C, Breukel C, van der Luijt R, Fidalgo P, Lage P, Slors FJ, Leitao CN, Fodde R, Smits R: **The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade.** *Hum Mol Genet* 2002, **11**:1549-1560.
25. Woerner SM, Benner A, Sutter C, Schiller M, Yuan YP, Keller G, Bork P, Doeberitz MK, Gebert JF: **Pathogenesis of DNA repair-deficient cancers: a statistical meta-analysis of putative Real Common Target genes.** *Oncogene* 2003, **22**:2226-2235.
26. Zhang L, Yu J, Willson JK, Markowitz SD, Kinzler KW, Vogelstein B: **Short mononucleotide repeat sequence variability in mismatch repair-deficient cancers.** *Cancer Res* 2001, **61**:3801-3805.
27. Duval A, Gayet J, Zhou XP, Iacopetta B, Thomas G, Hamelin R: **Frequent frameshift mutations of the TCF-4 gene in colorectal cancers with microsatellite instability.** *Cancer Res* 1999, **59**:4213-4215.
28. Ruckert S, Hiendlmeyer E, Brueckl WM, Oswald U, Beyser K, Dietmaier W, Haynl A, Koch C, Ruschoff J, Brabletz T, *et al*: **T-cell factor-4 frameshift mutations occur frequently in human microsatellite instability-high colorectal carcinomas but do not contribute to carcinogenesis.** *Cancer Res* 2002, **62**:3009-3013.
29. Yoo NJ, Kim S, Lee SH: **Mutational analysis of WTX gene in Wnt/beta-catenin pathway in gastric, colorectal, and hepatocellular carcinomas.** *Dig Dis Sci* 2009, **54**:1011-1014.
30. Nusse R: **Cancer. Converging on beta-catenin in Wilms tumor.** *Science* 2007, **316**:988-989.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2407/10/413/prepub

doi:10.1186/1471-2407-10-413
Cite this article as: Scheel *et al.*: Mutations in the *WTX* - gene are found in some high-grade microsatellite instable (MSI-H) colorectal cancers. *BMC Cancer* 2010 **10**:413.

3.2 Dickkopf-4 ist in kolorektalen Krebszellen oft herunterreguliert und inhibiert ihr Wachstum

Cancer Letters 276 (2009) 152–159

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Dickkopf-4 is frequently down-regulated and inhibits growth of colorectal cancer cells

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ARTICLE INFO

Article history:
Received 25 July 2008
Received in revised form 31 October 2008
Accepted 4 November 2008

Keywords:
β-catenin
Wnt
Dickkopf
DKK4
Colorectal cancer

ABSTRACT

Like Dickkopf-1 (DKK1), DKK4 is a target of β-catenin/Tcf-4 in colorectal cancer. However, as a negative regulator of Wnt signalling its function in colorectal cancer cells is not well understood. We report that DKK4 is frequently down-regulated in colorectal cancer cell lines with deregulated β-catenin/Tcf-4 and in primary colorectal cancers. Exposure of cancer cells to DKK4 strongly inhibits basal β-catenin/Tcf-4 signalling activity, cancer cell growth and cell cycle progression. Therefore, loss of this negative feed-back loop provides Wnt factor expressing cancer cells with a growth advantage. Our data demonstrate that DKK4 is an important negative regulator of colon cancer cell growth.

1. Introduction

Wnt/β-catenin signalling plays essential roles in embryogenesis, tissue homeostasis and tumor development [1,2]. Physiological signalling is initiated by binding of soluble Wnt ligands to members of the Frizzled family of seven-pass transmembrane receptors and LRP5/6 co-receptors. This in turn leads to inhibition of β-catenin phosphorylation by a multi protein complex consisting of CKIα, GSK-3β, Dishevelled, APC, and Axin, and consequently to the inhibition of β-catenin degradation by the ubiquitin/proteosome pathway [1]. β-catenin then translocates to the nucleus, binds to Tcf/LEF factors and orchestrates the transcription of multiple target genes. The coordinated regulation of this canonical Wnt pathway is essential for differentiation of stem cells and homeostasis of tissues including intestinal epithelium, skin, muscle, and hematopoetic cells [3]. Two groups of extracellular Wnt antagonists, the secreted Frizzled-related proteins (sFRPs), and the members of the Dickkopf (DKK) family, act to fine-tune the spatiotemporal patterns of Wnt activity [4]. sFRPs inhibit Wnt signalling by sequestering Wnts. Binding of Dickkopf proteins to the Wnt co-receptor LRP5/6 causes endocytosis of the complex and consecutively inhibition of the recruitment of LRP5/6 to Frizzled receptors which is essential for initiation of Wnt signalling at the cell membrane [5].

Constitutively active Wnt signalling is causally involved in the genesis of various cancers [3,6]. Most frequently, deregulation of Wnt/β-catenin signalling is found in colorectal cancer due to mutation of APC. This leads to cytoplasmic stabilization of β-catenin and in turn results in

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doi:10.1016/j.canlet.2008.11.003

the activation of β-catenin/Tcf mediated transcription. In a widely accepted linear view of constitutive activation of Wnt/β-catenin signalling in cancer, additional activating signals are thought to be ineffective and redundant [7]. However, several lines of evidence support a view in which activating and inhibiting signals act simultaneously on different levels of the pathway to modulate Wnt signalling in cancer cells [3]. Additionally, mutant APC retains some residual activity to regulate β-catenin [8], and thus activation of Wnt signalling at the membrane level in addition to APC mutation would allow for further growth advantage of cancer cells. In this model, extracellular Wnt factors might act as a second force to stabilize β-catenin [9]. On this background, silencing of the expression of sFRPs and DKK family members provides a growth advantage for cancer cells [10–12].

Here, we report that DKK4, which like DKK1 is a target gene of β-catenin/Tcf-4, and would therefore be expected to be strongly expressed in colorectal cancers carrying mutations of APC or β-catenin, is frequently down-regulated in colorectal cancer cell lines and primary tumors. We find that DKK4 acts as a potent inhibitor of Wnt-factor induced Tcf-dependent signalling. Ectopic re-expression of DKK4 or treatment of colorectal cancer cells with recombinant DKK4 resulted in inhibition of cell growth by attenuation of cell cycle progression. We find that down-regulation of DKK4 expression is caused by histone deacetylation. Our data demonstrate that colorectal cancer cells select against the expression of DKK4. Due to abolishment of the negative feed-back loop provided by DKK4 colorectal cancer cells gain further growth advantage. Therefore, DKK4 is a new tumor suppressor gene candidate in colorectal cancer.

2. Materials and methods

2.1. Plasmids

The generation of the pcDNA3-based plasmids S33Y-β-catenin, Tcf-4, and Tcf4ΔN31 has been described previously [14]. S33Y-β-catenin-pTRE2hyg and β-galactosidase-pTRE2hyg were generated by shuttling of the coding sequences from the pBMN constructs ([14]) into the corresponding restriction sites of pTRE2hyg (Clontech, Mountain View, CA). The reporter plasmids 8xTOPflash and 8xFOPflash were kindly provided by Randall T. Moon, University of Washington. The putative promoter region of DKK4 was amplified by PCR from the human BAC-clone B737B051025D (RZPD, Berlin, Germany) by using the primers 5′-ctc cca aag tgc tgg gat ta-3′ and 5′-GCA CGT CGT CTG TTT GTC AC-3′. The resulting fragment was subcloned into the luciferase reporter plasmid pGL3-basic (Promega, Mannheim, Germany) and the sequence was confirmed by direct sequencing.

To clone pcDNA3 DKK4, human DKK4 cDNA (source: SW480 cells) was amplified using the primers hDKK4_BamHI_S 5′-CGG GAT CCG CCG CCA CCA TGG TGG CGG CCG TCC TGC T-3′ and hDKK4_NotI_R 5′-ATA AGA ATG CGG CCG CTT ATA GCT TTT CTA TTT TTT GGC ATA C-3′. The resulting PCR product was digested with the restriction enzymes BamHI and NotI and cloned into the expression vector pcDNA3 (Invitrogen) digested with the same restriction enzymes. The sequence of this plasmid was verified by sequencing.

2.2. Cell culture

All parental cell lines were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM medium supplemented with 10% calf serum (PAA, Pasching, Austria). HEK293 Tet-On cells were purchased from Clontech. Short interfering RNAs were purchased from Dharmacon (Lafayette, CO, USA) as on-target validated siRNAs. These were transfected employing Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). To generate stably transfected HCT116 cells expressing human DKK4, 3×10^5 cells were transfected with the plasmid pcDNA3 DKK4 using Mirus LT1 transfection reagent (Mirus Bio). Transfected cells were selected using 250 μg/ml G418 (PAA) and the polyclonal population was used for subsequent experiments. For reporter assays cells were transfected using Nanofectin I (PAA) with 0.5 μg of luciferase reporter plasmids, 0.5 μg expression plasmids and as a control for transfection efficiency with 0.5 μg of constitutively active β-galactosidase reporter plasmid pCH110 (Amersham Pharmacia Biosciences, Freiburg, Germany). Fourty-eight hours after transfection, cells were collected and suspended in reporter lysis buffer (Promega) and luciferase activities were measured using the luciferase assay reagent (Promega) and a luminometer (model TD-20/20; Turner Corp., Sunnyvale, California, USA). β-galactosidase activity was determined by standard methods as a control for transfection efficiency.

Doxycycline-inducible HEK293 Tet/S33Y β-cat cells were generated by stable transfection of S33Y-β-catenin-pTRE2hyg and β-galactosidase-pTRE2hyg into HEK293 Tet-On cells. Cells were selected in G418 (0,1 mg/ml; PAA) and hygromycin B (0,1 mg/ml; PAA). Stimulations were performed with doxycycline (Fluka, Steinheim, Germany) at 2 μg/ml for 24 and 48 h. Recombinant human DKK4 and Wnt3a was purchased from R&D Systems (Minneapolis, MN, USA). Proliferation assays were performed with the CellTiter 96 One Solution Cell Proliferation Assay (Promega).

2.3. Functional assays

HCT116 cells were stimulated with 1 μg/ml recombinant DKK4 (R&D) for 72 h. 1×10^4 HCT116 cells were seeded in soft agar ((Agar noble, Becton Dickinson) mixed with 2× DMEM) and treated with 1 μg/ml recombinant DKK4 (R&D) as indicated. Similarly, 1×10^4 stably transfected HCT116 cells expressing DKK4 were seeded in soft agar. After four weeks, colonies were stained with methyleneblue (Sigma) in 25% glutaraldehyde (Sigma), photographed, and counted.

For flow cytometry analysis, cells were stimulated with 1 μg/ml recombinant DKK4 (R&D) for 72 h. Cells were harvested and stained with 50 mg/ml propidium iodide (Fluka, Steinheim, Germany) in 0.1 M sodium citrate buffer containing 0.1% (w/v) Triton X-100 (Bio-Rad, Munich, Germany) for 30 min at 4 °C and subsequently analyzed using

a Becton Dickinson FACS Calibur flow cytometer (B&D, Heidelberg, Germany). For the analysis of stably with DKK4 transfected HCT116 cells, 1×10^5 cells were seeded, synchronized by serum starvation for 48 h, and stimulated with 10% FCS for 10 h. Flow cytometry analysis was performed as described for cell lines treated with recombinant DKK4 protein.

DLD1, SW480, HT29, LoVo and HCT116 cells were treated with 5 μg/ml trichostatin A (TSA, Sigma) for 24 h. Cells were harvested and total RNA was isolated using TRIZOL (Invitrogen). Reverse transcribed DKK4 mRNA (cDNA) was quantified using real-time PCR.

The histone acetylation status was analyzed by chromatin immunoprecipitation (ChIP). After treatment of DLD1 cells with TSA, immunoprecipitation of acetylated histones H3 K9/14 and H4 K12 was performed using the anti-H3 K9/14ac (Millipore; #06-599) and anti-H4 K12ac (Millipore; #07-595) antibodies, respectively, as described (Acetyl-Histone H3 Immunoprecipitation (ChIP) Kit, Upstate, Lake Placid, NY, USA). The co-immunoprecipitated DNA was amplified by PCR (Thermocycler CG1-96, Corbett-Research, Sydney, Australia) and PCR products were analyzed on an agarose gel. The following PCR were used for the amplification of co-immunoprecipitated DNA: ChIP_DKK4_A_FW 5′-CCC TGC CTC TTC TCT CCT TC-3′, ChIP_DKK4_A_RV 5′-GAA ATC GGC TGA GCA AAG TC-3′, CHIP_GAPDH_prom_FW 5′-TGA GCA GAC CGG TGT CAC TA-3′, and CHIP_GAPDH_prom_RV 5′-AGG ACT TTG GGA ACG ACT GA-3′.

2.4. Western blots

Cell lysates were prepared with RIPA lysis buffer [Tris-buffered saline (TBS), 0.5% deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1% (v/v) Nonidet P-40] supplemented with proteinase inhibitors and equal amounts of protein were supplemented with 4× SDS loading buffer (0.35 M Tris, pH6.8; 30% (v/v) glycerol; 10% (w/v) SDS; 90 mM 2-mercaptoethanol; 0.01% (w/v) bromphenol blue) and separated by electrophoresis in discontinuous SDS–polyacrylamide gels. Antibodies specific for the Flag-tag (Sigma), β-catenin antibody (Transduction Labs, San Jose, CA, USA), β-actin (Sigma–Aldrich, St. Louis, MO, USA) and the secondary horseradish peroxidase-conjugated goat anti-mouse antibody (Amersham Biosciences) were used. Blots were subjected to enhanced chemiluminescence substrate (ECL; Amersham) and exposed to Hyperfilm ECL (Amersham).

2.5. Quantitative RT-PCR

RNA was extracted from cells and microdissected colorectal tissues using Trizol reagent (Invitrogen). RNA was reverse-transcribed after DNase treatment with random hexamer primers (final concentration 2.5 ng/μl) using SuperScript™ II reverse transcriptase (Invitrogen). Oligonucleotide primers and probes used for quantitative RT-PCR will be made available upon request. Resulting PCR-products were monitored by melting point analysis and agarose gel electrophoresis. PCR-efficiency was determined by analyzing serial dilutions of cDNA. Pair-wise fixed reallocation randomization test was used to determine the significance of differences between samples.

3. Results

3.1. Down-regulation of DKK4 expression in colorectal cancer cell lines and primary tumors

It has previously been described that DKK1 and DKK4 are regulated by β-catenin/Tcf-4. To directly compare β-catenin dependent regulation of DKK1 and DKK4 expression, a conditional expression was utilized, which inducibly expresses a stabilized mutant form of β-catenin, namely S33Y-β-catenin. Induction of the cells with doxycycline resulted in strong expression of the β-catenin transgene and activation of Tcf-dependent transcription in the TOPflash reporter assay (Fig. 1A and B). Interestingly, the induction of DKK4 was earlier and stronger than induction of DKK1 (Fig. 1C). Accordingly, as the majority of colorectal cancer cell lines is characterized by constitutive activation of the Wnt/β-catenin pathway due to either APC (LoVo, DLD1, Widr, SW480, SW620, HT29, Colo205, Colo320, CacoII) or β-catenin (Colo205, HCT116, LS174T, SW48) mutation and only few cell lines reveal no deregulation of this pathway (RKO), the expression of DKK1 and DKK4 would be expected to be strongly up-regulated in most of these cell lines. As expected, DKK1 expression was frequently up-regulated in colon cancer cell lines with deregulated β-catenin dependent signalling when compared to a pool of micro-dissected normal colonic epithelium (Fig. 1D). In contrast, expression of DKK4 could only be detected in SW480 and SW620 cells, while all other cell lines revealed no DKK4 expression irrespective of their APC and β-catenin mutational status (Fig. 1D). To test, whether the observed down-regulation of DKK4 in cancer cell lines can also be found in primary colorectal cancers, we determined the expression of DKK4 in a series of matched pairs of micro-dissected colorectal cancers and micro-dissected normal colorectal epithelium. Only 6 of 21 tumors tested revealed elevated DKK4 levels while 15 of the 21 primary colorectal cancers analyzed revealed reduced or unchanged expression of DKK4 compared to the matched normal epithelium (Fig. 1E). This suggests that the β-catenin target gene DKK4 is negatively regulated in the majority of colorectal cancers and cancer cell lines irrespective of the mutational status of APC and β-catenin. Therefore, despite DKK4 being regulated by β-catenin/Tcf-4 on transcriptional level, in colorectal cancer cells DKK4 is frequently down-regulated, suggesting that cancer cells might select against the expression of DKK4 in order to gain growth advantage.

3.2. Dickkopf-4. is a potent inhibitor of β-catein/Tcf-4-dependent signalling activity

The colorectal cancer cells line HCT116 carries a mutated allele of β-catenin resulting in constitutively active Tcf-dependent signalling as determined by the TOPflash/FOPflash reporter gene assay (Fig. 2A). Exposure of these cells to the transforming Wnt-factor Wnt3a resulted in further dose-dependent increase in reporter gene activity

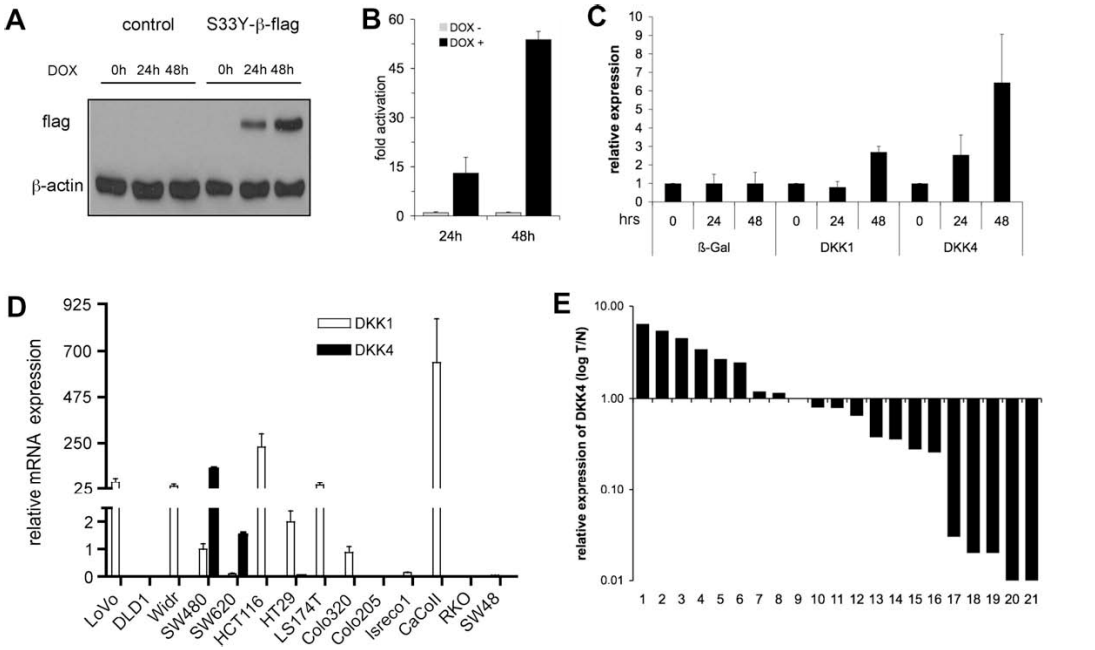

Fig. 1. Down-regulation of DKK4 expression in colorectal cancer cell lines and primary tumors. (A) Induction of flag-tagged S33Y-β-catenin in HEK293 Tet/S33Y β-cat cells after treatment of the cells with doxycycline. Western blot shows flag-tagged β-catenin and β-actin as loading control. (B) Expression of S33Y-β-catenin results in activation of Tcf-dependent transcription as determined by pTOPflash based luciferase reporter gene assay. Transfection efficiency was controlled for by co-transfection of a β-galactosidase expressing plasmid. (C) The activation of DKK1 and DKK4 mRNA expression after activation of S33Y-β-catenin expression was determined by quantitative RT-PCR. (D) The mRNA expression of DKK1 and DKK4 in colorectal cancer cell lines was determined by quantitative RT-PCR and compared to six independent samples of microdissected normal human colon epithelium. The ratios of tumor/normal mRNA levels are shown. (E) The mRNA expression of DKK4 was determined by quantitative RT-PCR in matched pairs of micro-dissected normal colorectal epithelium and colorectal cancers of the corresponding patients. The ratios tumor/normal are shown.

(Fig. 2A), demonstrating that Wnt/β-catenin signalling can be modulated on the membrane level also in the context of down-stream activating mutations. Co-incubation of these cells with Wnt3a and increasing amounts of DKK4 resulted in a strong suppression of Wnt3a-induced Tcf-dependent reporter activity (Fig. 2B), supporting DKK4's role as a negative regulator of Wnt-induced signalling. As all colorectal cancer cell lines endogenously express abundant levels of the transforming Wnt-ligands Wnt1, Wnt3, Wnt3a, and Wnt7 (Fig. 2C), we next tested whether DKK4 was also capable of inhibiting basal β-catenin/Tcf-dependent signalling in HCT116 cells, which we supposed is dependent on both extracellular Wnt-factors and mutation of β-catenin in HCT116 cells. This assumption was confirmed by exposure of HCT116 cells to DKK4, which resulted in a dose-dependent reduction of the activity of the TOPflash reporter by up to 60% (Fig. 2D).

3.3. Inhibition of cancer cell growth and cell cycle progression by Dickkopf-4

To analyze whether this potency of DKK4 to inhibit Wnt-factor induced Tcf-dependent signalling has functional consequences on tumor cell growth, HCT116 cells stably expressing DKK4 were plated in semi-solid agar medium. This revealed that DKK4 strongly inhibited colony formation reducing the colony number by more than 60% (Fig. 3A and B). To exclude a non-specific effect due to ectopic over-expression of DKK4, the parental HCT116 cells were plated in agar-medium and grown in the presence or absence of recombinant DKK4. This confirmed that extracellular DKK4 is a potent inhibitor of tumor cell growth (Fig. 3A and B). Fluorescence-activated cell sorting of HCT116 cells stably expressing DKK4 (Fig. 3C and D) or HCT116, DLD1, HT29, and LoVo cells exposed to recombinant DKK4 demonstrated that the growth inhibitory effect of DKK4 was attributable to G0/G1 arrest (Fig. 3E). This clearly demonstrates the anti-neoplastic function of DKK4 in colorectal cancer cells and provides a rationale for why cancer cells select against the expression of DKK4.

3.4. Epigenetic down-regulation of Dickkopf-4 expression

SW480 and SW620, which have been propagated from a primary tumor and a metastasis of the same patient, were the only colorectal cancer cell lines with detectable DKK4 expression. To test whether DKK4 expression was dependent on β-catenin, SW480 cells were transfected with siRNAs directed against β-catenin or a control siRNA. All gene-specific siRNAs led to silencing of β-catenin protein and mRNA expression by at least 90% and a strong reduction of TOPflash reporter activity (Fig. 4A and B and data not

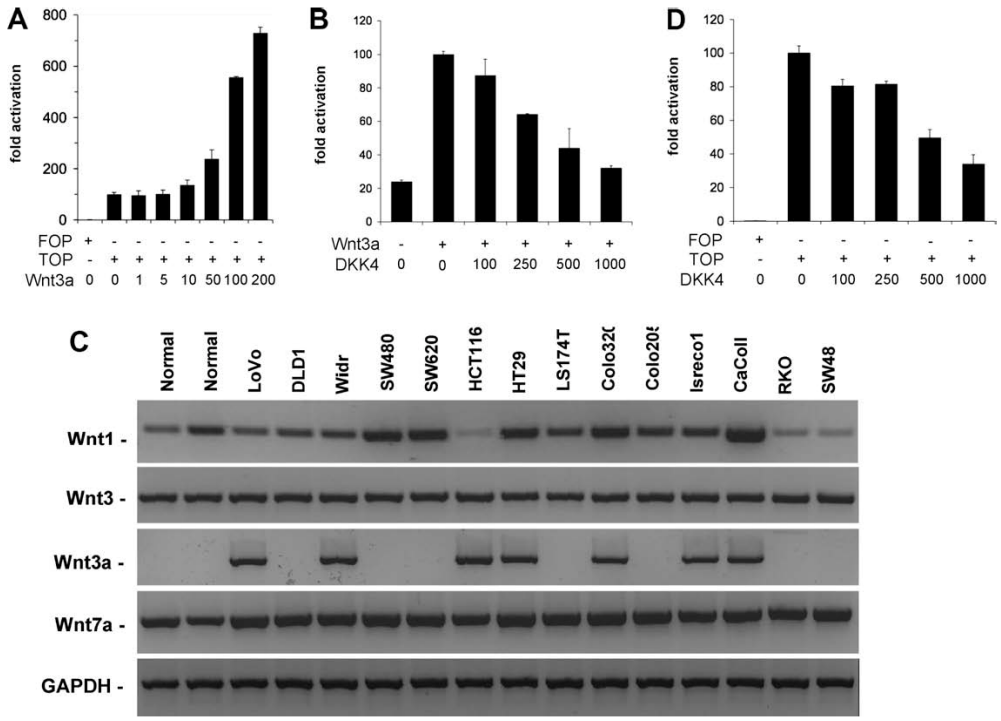

Fig. 2. Dickkopf-4 is a potent inhibitor of β-catein/Tcf-4-dependent signalling activity. (A and B) HCT116 colorectal cancer cells lines were transfected with pTOPflash and incubated with recombinant Wnt3a and/or recombinant DKK4 at concentrations as indicated and luciferase activity was determined after 24 h. The means and standard deviations of representative experiments performed in triplicates are shown. (C) The expression of Wnt1, Wnt3, Wnt3a, Wnt7a and GAPDH mRNA was analyzed by RT-PCR in microdissected normal human colonic epithelium and colorectal cancer cell lines. (D). Luciferase activity of the pTOPflash reporter was determined after treatment of HCT116 cells with increasing concentrations of DKK4. The means and standard deviations of an experiment performed in triplicates are shown.

shown). Silencing of β-catenin coincided with a strong inhibition of expression of DKK4 and the well-established target gene c-Myc (Fig. 4B). To confirm that this was mediated via the DKK4 promoter, a fragment of the DKK4 promoter containing eight putative Tcf-binding sites (5′-CTTTG(T/A)(T/A)-3′) was cloned into a luciferase reporter gene plasmid. Transfection of a dominant negative Tcf-4 allele, TCF4ΔN31, into SW480 cells led to inhibition of the Tcf consensus reporter construct TOPflash and dose-dependently reduced transcription from the DKK4 promoter (Fig. 4C). To test whether the loss of DKK4 expression in colorectal cancer cells was a consequence of a loss of transcriptional activators or expression of transcriptional repressors this experiment was also performed in HCT116 cells. This revealed that in a similar fashion Tcf-reporter activity as well as DKK4-promoter activity could be inhibited by dominant negative Tcf-4 in HCT116 (Fig. 4D).

Silencing of DKK1 expression in colorectal cancers has been found to be due to methylation of CpG islands of the promoter [10]. However, our analysis did not identify any relevant CpG islands in the DKK4 promoter and analysis of promoter methylation in DLD1 and HCT116 cells by bisulfite sequencing did not reveal any relevant methylation (data not shown). To test, whether modification of histone acetylation might contribute to the expression of DKK4 in tumors, DLD1, HCT116, HT29, LoVo and SW480 cells were treated with the histone deacetylase inhibitor trichostatine A. We found that DKK4 mRNA was strongly induced in DLD1, HCT116 and LoVo cells, which lack endogenous DKK4 expression, but expression was not further induced in HT29 and SW480 cells (Fig. 4E and data not shown). Chromatin immunoprecipitation of acetylated histones H3 K9/14 or H4 K12 demonstrated that treatment of DLD1 cells with TSA increased these activating histone modifications within the DKK4 promoter (Fig. 4F). Therefore, our data suggest that despite positive regulation by β-catenin/Tcf-4, DKK4 is negatively regulated in colorectal cancer cells on the epigenetic level.

4. Discussion

With the exception of DKK3, the three Dickkopf family members DKK1, DKK2, and DKK4 interact with LRP6 and Kremen-2 and have been described to be inhibitors of canonical Wnt signalling by inducing endocytosis of LRP-6 [5,15]. They contribute to tissue homeostasis by fine tuning of Wnt/β-catenin signalling as exemplified by studies on DKK1 [16,17]. However, only DKK1 and DKK4 suppress Wnt-induced secondary axis induction in Xenopus embryos [18] and a recent study suggested DKK4 to be in-

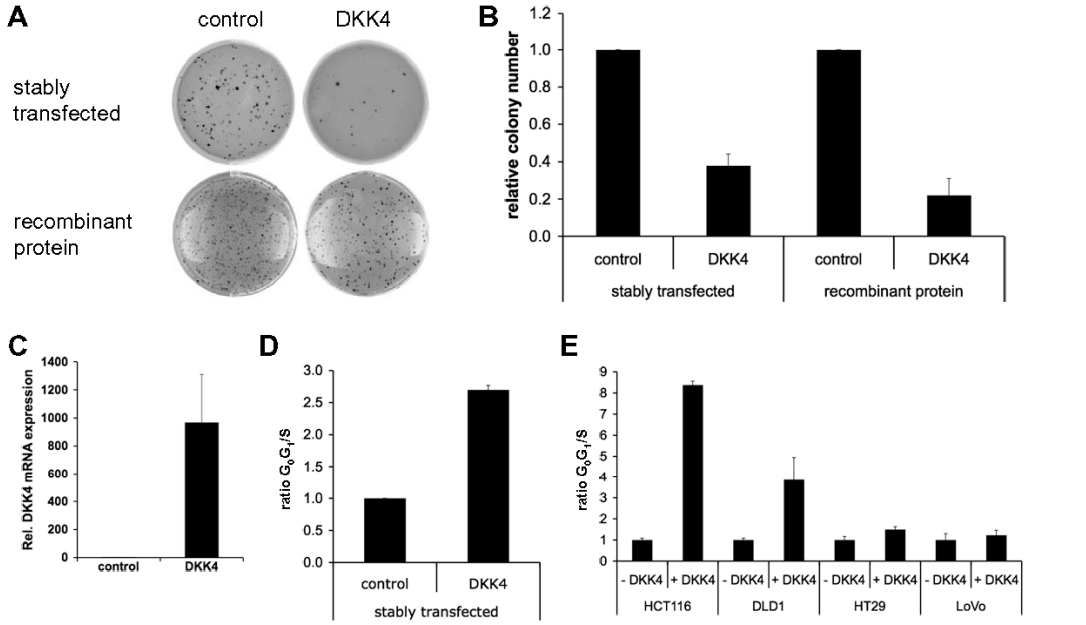

Fig. 3. Inhibition of cancer cell growth and cell cycle progression by Dickkopf-4. (A and B) DKK4 inhibits colony formation of HCT116 cells in semi-solid medium. HCT116 cells were either stably transfected with a plasmid encoding human DKK4 (top part) or treated with recombinant DKK4 protein (bottom part). Mean colony numbers and standard deviations of a representative experiment performed in triplicates are shown. (C) Expression of human DKK4 in stably with DKK4 transfected HCT116 cells. DKK4 mRNA expression was analyzed in stably transfected HCT116 cells using quantitative RT-PCR. The graph depicts the mean mRNA expression and standard deviation of a representative experiment performed in triplicates. (D) Effect of DKK4 on cell cycle distribution in stably with DKK4 transfected HCT116 cells. Cells were synchronized and cell cycle distribution was determined by flow cytometry. The graph depicts the ratio of cells in G_0G_1 versus S phase. The means and standard deviations of an experiment performed in triplicates are shown. (E) Effect of recombinant DKK4 on cell cycle distribution in colon carcinoma cell lines. Cells were treated with DKK4 or left untreated (−DKK4) and cell cycle distribution was determined by flow cytometry. The graph depicts the ratio of cells in G_0G_1 versus S phase. The means and standard deviations of an experiment performed in triplicate are shown.

duced by canonical Wnt signalling during ectodermal appendage morphogenesis in mice [19]. Like DKK1, DKK4 is also regulated by β-catenin/Tcf-4 on the transcriptional level [13,20,21]. As negative regulators of Wnt signalling, DKK1 and DKK4 are supposed to provide negative feed-back loops that have important roles in fine-tuning Wnt signalling in normal tissues.

It has previously been reported that DKK-1, -2, and -3 as well as members of the sFRP family are frequently down-regulated in colorectal cancer by promoter methylation [10–12,22]. Very recently, however, it was reported that DKK4 is up-regulated in primary colorectal cancers and promotes tumour cell invasion and angiogenesis [13]. In accordance with our data, this study reported high expression of DKK4 in SW480 cells, but no expression data on DKK4 were provided for other colorectal cancer cell lines. To exclude that our finding of frequent down-regulation of DKK4 in these cell lines was a secondary effect due to selection of cells in tissue culture, expression studies in primary colorectal cancers were performed. In contrast to Pendas-Franco et al., who reported up-regulation of DKK4 expression in 20 of 29 cancers analyzed, we only found DKK4 expression elevated over normal in 8 of 21 cases. This discrepancy might be explained by the different methods of tissue preparation, as only our study performed careful micro-dissection of colorectal cancers and colonic epithelium, thereby guaranteeing that no submucosa was dissected which might have different expression levels of DKK4 than the epithelium. Taken altogether, our data suggest that a relevant number of colorectal cancers select against the expression of DKK4.

sFRPs but not DKK1 can inhibit basal β-catenin/Tcf-dependent transcription in colorectal cancer cells with deregulated Tcf-dependent transcription [9,10,12]. However, here we present evidence that DKK4 is capable of inhibiting basal β-catenin/Tcf-dependent signalling in colorectal cancer cells carrying activating mutations in the Wnt/β-catenin signalling pathway. Moreover, as an inhibitor of Wnt signalling DKK4 is a negative regulator of cancer cell growth and cell cycle progression. This poses DKK4 as an important regulator of Wnt signalling and cell growth in cancer. Apart from this role as negative regulator of Wnt signalling, DKK4 has also been suggested to promote angiogenesis and invasion [13]. However, since the Wnt/β-catenin signalling pathway regulates DKK4 expression under physiological conditions, probably in order to fine-tune β-catenin activity, this DKK4-dependent negative feed-back loop provides an inhibitory signal that interferes with β-catenin mediated colorectal cancer cell growth. Therefore, loss of DKK4 expression by epigenetic inactivation of the DKK4 gene provides colon cancer cells with a growth advan-

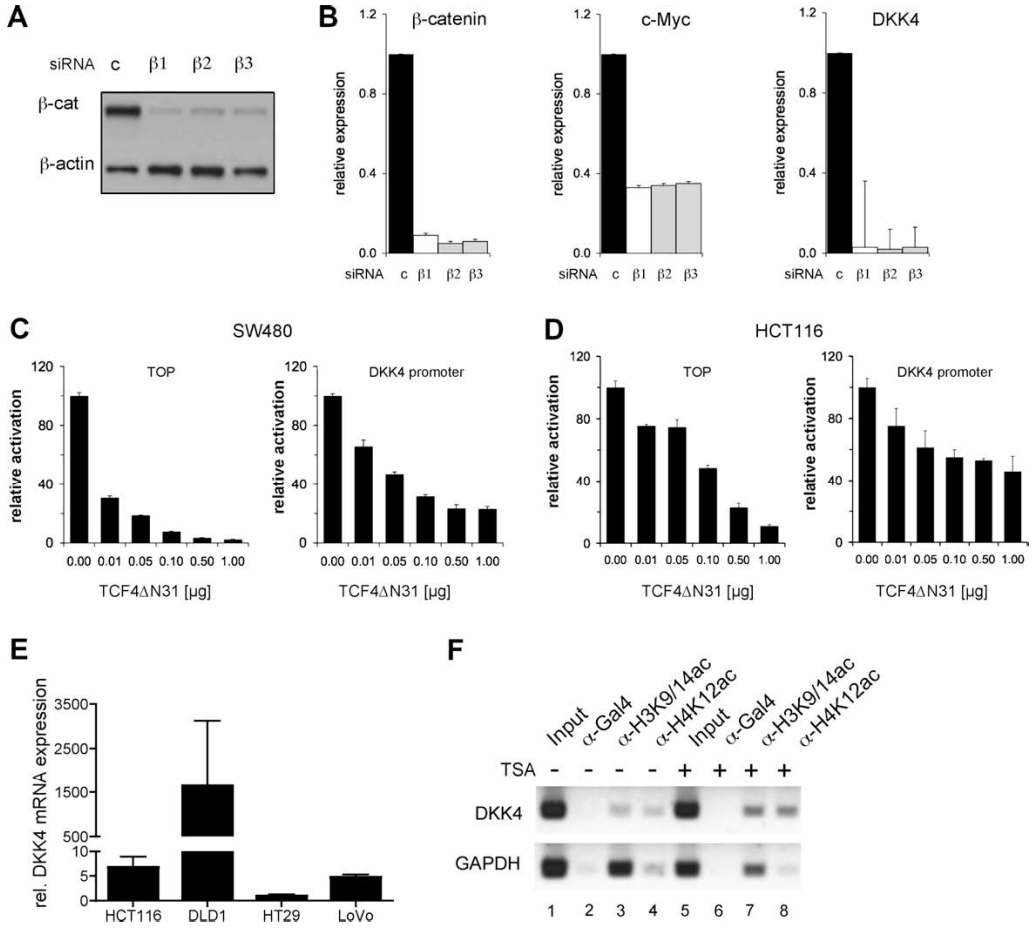

Fig. 4. Epigenetic down-regulation of Dickkopf-4 expression. (A and B) Transfection of different siRNAs resulted in silencing of β-catenin protein (A) and mRNA (B) expression in SW480 colorectal cancer cells (c: control siRNA; β1–β3: 3 siRNAs directed against β-catenin; β-actin: control for equal loading of lanes). Silencing of β-catenin expression resulted in down-regulation of the known β-catenin target genes c-Myc and DKK4. (C and D) SW480 and HCT116 colorectal cancer cells were transfected with increasing amounts of a plasmid expressing dominant negative Tcf-4 (TCF4ΔN31) and the luciferase reporter plasmids pTOPflash (TOP) or DKK4-promoter construct. Luciferase activity was measured and normalized to β-galactosidase activity. The means and standard deviations of a representative experiment performed in triplicate are shown. (E) Four colorectal cancer cell lines were treated with trichostatine A (TSA) for 24 h. Expression of DKK4 mRNA was determined by quantitative RT-PCR and normalized to untreated cells. The means and standard deviations of a representative experiment performed in triplicates are shown. (F) Re-expression of DKK4 after treatment of DLD1 cells with TSA correlates with activating histone modifications. DLD1 cells were treated with TSA ('+', lanes 5–8) or left untreated ('–', lanes 1–4) and total cell lysates were subjected to chromatin immunoprecipitation using antibodies recognizing acetylated histones H3 K9/14 (lanes 3/7) or H4 K12 (lanes 4/8). The α-Gal4 antibody was used as a negative control (lanes 2/6). Co-immunoprecipitated DNA was analyzed for the presence of DKK4 and GAPDH promoter sequences using PCR.

tage and offers an explanation why colon cancer cells select against the expression of DKK4.

Many cancer cells secret Wnt factors which have been classified according to their ability to transform the mouse mammary epithelial cell line C57MG [23,24]. We find that Wnt-1, Wnt-3, Wnt-3a, and Wnt-7a, which belong to the group of highly transforming Wnts and which are all thought to activate the canonical Wnt/β-catenin pathway, are abundantly expressed in colorectal cancer cell lines. This supports a view in which transforming Wnt factors have a significant role in the deregulation of β-catenin/Tcf-dependent transcription also in cells with mutations in down-stream components of the Wnt-pathway. This and the down-regulation of the expression of DKK4 and other antagonists and inhibitors of Wnt signalling provides colorectal cancer cells with further growth advantage due to deregulation of β-catenin/Tcf-4 mediated transcription in addition to mutation of β-catenin and APC.

In contrast to the promoters of DKK1, DKK2, and DKK3, which are targets for CpG-island methylation in colorectal cancers [10,11], the mechanism of down-regulation of DKK4 has so far been unclear. Our analyses and one recent report failed to identify relevant CpG islands within the regulatory regions of the DKK4 gene which could contribute to silencing of DKK4 expression [11]. Pendas-Franco et al. [13] reported that DKK4 expression could be repressed by 1α,25-dihydroxyvitamin D3 in SW480 cells. However, as colorectal cancer cells do not

express vitamine D3 and the culture medium does not contain relevant levels of this vitamine, this cannot be the mechanism of DKK4 down-regulation in the colorectal cancer cells. Our studies show that silencing of DKK4 expression is not likely due to loss of transcriptional activators or the presence of repressors of transcription, as transcription from a luciferase reporter gene driven by the DKK4 promoter is equally active in cultivated cells with high DKK4 expression and in those lacking endogenous DKK4 expression. We find that DKK4 expression can specifically be restored by treatment with a histone deacetylase inhibitor in cells lacking endogenous DKK4 expression, while this had no effect on DKK4 expression in DKK4 expressing SW480 cells. This demonstrates that all Dickkopf factors are frequently negatively regulated in colorectal cancers due to epigenetic mechanisms. However, the DKK4 gene is the only member of the Dickkopf family that is not regulated by DNA methylation but by histone modification.

In conclusion, our studies show that DKK4 is a potent inhibitor of Tcf-dependent signalling and growth in colorectal cancer cells. Loss of DKK4 expression which is facilitated by histone modification provides cancer cells with a growth advantage. Under physiological conditions DKK4 provides a negative feed-back loop for fine-tuning of Wnt-signalling. In colorectal cancer cells this loop is shut down resulting in a growth advantage of cancer cells. Therefore, loss of DKK4 expression and its function as a growth suppressor suggests that DKK4 is an important regulator of colorectal cancer cell growth.

Conflict of interest

The authors have no competing financial interests.

Acknowledgements

The authors thank Randall T. Moon for providing the 8xTOPflash reporter constructs. This work was supported by Grants from the Deutsche Forschungsgemeinschaft KO1826/3 to F.T.K. and GO417/4 to B.G.

References

[1] R.H. Giles, J.H. van Es, H. Clevers, Caught up in a Wnt storm: Wnt signaling in cancer, Biochim. Biophys. Acta 1653 (2003) 1–24.
[2] T. Reya, H. Clevers, Wnt signalling in stem cells and cancer, Nature 434 (2005) 843–850.
[3] H. Clevers, Wnt/beta-catenin signaling in development and disease, Cell 127 (2006) 469–480.
[4] Y. Kawano, R. Kypta, Secreted antagonists of the Wnt signalling pathway, J. Cell Sci. 116 (2003) 2627–2634.
[5] C. Niehrs, Function and biological roles of the Dickkopf family of Wnt modulators, Oncogene 25 (2006) 7469–7481.
[6] M. Ilyas, Wnt signalling and the mechanistic basis of tumour development, J. Pathol. 205 (2005) 130–144.
[7] P. Polakis, The many ways of Wnt in cancer, Curr. Opin. Genet. Dev. 17 (2007) 45–51.
[8] L.N. Spirio, W. Samowitz, J. Robertson, M. Robertson, R.W. Burt, M. Leppert, R. White, Alleles of APC modulate the frequency and classes of mutations that lead to colon polyps, Nat. Genet. 20 (1998) 385–388.
[9] A. Bafico, G. Liu, L. Goldin, V. Harris, S.A. Aaronson, An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells, Cancer Cell 6 (2004) 497–506.
[10] O. Aguilera, M.F. Fraga, E. Ballestar, M.F. Paz, M. Herranz, J. Espada, J.M. Garcia, A. Munoz, M. Esteller, J.M. Gonzalez-Sancho, Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer, Oncogene 25 (2006) 4116–4121.
[11] H. Sato, H. Suzuki, M. Toyota, M. Nojima, R. Maruyama, S. Sasaki, H. Takagi, Y. Sogabe, Y. Sasaki, M. Idogawa, T. Sonoda, M. Mori, K. Imai, T. Tokino, Y. Shinomura, Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors, Carcinogenesis 28 (2007) 2459–2466.
[12] H. Suzuki, D.N. Watkins, K.W. Jair, K.E. Schuebel, S.D. Markowitz, W.D. Chen, T.P. Pretlow, B. Yang, Y. Akiyama, M. Van Engeland, M. Toyota, T. Tokino, Y. Hinoda, K. Imai, J.G. Herman, S.B. Baylin, Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer, Nat. Genet. 36 (2004) 417–422.
[13] N. Pendas-Franco, J.M. Garcia, C. Pena, N. Valle, H.G. Palmer, M. Heinaniemi, C. Carlberg, B. Jimenez, F. Bonilla, A. Munoz, J.M. Gonzalez-Sancho, DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon, cancer promotes tumour cell invasion and angiogenesis and is repressed by 1alpha, 25-dihydroxyvitamin D3, Oncogene 27 (2008) 4467–4477.
[14] F.T. Kolligs, B. Kolligs, K.M. Hajra, G. Hu, M. Tani, K.R. Cho, E.R. Fearon, Gamma-catenin is regulated by the APC tumor suppressor and its oncogenic activity is distinct from that of beta-catenin, Genes Dev. 14 (2000) 1319–1331.
[15] B. Mao, C. Niehrs, Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling, Gene 302 (2003) 179–183.
[16] F. Kuhnert, C.R. Davis, H.T. Wang, P. Chu, M. Lee, J. Yuan, R. Nusse, C.J. Kuo, Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1, Proc. Natl. Acad. Sci. USA 101 (2004) 266–271.
[17] D. Pinto, A. Gregorieff, H. Begthel, H. Clevers, Canonical Wnt signals are essential for homeostasis of the intestinal epithelium, Genes Dev. 17 (2003) 1709–1713.
[18] V.E. Krupnik, J.D. Sharp, C. Jiang, K. Robison, T.W. Chickering, L. Amaravadi, D.E. Brown, D. Guyot, G. Mays, K. Leiby, B. Chang, T. Duong, A.D. Goodearl, D.P. Gearing, S.Y. Sokol, S.A. McCarthy, Functional and structural diversity of the human Dickkopf gene family, Gene 238 (1999) 301–313.
[19] H. Bazzi, K.A. Fantauzzo, G.D. Richardson, C.A. Jahoda, A.M. Christiano, The Wnt inhibitor, Dickkopf 4, is induced by canonical Wnt signaling during ectodermal appendage morphogenesis, Dev. Biol. 305 (2007) 498–507.
[20] J.M. Gonzalez-Sancho, O. Aguilera, J.M. Garcia, N. Pendas-Franco, C. Pena, S. Cal, A. Garcia de Herreros, F. Bonilla, A. Munoz, The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer, Oncogene 24 (2005) 1098–1103.
[21] A. Niida, T. Hiroko, M. Kasai, Y. Furukawa, Y. Nakamura, Y. Suzuki, S. Sugano, T. Akiyama, DKK1, a negative regulator of Wnt signaling is, a target of the beta-catenin/TCF pathway, Oncogene 23 (2004) 8520–8526.
[22] B. He, N. Reguart, L. You, J. Mazieres, Z. Xu, A.Y. Lee, I. Mikami, F. McCormick, D.M. Jablons, Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations, Oncogene 24 (2005) 3054–3058.
[23] M. Kuhl, L.C. Sheldahl, M. Park, J.R. Miller, R.T. Moon, The Wnt/Ca^{2+} pathway: a new vertebrate Wnt signaling pathway takes shape, Trends Genet. 16 (2000) 279–283.
[24] G.T. Wong, B.J. Gavin, A.P. McMahon, Differential transformation of mammary epithelial cells by Wnt genes, Mol. Cell Biol. 14 (1994) 6278–6286.

3.3 Der Krebsstammzellmarker CD133 hat eine hohe prognostische Aussagekraft aber unbekannte funktionelle Relevanz für die Metastasierung von humanem Dickdarmkrebs

Journal of Pathology
J Pathol 2009; **219**: 427–434
Published online 25 June 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/path.2597

Original Paper

The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer

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No conflicts of interest were declared.

Received: 17 December 2008
Revised: 29 May 2009
Accepted: 18 June 2009

Abstract

In colon cancer, CD133 has recently been used to enrich for a subset of tumour cells with tumour-initiating capabilities and was therefore suggested to mark colon cancer stem cells. However, this molecule has surprisingly been shown to lack functional importance for tumour initiation itself. Herein, we investigated whether CD133 may be relevant for colon cancer metastasis in patients, and as metastasis requires several additional biological characteristics besides tumour initiation, we examined the effects of knocking down CD133 expression in colon cancer cell lines on proliferation, migration, invasion, and colony formation. We demonstrate that high CD133 expression correlates strongly with synchronous liver metastasis in a matched case–control collection, while siRNA-mediated knock down of this factor has no significant effect on the mentioned biological characteristics. Thus, we conclude that CD133 expression is a marker with high prognostic impact for colon cancer, while it seems to have no obvious functional role as a driving force of this malignancy.

Keywords: CD133; Prominin-1; knock down; colon cancer; metastasis; invasion; migration; transformation; proliferation; prognosis

Introduction

In colon cancer, the cell surface marker CD133 has recently been used in several studies to enrich for a subset of tumour cells that have the ability to initiate tumour growth when xenografted into immunocompromised mice [1–4]. Furthermore, these cells were able to retain tumourigenicity *in vitro* as spheroid cultures and were resistant to chemotherapeutic drugs [2,3]. As CD133-negative colon cancer cells had none of these features, CD133 was suggested to mark tumour-initiating colon cancer stem cells (Co-CSCs). However, in one of the initial studies that used this marker for enrichment of tumour-initiating cells, it was estimated that only about 1 in 262 CD133-positive cells actually had such tumour-initiating capabilities [1], which suggests that CD133 is not highly specific for these cells. Moreover, in a recent study, knock down of CD133 did not compromise the tumour-initiating capabilities of colon cancer cells, questioning a functional role of this molecule for the Co-CSC phenotype [5]. Taken together, CD133 may roughly enrich for Co-CSCs but may lack functional relevance for their tumour-initiating capabilities.

Clinical relevance of Co-CSCs arises as these cells are thought to contribute substantially to tumour progression and metastasis in the patient [6–9] and in support of this hypothesis, we and others previously demonstrated that high CD133 expression, suggestive of large amounts of Co-CSCs in the tumours, correlated with poor patient survival [10,11]. As CD133 may lack a functional role for tumour initiation [5] but had an impact on survival, it is possible that CD133 has functional properties for the classical biological hallmarks of tumour progression, which include increased cell motility, invasiveness, colonization of distant organs, and growth of metastatic tumours [12–14].

We therefore investigated the impact of CD133 expression on colon cancer metastasis and tested the functional importance of this molecule for the mentioned biological characteristics of tumour progression. Using a matched case–control collection, we demonstrated that high CD133 expression correlated strongly with synchronous liver metastasis, while in

cultured colon cancer cell lines, knock down of CD133 expression did not affect cell proliferation, invasion, migration or colony formation. Thus, we conclude that CD133 has a high prognostic relevance for colon cancer progression and metastasis but it is unlikely to contribute functionally to the malignant phenotype of colon cancer cells.

Materials and Methods

Unless otherwise stated, methods were performed following the instructions given in the user's manuals.

Clinical samples and statistical analysis

CD133 expression was evaluated using formalin-fixed, paraffin-embedded (FFPE) colon cancer samples of patients who underwent surgical tumour resection at the Ludwig-Maximilians-Universität München between 1994 and 2005. Follow-up data were recorded by the Tumorregister München. Samples had not been previously evaluated for CD133 expression or considered for survival analyses [10]. The collection consisted of colon cancers with synchronous liver metastasis, where metastasis was diagnosed by clinical imaging, liver biopsy, or both. Controls had no distant metastasis at the time of diagnosis and disease-free survival of at least 5 years after primary resection. Controls and cases were matched on a one-to-one basis for tumour grade (according to WHO), T stage (according to UICC), and tumour localization (all tumours were located on the right side), resulting in 54 matched pairs. FFPE samples of corresponding liver metastases, available for 16 cases, were used for a direct comparative analysis. Frequency data were analysed using the χ^2-test. Statistical procedures were done using SPSS version 16.0 (SPSS Inc). $p < 0.05$ was considered statistically significant. The study was approved by the local ethics committee of the Medical Faculty of the Ludwig-Maximilians-Universität München.

Immunohistochemistry

Immunohistochemical staining was carried out on 5 µm sections of FFPE tumour samples. Staining was done on a Ventana Benchmark XT autostainer using anti-CD133 rabbit monoclonal antibody (clone C24B9; Cell Signaling Technologies, 1 : 100) together with XT UltraView DAB Kits (Ventana Medical Systems). Slides were counterstained with haematoxylin (Vector). System- and isotype-controls were included. CD133 staining intensity was categorized semi-quantitatively as CD133 low (0% to <50% CD133-positive glands) or CD133 high (≥50% CD133-positive tumour glands), evaluating five medium-power fields of each tumour tissue. Positivity was defined as membranous staining or staining of shed cellular debris in the tumour glands, as previously described [10]. Staining was evaluated independently by two observers (DH and JN), and their inter-observer agreement was calculated using the κ-statistics [15].

Cell lines, cell culture, and transfections

Caco-2 and LoVo cells (ATCC/LGL Promochem GmbH, Wesel, Germany) were maintained in DMEM with 7.5% (v/v) fetal bovine serum (FBS; Biochrom). Double-stranded siRNAs (50 nM) specific for *CD133* (Dharmacon, OnTarget Plus Smartpool) or *GFP* (AAGCUACCUGUUCCAUGGCCAdTdT) were transfected overnight in six-cluster well plates using 7.5 µl of Lipofectamine RNAiMax (Invitrogen). Twenty-four or 72 h later, cells were used in functional assays and for isolation of proteins and RNA. For determination of the knock-down duration, cell lysates were generated for up to 10 days post-transfection.

RNA isolation, reverse transcription, qRT-PCR

RNA was prepared employing RNeasy Kits (Qiagen). RNA was reverse-transcribed using a QantiTect Reverse Transcription Kit (Qiagen). For subsequent PCR, dilutions of RT reactions were incubated with either 5 µl of 2× iQ-SYBR-green (Biorad) for the detection of *β-Actin* (900 nM TTGCGGATGTCCACGTCA, 900 nM GCCCTGAGGCACTCTTCCA) or 5 µl of LightCycler 480 Probes Master and 100 nM universal probe human #83 for *CD133* (300 nM TCCACAGAAATTACCTACATTGG, 300 nM CAGCAGAGAGCAGATGACCA). Real-time PCR was done three times in duplicate using a LightCycler 480 (Roche Diagnostics). *CD133* expression was normalized on the corresponding values of the housekeeping gene *β-Actin*. Normalized values resulting from *CD133*-specific siRNA experiments were referred to those from *GFP*-specific knock-down experiments, giving rise to relative copy numbers.

Immunoblotting

Total protein was isolated using protein lysis buffer supplemented with PMSF (Maniatis, triple lysis buffer) and protease inhibitor (Complete, Sigma). Protein concentrations were determined using DC-protein reagent (BioRad). 20 µg of protein was used for gel electrophoresis. After blotting onto PVDF membranes (Millipore), anti-CD133 (clone C24B9; 1 : 1000) or anti-cleaved Caspase 3 (Asp175, Cell Signaling; 1 : 1000) and after stripping, anti-β-Actin-specific antibodies (Sigma, Ac15; 1 : 3000) were added. HRP-conjugated secondary antibodies (Pierce; 1 : 20 000) were used together with ECL or ECL Advance Western Blotting Detection Kits (Amersham) for the visualization of bands.

Functional assays

Assessment of proliferation, migration, and invasion was carried out three times each, 72 h after transfection with siRNA. For proliferation, the Cell Proliferation ELISA, BrdU Kit (Roche) was used. Triplicates of

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10^4 cells were seeded onto 96-well plates; BrdU was added 24 h later; and non-confluent cells were fixed and stained the next day. Relative BrdU incorporation, reflecting the number of labelled cells, was determined by colorimetric analysis. The presence of cleaved Caspase 3 was determined by immunoblotting. Migration was tested in wound-healing assays using Culture Inserts (ibidi). After cell adherence, Culture Inserts were removed and pictures of the remaining gaps were taken every 24 h. Migration distances were measured at three random points for each replicate and wound areas were assessed on photo-images using Photoshop's Lasso-Tool (Adobe). *In vitro* invasion was assessed by adding serum-starved cells to Matrigel-coated transwells as previously described [16]. Levels of invasion were determined after 5 (LoVo) or 7 days (Caco-2) by photographs and colorimetric determination of crystal violet staining. Anchorage-independent growth was assessed twice in triplicates by embedding 500 cells into culture medium containing 0.9% methylcellulose (Fluka, Deisenhofen, Germany) in 35 mm culture dishes (Greiner, Germany). After 10 days, colonies were counted microscopically with an inverted microscope (Zeiss).

Results

CD133 expression correlates strongly with liver metastasis of colon cancer

We investigated CD133 expression in a matched case–control collection of 54 pairs of colon cancers with and without synchronous liver metastasis. In normal mucosa adjacent to tumours, immunohistochemical staining for CD133 was always negative (Figure 1). CD133 expression in tumours was assessed as either low or high by two observers, giving an inter-observer agreement coefficient κ of 0.88. In the case–control collection, high CD133 expression was significantly associated with liver metastasis ($p = 0.0006$, OR = 5.0, Table 1). Among other clinical variables, only lymph node metastasis also correlated with CD133 expression, being only marginally significant ($p = 0.035$). Age, gender, tumour size, and histological grade were independent of CD133 expression levels. In 16 cases where tissue samples of both tumour sites were available, we investigated whether CD133 expression in primary colon cancers was comparable to expression in corresponding liver metastases. In 15 cases (94%), the expression

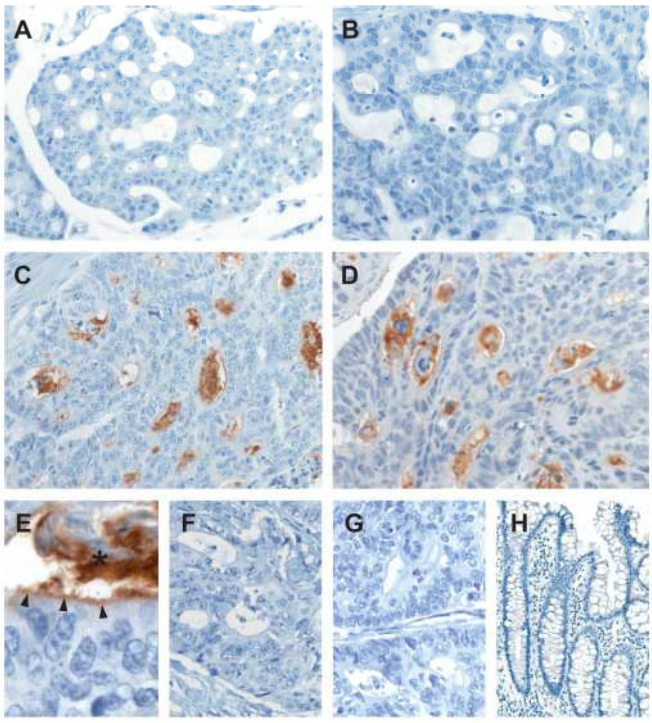

Figure 1. CD133 expression in colon cancer primary tumours and their liver metastases is usually similar. Primary tumours with low CD133 expression (A) usually form liver metastases which also display low CD133 expression (B). Accordingly, CD133 high primary colon cancers (C) form liver metastases with high CD133 expression (D). High-power magnification (E) of a CD133-positive tumour gland showing apical luminal staining of the tumour cells (arrowheads) and staining of intra-glandular debris (asterisk). Negative system control (F) and isotype control (G) of the same primary tumour shown for high CD133 expression. Normal mucosa (H) did not stain for CD133

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level in the primary tumour and metastasis was the same (Figure 1). In one case, the metastasis showed high CD133 expression, while the corresponding primary tumour was CD133 low. Taken together, CD133 expression correlates strongly with synchronous liver metastasis in colon cancer, while the expression levels in the primary and metastasis are usually similar.

CD133 siRNA knocks down CD133 expression in cultured colon cancer cell lines

To investigate the functional role of CD133 in colon cancer for proliferation and metastasis-associated hallmarks, we depleted CD133 in the cultured colon cancer cell lines Caco-2 and LoVo, which express high and moderate endogenous amounts of CD133, respectively (data not shown). Both cell lines were treated with a *CD133*-specific or — as a control — *GFP*-specific siRNA. *CD133* siRNA knocked down *CD133* mRNA levels by factors of 6.7 (Caco-2) and 4.8 (LoVo), respectively (Figure 2A). Comparable effects were observed on CD133 protein expression levels (Figure 2B), with no substantial recovery within 10 days (Supporting information, Supplementary Figure 1). Among the different experiments used for this study, CD133 baseline expression levels and knock-down efficiencies were comparable (Supporting information, Supplementary Figure 2). The selected *CD133* siRNA therefore worked properly, with long-lasting effects on CD133 protein levels.

Table 1. Clinicopathological variables and correlation with CD133 expression

		CD133 expression level		
Characteristic	**Total**	**Low**	**High**	***p***
All patients	108 (100)	78 (72.2)	30 (27.8)	
Age (median 66.5), years				
≤66	54 (50)	37 (34.3)	17 (15.7)	0.39
≥67	54 (50)	41 (38.0)	13 (12.0)	
Gender				
Male	57 (52.8)	39 (36.1)	18 (16.7)	0.35
Female	51 (47.2)	39 (36.1)	12 (11.1)	
Tumour size (UICC)				
T1	2 (1.9)	2 (1.9)	0 (0)	0.14
T2	10 (9.3)	10 (9.3)	0 (0)	
T3	80 (74.1)	56 (51.9)	24 (22.2)	
T4	16 (14.8)	10 (9.3)	6 (5.6)	
Nodal status				
N0	50 (46.3)	41 (38.0)	9 (8.3)	**0.035**
N+	58 (53.7)	37 (34.3)	21 (19.4)	
Metastasis (liver)				
M0	54 (50)	47 (43.5)	7 (6.5)	**0.0006**
M1	54 (50)	31 (28.7)	23 (21.3)	
Tumour grade (WHO)				
G2	42 (38.9)	27 (25.0)	15 (13.9)	0.20
G3	62 (57.4)	47 (43.5)	15 (13.9)	
G4	4 (3.7)	4 (3.7)	0 (0)	

Per cent values are given in parentheses.

CD133 knock down does not affect the proliferation of cultivated colon cancer cells

To assess cell proliferation, we compared the incorporation of BrdU-labelled thymidine between cells treated with *CD133* siRNA and those treated with control *GFP* siRNA. No significant differences in BrdU incorporation were observed in Caco-2 and LoVo cells (Figure 3). To exclude apoptosis as a confounding variable, we monitored the cleavage of Caspase 3 for each group over several days after knock down and observed no significant cleaved Caspase 3 in either cell line (Supporting information, Supplementary Figure 3). Thus, CD133 had no significant effect on the proliferation of Caco-2 and LoVo colon cancer cells.

CD133 expression is not linked to either migration or invasion of colon cancer cells

To investigate the effect of CD133 on migration, we performed wound-healing assays. For neither cell line, transfected with either *CD133*- or control *GFP*-siRNA, did we observe differences in the time required for closure of the gap or significant differences in migrated distances towards the centre of the gap (data not shown), or significant differences among the wound areas (Figure 4). We also analysed transwell migration and recorded no significant difference in the number

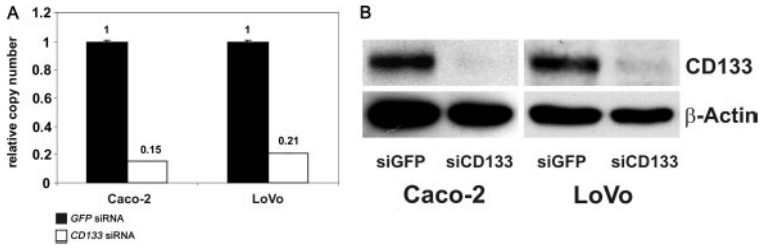

Figure 2. Specific siRNA-mediated knock down of CD133 in Caco-2 and LoVo colon cancer cells. Both cell lines were transiently transfected using siRNA specific for *CD133* (siCD133) or with siRNA against *GFP* (siGFP) as a control. Transfection significantly down-regulated CD133 expression on mRNA (A) and protein levels (B). Due to lower endogenous expression, a more sensitive detection system was used to illustrate CD133 protein levels of LoVo cells (B)

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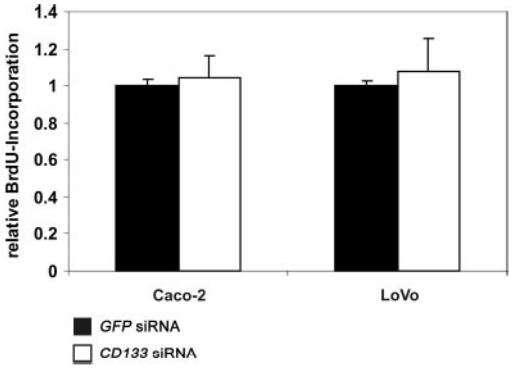

Figure 3. Knock down of CD133 in Caco-2 and LoVo cells has no influence on proliferation. No significant differences in BrdU incorporation were observed after *CD133* RNAi in either cell line

of migrated cells for either cell line (data not shown). Next, we investigated cell invasion, applying Matrigel-coated transwell assays. Although we observed a trend towards greater invasion of *CD133*-siRNA-treated cells, there was no significant difference between *CD133*- and *GFP*-siRNA-treated colon cancer cell lines (Figure 5). These results collectively suggest that CD133 knock down does not significantly affect migration or invasion of these cultured colon cancer cell lines.

CD133 knock down does not alter the colony formation capabilities of colon cancer cells

To test whether CD133 might be important for anchorage-independent colony formation, *CD133*- or *GFP*-siRNA-treated LoVo or Caco-2 cells were grown in methylcellulose and the formation of colonies was assessed. Both cell lines formed comparable numbers of colonies under these conditions and these numbers did not differ significantly between the *CD133*-siRNA and *GFP*-siRNA treatment groups (Figure 6). As the time window for the analysis was 10 days only, the colony growth had to be inspected microscopically. These results imply that CD133 does not significantly affect the colony formation of both colon cancer cell lines.

Discussion

In this study, we demonstrate that high CD133 expression in colon cancer correlates strongly with synchronous liver metastasis. This result is in support of the previous finding that high CD133 expression is a marker for low patient survival [10,11] in this tumour entity, when considering that the risk of colon cancer-specific death may be mainly attributable to metastasis formation [17,18]. CD133-positive tumours expressed this antigen on the luminal surface of glandular differentiated cancer cells and on cellular debris inside these glands. Although we have previously demonstrated that this staining pattern is specific and that staining of intra-glandular debris mirrors staining of surrounding tumour cells [10], this pattern creates some unease in enumerating CD133-positive cells. We therefore applied an empirically determined semi-quantitative score of 50% or more positive tumour glands, which previously correlated significantly with low patient survival [10]. As we found almost perfect inter-observer agreement using this scoring approach [15], we suggest that it is a robust and reproducible way to define CD133 expression status in colon cancers. We did not see CD133 staining in normal mucosa, which agrees with a previous study [19] but diverges from a more recent report [20]. This may be explained by the different sensitivity levels of CD133 detection; we seem to detect CD133 overexpression only within tumours. Regardless of expression in normal mucosa, we add further validity to the suggestion that high CD133 expression may be a reliable and clinically relevant marker to identify high-risk colon cancer cases.

CD133 gained attention as a marker to enrich for a subset of colon cancer cells with tumour-initiating capabilities [1–4]. Such cells were also termed colon cancer stem cells (Co-CSCs) and are assumed to promote progression and metastasis of

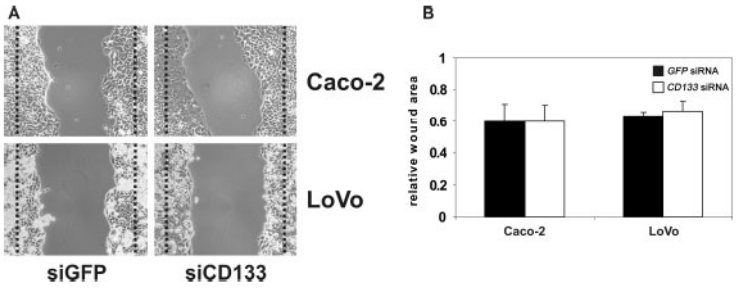

Figure 4. Wound-healing assays showed no altered migratory behaviour of Caco-2 or LoVo cells as a result of CD133 depletion. (A) No significant difference in the closure of gaps was observed for cells transfected with *CD133* (siCD133) or *GFP* (siGFP) control siRNA. Dashed lines indicate borders of gaps at the start of the experiment. (B) Wound areas from experimental triplicates are shown in relation to wound areas at the start of the experiment, demonstrating lack of significant differences in cell migration

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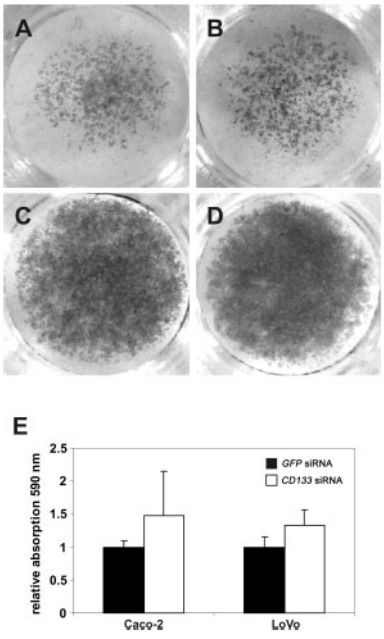

Figure 5. CD133 knock down does not inhibit the invasion of Caco-2 or LoVo cells. Cells treated with siRNA specific for *GFP* (A, C) or *CD133* (B, D) were tested for their ability to invade Matrigel-coated transwells. Membranes were stained with crystal violet. Comparable densities of invaded cells are exemplarily shown for Caco-2 (A, B) and LoVo (C, D) colon cancer cell lines. Additionally, membranes with invaded cells were dissolved in acetic acid and analysed colorimetrically for quantification of invasion (E). Trends towards a greater invasion of *CD133*-RNAi-treated cells were not significant. The data represent one triplicate of three independent experiments

the disease [6–9]. Under these presumptions, high CD133 expression in colon cancer may indicate a large amount of Co-CSCs in the tumour, with an increased risk that these Co-CSCs might disseminate from the primary and initiate tumour growth at the metastatic site. Our additional finding that CD133 expression in the primary tumour and its corresponding liver metastases is usually comparable suggests a similar ratio of Co-CSCs at both tumour sites, which is in further agreement with the cancer stem cell model [6,21]. Nevertheless, these presumptions should be taken with care until the controversy that questions the use of CD133 to enrich for Co-CSCs [20] is resolved.

The relevance of CD133 as a marker to enrich for Co-CSCs rests mainly on the potential to initiate tumour growth in mouse xenografts [1,2]. Colon cancer metastasis in the human patient, however, requires several additional biological characteristics, such as increased cell motility, invasiveness, and colonization of distant organs, followed by proliferation and growth at the new site [12–14]. Although CD133 has been shown to lack functional importance for tumour initiation [5], it may still contribute functionally to tumour progression. To clarify the role of CD133 for this process, we used a siRNA knock-down strategy in two cultured colon cancer cell lines expressing high and moderate levels of CD133, respectively. We achieved considerable knock down of CD133 on both mRNA and protein levels and showed that CD133 protein levels remained depleted for at least 10 days. Thus, proliferation, migration, invasion, and colony formation were evaluated within this time frame. We did not find a significant functional impact of the CD133 knock down on any of these cellular characteristics in either cell line. Together with the observation that CD133 knock down had no effect on the tumour-initiating properties of colon cancer cells [5], we therefore suggest that, at least in cultivated colon cancer cell lines, CD133 itself is unlikely to be essential for these specific biological cellular characteristics that are assumed to promote colon cancer progression and metastasis. Moreover, as CD133 is encoded by the *PROM1* gene [22,23], recent findings demonstrating the dispensability of *Prom1* in mice for normal development [24] even more generally question an important function of this factor.

CD133 was initially recognized as a marker for human haematopoietic stem cells using the monoclonal antibody AC133, which detected a glycosylated form of this protein and which later proved useful

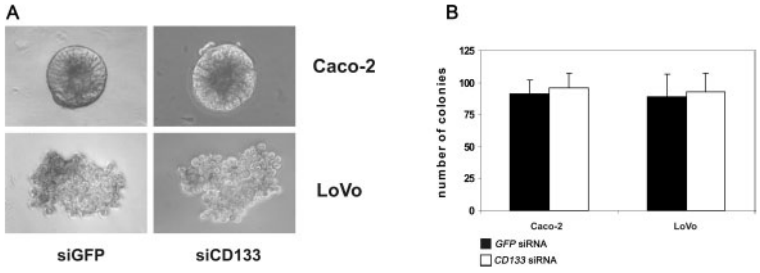

Figure 6. CD133 knock down does not compromise the colony formation of Caco-2 and LoVo colon cancer cells in methylcellulose assays. (A) For both cell lines, similar numbers of colonies of comparable size and appearance formed after RNAi-mediated knock down of CD133 (siCD133) or GFP (siGFP) as a control. Representative colonies are shown (original magnification ×100). (B) Colonies had to be microscopically counted due to the short growth interval of 10 days, showing no significant differences among the treatment groups for either cell line. The data represent one triplicate of two experiments

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for isolating tumour-initiating cells from solid neuronal malignancies [25,26]. The usage of CD133 for the prospective isolation of Co-CSCs was thus not based on any known role or expression in the normal colon. Nevertheless, when assuming that Co-CSCs may derive from normal colonic epithelial stem cells, recent results showing widely distributed expression of *Prom1* in the colon of mice [20,24] rather than expression more restricted to the stem cell and progenitor cell compartment — as suggested for the small intestinal epithelium [24,27] — are surprising. Whether in addition to the detection of glycosylation-dependent epitopes in Co-CSC-related studies, splice variants of CD133 [23] contribute to such alleged discrepancies still remains to be determined.

In humans, the expression of two CD133 splice variants, CD133.s1 and CD133.s2, has been characterized in several tissues: CD133.s2 is mainly found in neuronal structures, whereas the predominant form in abdominal organs and various cancer cell lines, including colon cancer, is CD133.s1 [28,29], suggesting that this isoform is the one relevant to colon cancer. In our study, however, we used an antibody (C24B9) generated against a peptide corresponding to a region surrounding Asp562 of human CD133 [30], encoded by exon 15 of the *PROM1* gene. We previously demonstrated that both the C24B9 and the AC133 antibody detect the same sized protein and give the same staining pattern in colon cancer tissue [10], suggesting detection of the same glycosylated CD133 variant. Nonetheless, the exon encoding the antigen detected in the present study is invariable among known human splice variants of CD133 [23], so that more than one variant may have been detected. Which CD133 variants are expressed in colon cancer and whether they are distinct in their prognostic relevance for this malignancy thus also remain to be established.

In conclusion, we suggest that CD133 is a valuable prognostic marker for assessing the risk of disease progression, metastasis, and death of patients with colon cancer. However, this molecule seems unlikely to contribute directly to the aggressiveness of the disease, as colon cancer cell lines do not seem to depend on CD133 expression in cellular assays related to metastasis. It is therefore critical to distinguish between CD133 as a molecular marker and its functional role, which are independent variables, and the latter of which in colon cancer still remains elusive.

Acknowledgements

We thank G Janssen, A Sucic, S Pfeiffer, and S Sagebiel-Kohler for their experimental assistance; Professor Dr J Engel for providing clinical information on cases; and Professor Dr R Shivdasani for critical reading of the manuscript. This study was supported in part by the Friedrich-Baur-Stiftung, Germany (grant No 0015/2007, to DH), and the Wilhelm Sander-Stiftung, Germany (grant No 2004-111.2, to AJ and TK).

References

1. O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007;**445:**106–110.
2. Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, *et al.* Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007;**445:**111–115.
3. Todaro M, Alea MP, Di Stefano AB, Cammareri P, Vermeulen L, Iovino F, *et al.* Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. *Cell Stem Cell* 2007;**1:**389–402.
4. Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, *et al.* Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci U S A* 2008;**105:**13427–13432.
5. Du L, Wang H, He L, Zhang J, Ni B, Wang X, *et al.* CD44 is of functional importance for colorectal cancer stem cells. *Clin Cancer Res* 2008;**14:**6751–6760.
6. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells — an integrated concept of malignant tumour progression. *Nature Rev Cancer* 2005;**5:**744–749.
7. Burkert J, Wright NA, Alison MR. Stem cells and cancer: an intimate relationship. *J Pathol* 2006;**209:**287–297.
8. Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, *et al.* Cancer stem cells — perspectives on current status and future directions: AACR Workshop on Cancer Stem Cells. *Cancer Res* 2006;**66:**9339–9344.
9. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. *Nature* 2001;**414:**105–111.
10. Horst D, Kriegl L, Engel J, Kirchner T, Jung A. CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br J Cancer* 2008;**99:**1285–1289.
11. Kojima M, Ishii G, Atsumi N, Fujii S, Saito N, Ochiai A. Immunohistochemical detection of CD133 expression in colorectal cancer: a clinicopathological study. *Cancer Sci* 2008;**99:**1578–1583.
12. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006;**127:**679–695.
13. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;**100:**57–70.
14. Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. *Cancer* 1997;**80:**1529–1537.
15. Viera AJ, Garrett JM. Understanding inter-observer agreement: the kappa statistic. *Fam Med* 2005;**37:**360–363.
16. Fang D, Hawke D, Zheng Y, Xia Y, Meisenhelder J, Nika H, *et al.* Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. *J Biol Chem* 2007;**282:**11221–11229.
17. O'Connell JB, Maggard MA, Ko CY. Colon cancer survival rates with the new American Joint Committee on Cancer sixth edition staging. *J Natl Cancer Inst* 2004;**96:**1420–1425.
18. Stangl R, Altendorf-Hofmann A, Charnley RM, Scheele J. Factors influencing the natural history of colorectal liver metastases. *Lancet* 1994;**343:**1405–1410.
19. Miraglia S, Godfrey W, Yin AH, Atkins K, Warnke R, Holden JT, *et al.* A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* 1997;**90:**5013–5021.
20. Shmelkov SV, Butler JM, Hooper AT, Hormigo A, Kushner J, Milde T, *et al.* CD133 expression is not restricted to stem cells, and both CD133+ and CD133− metastatic colon cancer cells initiate tumors. *J Clin Invest* 2008;**118:**2111–2120.
21. Ricci-Vitiani L, Pagliuca A, Palio E, Zeuner A, De Maria R. Colon cancer stem cells. *Gut* 2008;**57:**538–548.
22. Corbeil D, Roper K, Hellwig A, Tavian M, Miraglia S, Watt SM, *et al.* The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions. *J Biol Chem* 2000;**275:**5512–5520.
23. Fargeas CA, Huttner WB, Corbeil D. Nomenclature of prominin-1 (CD133) splice variants — an update. *Tissue Antigens* 2007;**69:**602–606.

24. Zhu L, Gibson P, Currle DS, Tong Y, Richardson RJ, Bayazitov IT, *et al*. Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. *Nature* 2009;**457:**603–607.
25. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, *et al*. Identification of human brain tumour initiating cells. *Nature* 2004;**432:**396–401.
26. Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, *et al*. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* 1997;**90:**5002–5012.
27. Snippert HJ, van Es JH, van den Born M, Begthel H, Stange DE, Barker N, *et al*. Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. *Gastroenterology* 2009;**136:**2187–2194.e1.
28. Fargeas CA, Corbeil D, Huttner WB. AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in need of a rational nomenclature. *Stem Cells* 2003;**21:**506–508.
29. Yu Y, Flint A, Dvorin EL, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. *J Biol Chem* 2002;**277:**20711–20716.
30. Cell Signaling Technology Inc. CD133 (C24B9) rabbit mAb #3663. http://www.cellsignal.com/products/3663.html [accessed 1 December 2008].

SUPPORTING INFORMATION ON THE INTERNET

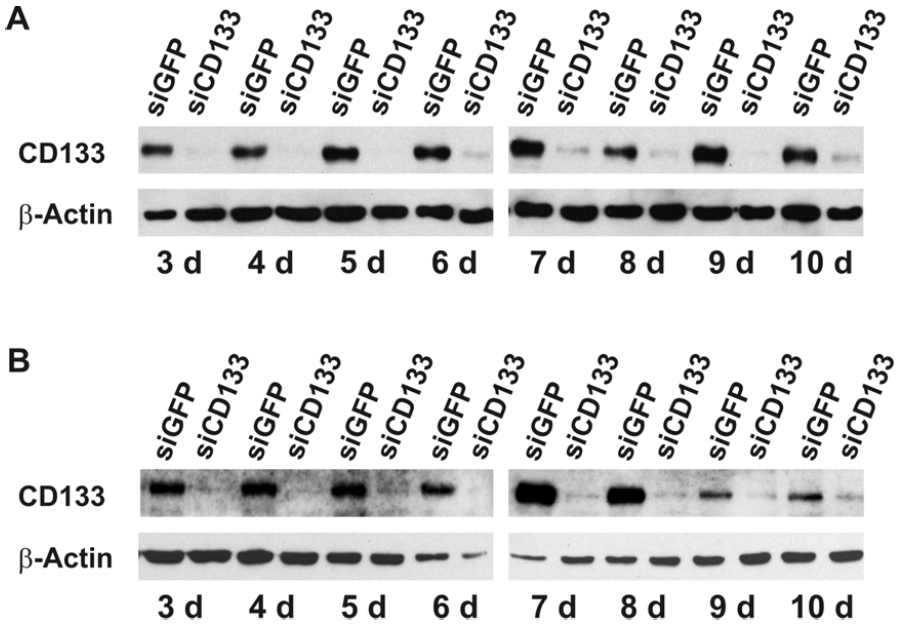
The following supporting information may be found in the online version of this article.

Figure S1. CD133 knock down is effective for at least 10 days in Caco-2 and LoVo cells.

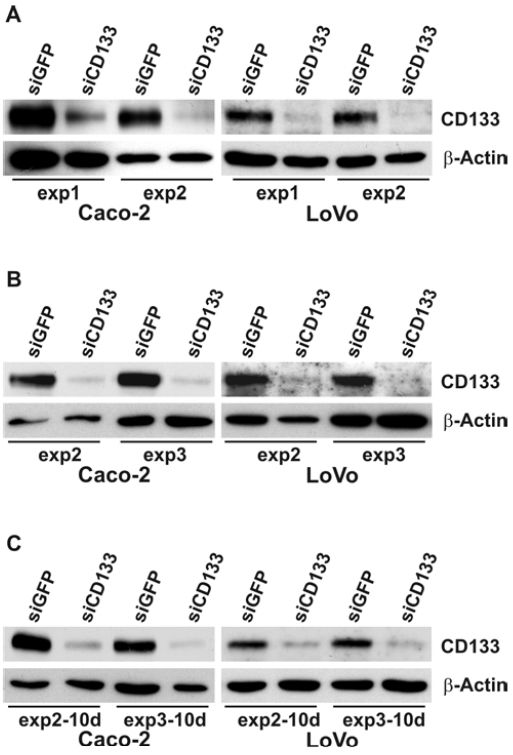
Figure S2. Proper function of siRNA-mediated knock down in cells used for the different experiments.

Figure S3. Knock down of CD133 does not induce apoptosis in Caco-2 or LoVo cells.

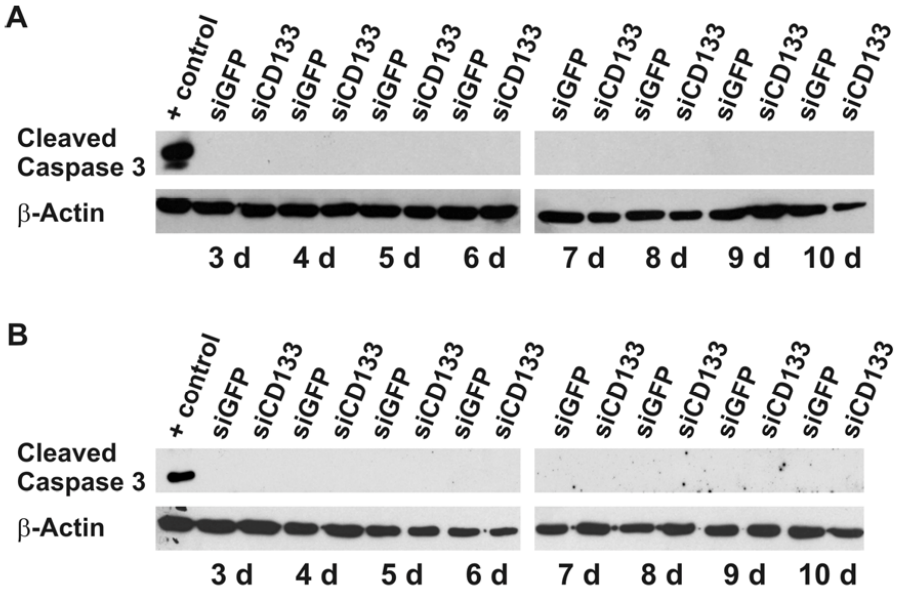
J Pathol 2009; **219**: 427–434 DOI: 10.1002/path



Supplementary Figure 1. CD133 knock down is effective for at least 10 days in Caco-2 and LoVo cells. To exclude quick recovery of CD133 protein levels after knock down, cell lysates of *CD133*- (siCD133) or *GFP*-siRNA (siGFP) transfected cells were obtained for up to 10 days after transfection. No significant CD133 protein levels were regained within this time frame in *CD133* siRNA-treated Caco-2 (A) and LoVo (B) cells



Supplementary Figure 2. Proper function of siRNA-mediated knock down in cells used for the different experiments. (A) Comparing cells that were used for duration of knock-down assessment (exp2) with those used for migration, invasion, and proliferation assays (exp1), and (B) with those used for colony formation experiments (exp3) demonstrate similar CD133 expression levels under control *GFP*-siRNA treatment (siGFP) and similar knock-down efficiencies under *CD133*-siRNA treatment (siCD133). Lysates were obtained at the starting point of each assay (72 h after knock down for exp1 and exp2; 24 h after knock down for exp3). (C) After 10 days, CD133 still remained similarly depleted in cells used for colony formation assessment (exp3-10d) and in cells used for duration of knock-down assessment (exp2-10d)



Supplementary Figure 3. Knock down of CD133 does not induce apoptosis in Caco-2 or LoVo cells. To exclude apoptosis as a confounder for the investigated cellular properties, cleaved Caspase 3 levels were evaluated in *CD133*- (siCD133) and *GFP*-siRNA (siGFP) treated cells for up to 10 days after transfection. No significant apoptosis was seen, either in Caco-2 cells (A) or in LoVo cells (B). Caspase-3 control cell extracts (Cell Signaling) were used as a positive control

3.4 Die humane TERT (Telomerase RT-Komponente) ist in kolorektalen Tumoren ein Zielgen von β-CATENIN

The human *TERT* (*telomerase RT-component*) is a β-catenin target gene in colorectal cancer

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Abstract

Background

A characteristic of most colorectal cancers is the dysregulation of the canonical WNT-pathway that leads to the stabilization and thus cellular increase of β-catenin. When β-catenin is found at the membrane as part of the zonula adhaerens the epithelial phenotype is enhanced. When β-catenin is expressed in the nucleus it works as a transcription factor. In this context β-catenin transactivates genes conferring the hallmarks of cancers like proliferation, anti-apoptosis, invasion and migration, or angiogenesis. Additionally, β-catenin imposes a progenitor phenotype on colorectal cells which is in support with β-catenin's ability to induce an EMT (epithelio-mesenchymal transition) because EMT is associated with stemness in tumor cells. Finally, β-catenin regulates the transcription of the cancer stem cell markers CD44 and CD166. Thus, β-catenin seems to be centrally involved in the maintenance of stemness in colorectal tumor cells. Therefore, we investigated if β-catenin might also be involved in the transcriptional regulation of the functional stem cell marker TERT (telomerase RT-component).

Results

We demonstrate that the human TERT (hTERT) is expressed in tumor cells where β-catenin is localized in the nucleus. Recombinant TCF-4 bound to the promoter/enhancer of the hTERT gene *in vitro* and β-catenin *in vivo*. β-catenin functionally regulated the transcriptional activity of the hTERT promoter/enhancer in luciferase gene reporter assays. The downregulation of β-catenin resulted in a loss of the expression of hTERT.

Conclusions

Our findings add another piece of evidence that β-catenin is involved in the regulation of stemness in colorectal tumor cells. Additionally, β-catenin is involved in the regulation of the cancer hallmark eternal life which underscores the important role of β-catenin in the process of colorectal carcinogenesis and might help to understand

why the WNT/β-catenin signaling pathway is mutated in so many tumors. Next, our data might help to explain why telomerase activity is found to be upregulated in colorectal cancers albeit the telomeres are shorter than in normal tissue as β-catenin is found to be expressed in only a small population of tumor cells. Finally, hTERT might indicate the colorectal cancer stem cells compartment in human tissue.

Background

Most colorectal carcinomas (CRCs) are characterized by a dysregulation of the canonical WNT-pathway due to mutations of its components *adenomatous polyposis coli* (*APC*), *β-catenin* or *axin*. As a consequence β-catenin accumulates in tumor cells [1] which has an ambivalent consequence [2]: on the one hand β-catenin is an integral part of the zonula adhaerens by linking E-cadherin and α-catenin, thus stabilizing the epithelial phenotype. In this context β-catenin works a tumorsuppressor. On the other hand, β-catenin induces gene expression in conjunction with DNA binding factors of the T-cell factor (TCF) / lymphocyte enhancing factor (LEF-1) family [1], if such dimers are present in the nucleus [3]. The β-catenin target gene products drive colorectal carcinogenesis by regulating proliferation, survival, angiogenesis, invasion and migration which represent hallmarks of cancer [4, 5]. Moreover, nuclear β-catenin induces EMT (epithelio-mesenchymal transition) [6] which is highly associated with stemness of tumor cells [7, 8]. Next, β-catenin/TCF-4 activity is essential for maintaining the regenerative capacity of colonic crypts [9] and is driving the progenitor phenotype of colorectal tumor cells [10]. Finally, β-catenin/TCF-4 activate the expression of the colorectal tumor stem cell markers *CD44* [11] and *CD166* [12]. In this context β- catenin works as an oncogene.

Another marker and vital component of stem cells is TERT (telomerase RT-component) [13] which is an essential component of the telomerase, an enzymatic complex that is important for the long term survival of cells [14]. This view is supported by the fact that TERT is not associated with proliferation in CRCs as the patterns of TERT expression and proliferation differ from each other [15].

Interestingly in human CRCs, the expression patterns of TERT [15] and nuclear β-catenin which also correlates with low proliferation [16] seem to be overlapping. Thus, we argued that *TERT* might be another target gene of β-catenin. Here we show that a small fraction of CRC cells at the invasive front expressed nuclear β-catenin and TERT at the same time. The promoter/enhancer of the human *TERT* (*hTERT*)-gene harbors four TCF-4 binding elements (TBEs) which conferred β-catenin and

TCF-4 binding as well as transcriptional activation of the *hTERT* promoter/enhancer. Finally, RNA interference (RNAi) of the expression of β-catenin led to the loss of hTERT-expression. Thus, *hTERT* seems to be another direct target gene of β-catenin which is in support that β-catenin is an active player in the biology of immortalization and stemness.

Results and Discussion

hTERT and β-catenin are found coexpressed in human colorectal cancers

CRC cells at the invasive front are characterized very often by the nuclear expression of β-catenin [3, 17] and expression of hTERT [15]. Thus, we investigated if nuclear β-catenin and hTERT displayed overlapping expression patterns using serial sections of a selected collection of 24 cases of well differentiated CRCs with well developed invasive fronts which displayed strong nuclear expression of β-catenin. As expected, it turned out that expression of hTERT (Figure 1 A) was found in tumor cells expressing nuclear β- catenin (Figure 1 B). In contrast, tumor cells lacking hTERT expression (Figure 1 C) also did not display nuclear β-catenin (Figure 1 D) expression. This correlation suggested that β-catenin might be involved in the transcriptional regulation of the *hTERT* gene.

The hTERT promoter/enhancer harbours TCF-4 binding elements (TBE) that bind TCF-4 and β-catenin

Next, we looked if the promoter/enhancer region of the *hTERT* gene [GenBank: AB016767] harbored consensus TCF-4 binding elements (TBEs, WWCAAAG) [12] as a prerequisite for the transcriptional regulation by β-catenin /TCF-4. Four TBEs were found at positions -1,627 (TBE1), -2,149 (TBE2), -2,488 (TBE3), and -2,974 (TBE4) upstream of the transcription start (+1). They are flanked by binding elements for other transcription factors like AP2 (activation protein 2), MAD (MAX dimerization protein), cMYC, MZF2 (myeloid zinc finger protein 2), SP1 (specificity protein 1) or

WT1 (Wilms tumor 1 gene) (Figure 2 A) [18]. Therefore, we tested if TCF-4 or β-catenin specifically interacted with TBEs of the *hTERT* promoter/enhancer. In a first approach the specific binding of the DNA binding domain (DBD) of a recombinant GST/TCF-4 (glutathione-S-transferase) fusion protein to a radioactively end-labeled DNA probe containing TBE3 of the *hTERT* gene (Table 1) was investigated applying electric mobility shift assays (EMSAs). GST/TCF-4 bound the TBE2 of the *hTERT* promoter/enhancer (Figure 2 B, lane 1). This binding was competed by adding unlabeled TBE4 (Figure 2 B TBE3, TBE4) or TBE3 containing fragments (Figure 2 B lane 4) of the *hTERT*- or the second TBE of the *cMYC* promoter/enhancers (MYC, Figure 2 B, MYC) but not when adding mutant variants of the TBE4 or TBE3 of the *hTERT* promoter/enhancer (Figure 2 B, MUT3, MUT4). Moreover as a control, GST alone did not bind to the TBE of the *hTERT* promoter/enhancer (Figure 2 B GST). In a second approach, chromatin immune-precipitations (ChIPs) were done to prove if β-catenin directly interacts with the region of the *hTERT* promoter/enhancer containing the TBEs in the context of native chromatin. Therefore, chromatin of DLD-1 cells was prepared for ChIP experiments using two different antibodies specific for β-catenin (Figure 2 C, β-CATI, β-CATII) or transcription factor II B (TFIIB), the TATA-box binding component of the RNA-polymerase II holo-enzyme as well as immunoglobulins as controls (Figure 2 C, TFIIB, IgG). Binding of β-catenin was analyzed by PCRs using primer-pairs that bind to the genomic region of the TBEs containing regions of both the *hTERT*- and *cMYC*-promoter/enhancers (Figure 2 C, hTERT, cMYC). To exclude unspecific binding of chromatin to the antibodies a region of the *GAPDH*-promoter/enhancer covering the TATA-box without a TBE was used as a control (Figure 2 C, GAPDH). Moreover, unspecific binding of DNA to immunoglobulins per se was excluded by the IgG control. To exclude that the background amounts of PCR products received with chromatin precipitated with β-catenin specific antibodies (β-CATI, β-CATII) applying the *GAPDH* specific PCR were only due to technical reasons TFIIB specific ChIPs were done (Figure 2 C, TFIIB). Here, a clear signal was received indicating that this system worked well and that the low signals obtained with the β-catenin specific antibodies were due to missing binding. Both experiments demonstrated that the TBEs but at least TBE3 of the *hTERT* promoter/enhancer interacted specifically with β-catenin/TCF-4. Moreover,

our data are in support with results from a genome-wide TCF-4 specific ChIP experiment. Here, TCF-4 was shown to interact with the *hTERT* promoter/enhancer [12].

β-catenin transcriptionally regulates the expression of the hTERT gene

Now, we wanted to know if β-catenin/TCF-4 exerted a role in the transcriptional regulation of the *hTERT* gene. Therefore, transient luciferase-reporter gene transfection assays were done first of all. A fragment of the *hTERT* promoter/enhancer (–3,328/+19) driving the firefly luciferase gene [18] (Figure 2 D) was transiently transfected together with increasing amounts of an expression plasmid encoding a dominant negative form of the TCF-4 gene (dnTCF-4) which is an inhibitor of β-catenin /TCF-4 transcriptional activity [19] into cultured colorectal SW480 cells. dnTCF-4 suppressed the activity of the *hTERT*-promoter/enhancer luciferase reporter gene construct in a dose-dependent manner to residual amounts of about 40 % (Figure 2 E) thus indicating a role of β-catenin/TCF-4 in the transcriptional regulation of the *hTERT* gene. Second, β-catenin expression was knocked down in cultured colorectal SW480 and DLD-1 cells using siRNAs specific for *β-catenin* [20] or *cdc2* as a control (Figure 3 B, β-catenin: ■, cdc2: □). As *cMYC* is a well known target gene of β- catenin [19] and is also known to regulate the *hTERT* gene transcription [18] conditions had to be selected to exclude effects of cMYC on the transcriptional regulation of the *hTERT* gene. Therefore, siRNA transfected cells were harvested at different timepoints (2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 12 h) after transfection and investigated for the expression of *β-catenin* and *cMYC* on the levels of mRNA using qRT-PCR and protein employing Western-Blotting. Thus, situations were selected where β-catenin expression was reduced as well on the protein- (Figure 3 A) as on the mRNA-level (Figure 3 B, C) but the expression of *cMYC* only on the mRNA- (Figure 3 B, C) but not yet on the protein level (Figure 3 A). This time point described a situation in which β-catenin but not cMYC should exert transcriptional activity. Under these conditions the *hTERT* mRNA levels were reduced (Figure 3 B, C) thus indicating a direct effect of β-catenin on the transcriptional regulation of the *hTERT* gene.

Conclusions

Our finding that β-catenin regulates the expression of the *hTERT*-gene indicates that β-catenin is also involved in the hallmark eternal life [5] and thus affects all hallmarks of carcinogenesis[4]. In this sense, the WNT/β-catenin signal pathway is a master switch of carcinogenesis and this might explain why this pathway is frequently deregulated in CRCs. On the other hand the question arises why additional mutations are then needed in the process of colorectal carcinogenesis.

Additionally, our data manifest that β-catenin is involved in the regulation of stemness of colorectal cancer stem cells (CSCs) as CSC-markers like *CD44* [11] or *CD166* [12] and *hTERT* are all transcriptionally regulated by β-catenin. Thus, colorectal CSCs might be defined as the tiny amount of hTERT expressing cells at the invasive front which had already been proposed [7]. These cells are characterized by low proliferation which correlates with the expression of the β-catenin target gene $p16^{INK4a}$ [16, 17] and EMT indicated by the loss of the expression of E-cadherin and gain of expression of vimentin and fibronectin [3]. Moreover, the number of these cells correlates with low survival [21] as does high TERT activity [22]. Yet, the distribution of cells expressing CD133 which is a well accepted maker of CSCs [23, 24] differs completely [25]. Thus, the localization of colorectal CSCs in the histological context still remains unsolved and needs more investigation.

Finally, our data might shed light on the conundrum that the length of the telomeres in colorectal tumor cells is shorter than their counterparts in normal cells [26] although the TERT activity of the tumor cells was found to be higher [22]. This might be explained by the observed fact that hTERT expression is found only in some tumor cells. Thus, one should expect that the tumor cells at the invasive front should be characterized by longer telomeres.

Methods

Immunohistochemistry

Immunohistochemical staining of serial sections was done for 24 well differentiated colorectal adenocarcinomas as described [17]. Briefly, 5 μm thick sections were subjected to antigen retrieval using citrate buffer (DAKO) employing microwave treatment. Antibodies specific for hTERT (1:100 - PC563T; Calbiochem) or β-catenin (1:750 - C2206; Sigma) were applied over night at 4°C. After washing, immunohistochemistry was completed using Envision AP (DAKO) or Envision HRP (DAKO) according to the user's instructions followed by counterstaining using methyleneblue. Pictures were done employing the AnalySis photo system (AnalySis).

Electric Mobility Shift Assay (EMSA)

Unlabeled competitor, or water in the case of controls, were incubated with 1 μl of crudely purified recombinant glutathione S-transferase (GST)-TCF-4 (amino acid 265–496) comprising the DNA binding domain or GST in binding buffer (10 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 4 % (w/v) Ficoll, 12.5 ng/micol poly-(deoxy-inosinic-deoxy-cytidylic)-acid, 62.5 μg/ml BSA) in a volume of 16 μl. After incubating for 5 min, 0.5 ng of [^{32}P]ATP end-labeled double-stranded oligonucleotides (Table 1) with a specific activity of $3 \cdot 10^8$ dpm/μg (Hartmann Analytik, Braunschweig, Germany) were added and the mixture incubated for further 20 min. A 30-fold molar amount of unlabeled oligonucleotides (15 ng, Table 1) was used for competition. Products of this binding procedure were separated with the help of 5% (w/v) 0.25x TBE (1xTBE: 89 mM Tris-HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA) polyacrylamide gels.

Cell lines, cell culture, luciferase assays

DLD-1, SW480 and HCT116 (American Type Culture Collection, Manassas, VA) were maintained in DMEM 4,500 mg/l glucose and 10 % (v/v) fetal bovine serum (Invitrogen). For luciferase assays 30 % confluent cultures of cultured colorectal SW480 cells were transiently transfected 24 h after seeding in 12 cluster well plates

adding 500 ng *hTERT*-promoter/enhancer-luciferase-reporter constructs together with increasing amounts of dnTCF-4 expression plasmids (0 ng, 120 ng, 240 ng, and 360 ng respectively), as well as 50 ng of pCMV-Renilla (Promega) for normalization of results, and finally filled up to a constant amount of 1.25 μg using the expression plasmid pcDNA3-CAT (Invitrogen) together with 2.5 μl Superfect (Qiagen) following the instruction manual. 36 h later, cells were harvested and luciferase activities were determined applying the Dual-Luciferase Reporter Assay System (Promega) following essentially the manufacturer's protocol. Triplicates were normalized on the activity of renilla-luciferase.

Chromatin immunoprecipitation (ChIP)

ChIPs were done using ChIP-IT kits (Active Motif) following essentially the instruction manual. Briefly, chromatin of cultured colorectal HCT116 cells was immunoprecipitated using 2 μg of two different antibodies specific for β-catenin (β-CATI: C2,206; Sigma, βCATII: clone 14; BD) or TFIIB specific antibodies (Active Motif, Belgium) or mouse IgG (Santa Cruz) as controls. Analysis of the reverse crosslinked chromatin precipitates was done employing PCRs using 10 % of these precipitates together with 400 nM of each primer spanning the region of the *hTERT* enhancer containing TBE3 and TBE4 (Table 1). 1 % of the input chromatin (input) or water (no template) were used as the positive or negative control, respectively.

RNA interference, RNA isolation and quantitative real-time PCR

For RNA interference (RNAi) DLD-1 or SW480 cells were transiently transfected with 3.9 μg siRNA specific for either β-catenin or cdc2 (Table 1) using 9.6 μl TransMessenger (Qiagen) following the user's instructions. Cells were harvested 24 h after transfection and total RNA was isolated using RNeasy kits (Qiagen). 1 μg of this RNA isolate was reverse transcribed using 0.2 μg random hexamer primers (Fermentas) in the presence of 1 U Superscript II (Invitrogen) following the user's manuals. As a control RT-reactions were done in parallel without adding Superscript II. Copy numbers of *β-catenin, hTERT* and *c-MYC* encoding cDNA representing mRNA steady state levels were determined employing real-time PCR (ABI Prism 7700; Applied Biosystems) together with specific primer-pairs (table 1).

2.5 μl of the RT-reactions with or without reverse transcriptase were analyzed employing SYBR-Green Master Mix kits (Applied Biosystems) following the manufacturer`s instructions. All experiments were done in triplicates and repeated at least twice.

Western-Blotting

20 μg of protein-lysate were separated using discontinuous polyacrylamide gelelectrophoresis, transferred onto PVDF-membranes (Millipore) following the user´s instructions. The membranes were incubated in the presence of antibodies specific for β-catenin (clone 14; BD), c-MYC (clone 9E10; BD, Germany) or β-actin (Pierce) and developed using a chemi-luminescence based system (SuperSignal® West; Pierce) together with Kodak Bio-Max films after stripping the membranes employing stripping buffer (Restore™ Western Blot; Pierce) following essentially the recommendations given in the user´s manuals.

Abbreviations

(AP1): activation protein 2, (β-CAT): β-catenin specific antibody, (CD): cluster of differentiation, (ChIP); chromatin immune-precipitation, (CRC): colorectal cancer, (CSC): cancer stem cell, (dnTCF4): dominant negative TCF-4, (EMSA): electric mobility shift assay, (EMT): epithelio-mesenchymal transition, (GAPDH): glycerol-aldehyde-3-phosphate dehydrogenase, (GST): glutathione-S-transferase, (hTERT): human TERT, (LEF-1): lymphocyte enhancing factor-1, (MAD): MAX dimerization protein, (MZF2): myeloid zinc finger protein 2, (qRT-PCR): quantitative RT-PCR, (RNAi): RNA interference, (RT): reverse transcriptase, (siRNA): small interfering RNA, (SP1): specificity protein 1, (TBE): TCF-4 binding element, (TCF): T-cell factor, (TERT): telomerase RT-component, (TFIIB): transcription factor IIB, (WT1): Wilms tumor 1 gene

Competing interests

There are no conflicts of interest.

Authors' contributions

SKS participated in the experimental work, helped to prepare the figures and drafted the manuscript. EH did part of the experimental work. TB was involved in the design of the study. AN did some of the experiments. HH made the immunohistochemical staining for TERT expression. TK helped in the design of the study and discussed the manuscript. AJ supervised SKS, EH, and AN, was mainly involved in the design of this study, contributed in making the figures and was deeply involved in writing the manuscript.

Authors' information

This project is Part of the PhD thesis of SKS.

Acknowledgements

We thank Dr. Masaki Inoue (Kanazawa University, Ishikawa, Japan) for giving us his pGL3-hTERT(-3,328) luciferase reporter construct, Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, U.S.A.) for kindly supplying pdnTCF-4 DNA expression plamids and Dr. Kerstin Amann for providing her digital photo-equipment. This work was supported by grants of the Wilhelm-Sander Stiftung (Az.: 2004.111) to AJ and TK and LMU*excellent* ZUK22 to AJ and TK.

References

1. Bienz M, Clevers H: **Linking colorectal cancer to WNT signaling. *Cell* 2000, 103:311-320.**

2. Harris TJ, Peifer M: **Decisions, decisions: beta-catenin chooses between adhesion and transcription. *Trends Cell Biol* 2005, 15:234-237.**

3. Brabletz T, Jung A, Reu S, Porzner M, Hlubek F, Kunz-Schughart LA, Knuechel R, Kirchner T: **Variable β-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* 2001, 98:10356-10361.**

4. Brabletz T, Hlubek F, Spaderna S, Schmalhofer O, Hiendlmeyer E, Jung A, Kirchner **T: Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and β-catenin. *Cells Tissues Organs* 2005, 179:56-65.**

5. Hanahan D: **Benefits of bad telomeres. *Nature* 2000, 406:573-574.**

6. Brembeck FH, Schwarz-Romond T, Bakkers J, Wilhelm S, Hammerschmidt M, Birchmeier W: **Essential role of BCL9-2 in the switch between β-catenin's adhesive and transcriptional functions. *Genes Dev* 2004, 18:2225-2230.**

7. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T: **Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* 2005, 5:744-749.**

8. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, Brooks M, Reinhard F, Zhang CC, Shipitsin M, et al**: The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 2008, 133:704-715.**

9. Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters PJ, Clevers H: **Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* 1998, 19:379-383.**

10. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, et al: **The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002, 111:241-250.**

11. Wielenga VJ, Smits R, Korinek V, Smit L, Kielman M, Fodde R, Clevers H, Pals ST: **Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. *Am J Pathol* 1999, 154:515-523.**

12. Hatzis P, van der Flier LG, van Driel MA, Guryev V, Nielsen F, Denissov S, Nijman IJ, Koster J, Santo EE, Welboren W, et al: **Genome-wide pattern of**

TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. *Mol Cell Biol* 2008, 28:2732-2744.

13. Melton DA, Cowan C: ***"Stemness": Definitions, Criteria, and Standards.* Elsevier; 2006.**

14. Blackburn EH: **Telomere states and cell fates. *Nature* 2000, 408:53-56.**

15. Kolquist KA, Ellisen LW, Counter CM, Meyerson M, Tan LK, Weinberg RA, Haber DA, Gerald WL: **Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. *Nat Genet* 1998, 19:182-186.**

16. Wassermann S, Scheel SK, Hiendlmeyer E, Palmqvist R, Horst D, Hlubek F, Haynl A, Kriegl L, Reu S, Merkel S, et al: **$p16^{INK4a}$ is a β-catenin target gene and indicates low survival in human colorectal tumors. *Gastroenterology* 2009, 136:196-205 e192.**

17. Jung A, Schrauder M, Oswald U, Knoll C, Sellberg P, Palmqvist R, Niedobitek G, Brabletz T, Kirchner T: **The invasion front of human colorectal adenocarcinomas shows co- localization of nuclear β-catenin, cyclin D1, and p16INK4A and is a region of low proliferation. *Am J Pathol* 2001, 159:1613-1617.**

18. Takakura M, Kyo S, Kanaya T, Hirano H, Takeda J, Yutsudo M, Inoue M: **Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. *Cancer Res* 1999, 59:551-557.**

19. Tang R, Cheng AJ, Wang JY, Wang TC: **Close correlation between telomerase expression and adenomatous polyp progression in multistep colorectal carcinogenesis. *Cancer Res* 1998, 58:4052-4054.**

20. Verma UN, Surabhi RM, Schmaltieg A, Becerra C, Gaynor RB: **Small Interfering RNAs Directed against beta-Catenin Inhibit the in Vitro and in Vivo Growth of Colon Cancer Cells. *Clin Cancer Res* 2003, 9:1291-1300.**

21. **U**eno H, Murphy J, Jass JR, Mochizuki H, Talbot IC: **Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology* 2002, 40:127-132.**

22. Tatsumoto N, Hiyama E, Murakami Y, Imamura Y, Shay JW, Matsuura Y, Yokoyama T: **High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. *Clin Cancer Res* 2000, 6:2696-2701.**

23. O'Brien CA, Pollett A, Gallinger S, Dick JE**: A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007, 445:106-110.**

24. **Ric**ci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R: **Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007, 445:111-115.**

25. **Ho**rst D, Kriegl L, Engel J, Kirchner T, Jung A: **CD133 expression is an independent prognostic marker for low survival in colorectal cancer. *Br J Cancer* 2008, 99:1285-1289.**

26. **H**astie ND, Dempster M, Dunlop MG, Thompson AM, Green DK, Allshire RC: **Telomere reduction in human colorectal carcinoma and with ageing. *Nature* 1990, 346:866-868.**

Tables

Table 1 Sequence of Oligonucleotides

Name	Sequence	length [bp]	amount concentrations
EMSA			
TBE3	ATTATTTCAAAACAAAGGTTTACAGAAA		
TBE4	GAGTTACCCTCCTTTGATATTTTCTGTA		
MUT1	ATTATTTCAACGCAGAGGTTTACAGAAA		
MUT2	GAGTTACCCTCCTGTGCGATTTTCTGTA		
MYC	CTAGCGCACCTTTGATTTCTGCACCTTTGATTTCTG		
ChIP-PCR			
TERT	ACTCGCGCTGCCCTTCTAGC ACGGTGTATCCCCAGTCTACGAAG	618	400 nM 400 nM
cMYC	ACAGACGCCTCCCGCACGGG CCACACCGAGAACGCACTGC	451	400 nM 400 nM
GAPDH	TACTAGCGGTTTTACGGGCG TCGAACAGGAGGAGCAGAGAGCGA	165	400 nM 400 nM
RNAi			
β-catenin	CAGUUGUGGUUAAGCUCUUdTdT		3.9 μg
cdc2	GAUGUAGCUUUCUGACAAAdTdT		3.9 μg
qRT-PCR			
β-catenin	CTTGGAATGAGACTGCTGATCTTG CACCAGAGTGAAAAGAACGATAGCTA	102	900 nM 900 nM
cMYC	GTGCTCCATGAGGAGACAC CCTCTTTTCCACAGAAACAACATC	96	300 nM 300 nM
hTERT	ACGGCCTCCCTCTGCTACTC CGACATCCCTGCGTTCTTG	51	900 nM 900 nM
β-actin	TTGCGGATGTCCACGTCA GCCCTGAGGCACTCTTCC	103	300 nM 300 nM

TBEs either WT or mutated are underlined.

Figures

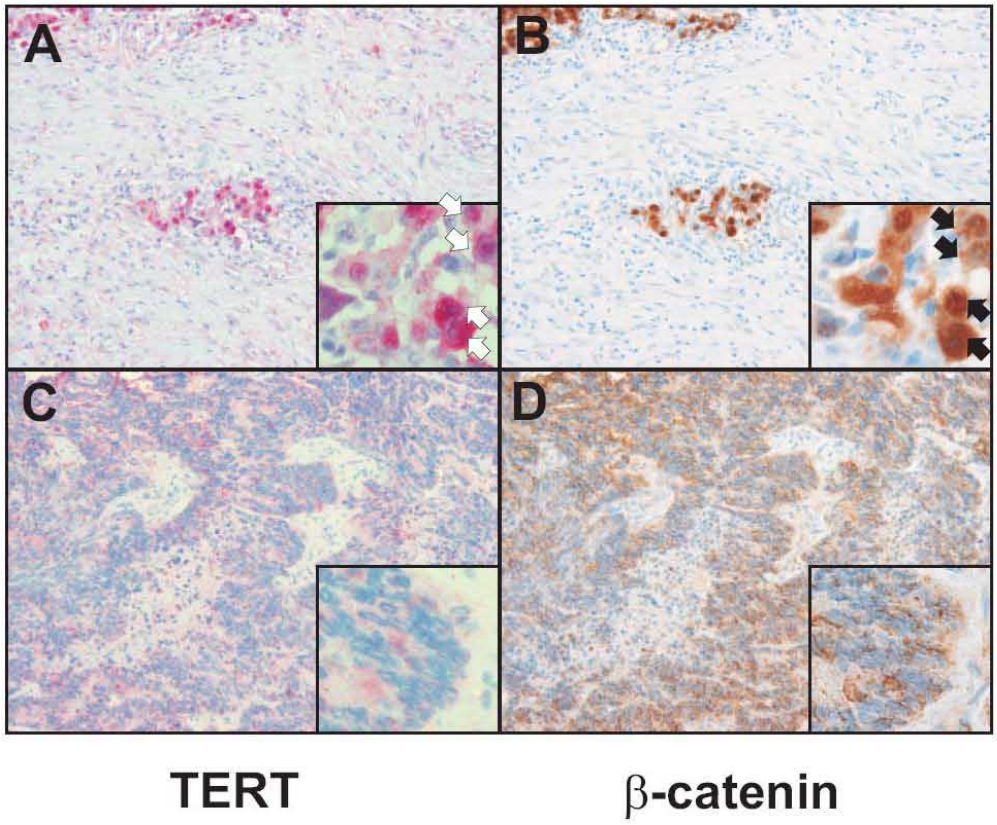

Figure 1 - Coexpression of hTERT and β-catenin in tumor cells at the invasive front of colorectal tumors.

(A): Tumor cells expressing hTERT (white arrows) are found at the invasive front. **(B):** Corresponding cells express β-catenin in their nuclei (black arrows). **(C):** Tumor cells without or with weak expression of hTERT are found in central areas (inlet picture) which are characterized by epithelial differentiation. **(D):** These tumor cells display no nuclear β-catenin (inlet picture).

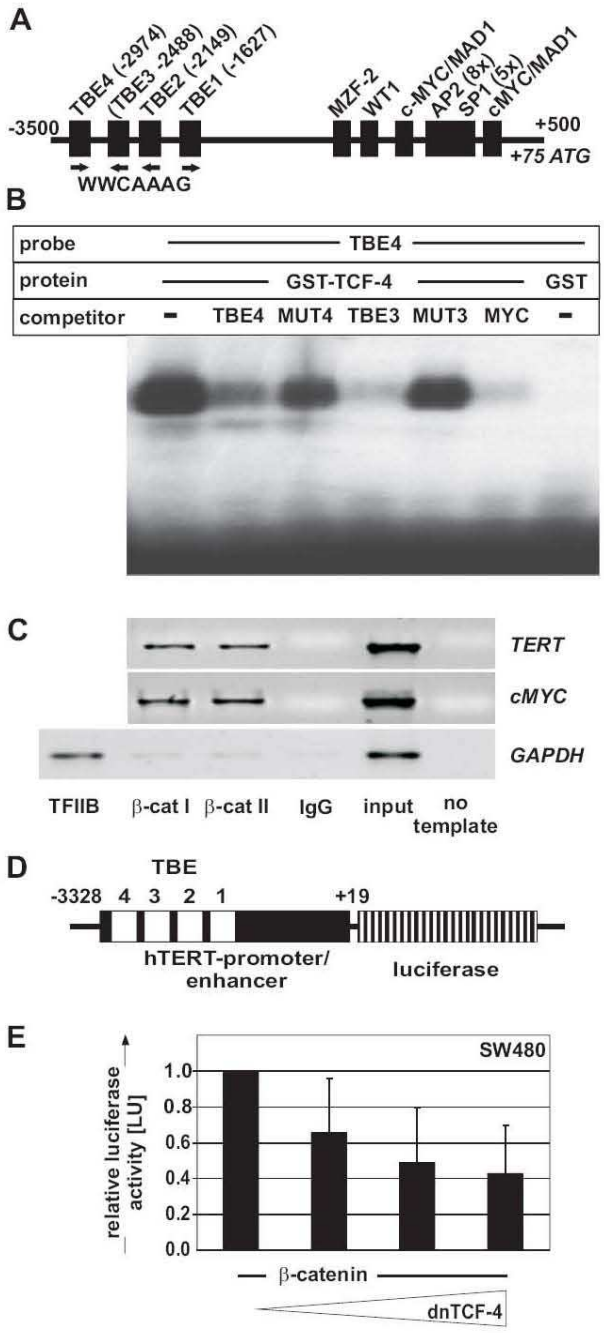

Figure 2 - β-catenin interacts with TBEs in the hTERT promoter/enhancer and confers transcriptional activation

(A): Schematic representation of the hTERT-promoter/enhancer showing several known responsive elements: AP2 (activation protein 2), MAD (MAX dimerization protein), MZF2 (myeloid zinc finger protein 2), SP1 (specificity protein 1), TBE (TCF

binding element), WT1 (Wilms tumor 1 gene). The arrows below the TBEs represent the orientation of the consensus sequence WWCAAAG. Figure is not drawn to scale. **(B):** Specific binding of a recombinant GST-TCF-4 (glutathion-S-transferase-T-cell - factor-4) fusion protein to the second TBE (TBE2) of the hTERT promoter/enhancer using Electric Mobility shift assays (EMSA). **(C):** β-catenin specifically binds the genomic region of the hTERT gene containing TBE3 and TBE4 applying ChIP (chromatin immune precipitation). **(D):** The hTERT-promoter/enhancer luciferase reporter gene construct consists of a 3,347 bp fragment of the hTERT promoter/enhancer harboring the four TBEs (1, 2, 3, 4) driving the firefly luciferase encoding gene. Not drawn to scale. **(E):** dnTCF-4 suppresses the transcriptional activity of the hTERT-promoter/enhancer luciferase reporter construct in a dose dependent manner.

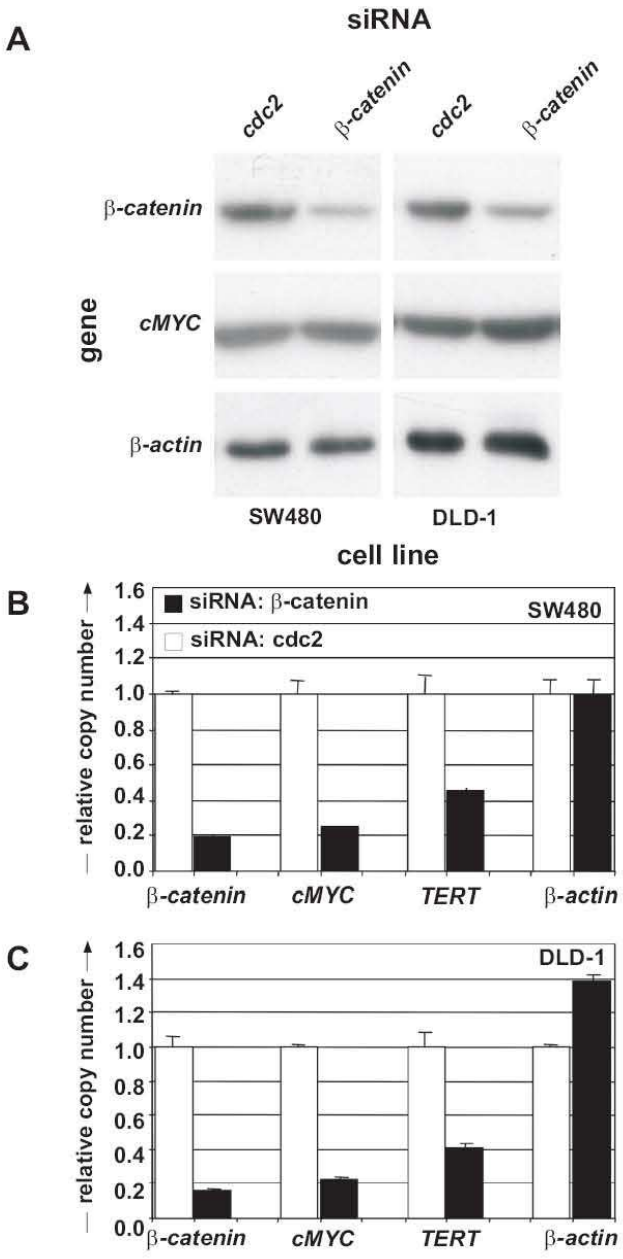

Figure 3 - β-catenin specific RNAi results in the downregulation of hTERT mRNA under conditions where cMYC protein is still present

(A): Time point when β-catenin specific siRNA leads to the downregulation of β-catenin but not cMYC proteins. (**B):** Here, β-catenin- in contrast to cdc2-specific siRNA leads to the downregulation of hTERT and cMYC in SW480 or (**C):** DLD-1 cells.

3.5 $p16^{INK4A}$ ist ein β-Catenin Zielgen und korreliert mit schlechtem Überleben in humanen kolorektalen Tumoren

GASTROENTEROLOGY 2009;136:196–205

BASIC—ALIMENTARY TRACT

p16^{INK4a} Is a β-Catenin Target Gene and Indicates Low Survival in Human Colorectal Tumors

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Background & Aims: **Human colorectal carcinomas display an infiltrative front of invasion where tumor cells undergo an epithelomesenchymal transition associated with low survival. Epithelomesenchymal transition is regulated by a nuclear β-catenin accumulation, and subsequently, activation of β-catenin/TCF4 target genes similar to *CYCLIN D$_1$*. Unexpectedly, these tumor cells are characterized by low proliferation, which correlates with the expression of the cell cycle inhibitor p16^{INK4A}. Therefore, we investigated the molecular mechanism of the transcriptional regulation of p16^{INK4A} in colorectal cancer and its correlation with survival.** ***Methods:*** **Molecular biological techniques were used for investigating the transcriptional mechanisms of the *p16^{INK4A}* gene regulation. Moreover, p16^{INK4A} expression was correlated with the 10-year survival of patients with colorectal carcinomas.** ***Results:*** **In colorectal carcinomas, expression of the *p16^{INK4A}* gene is regulated by β-catenin/TCF4 and correlates with low survival rates of patients with tumors displaying an infiltrative front of invasion.** ***Conclusions:*** **β-catenin/TCF4 regulates cell cycle promoting (*c-MYC, CYCLIN D$_1$*) and inhibiting genes (*p16^{INK4A}*) at the same time in the mesenchymally differentiated tumor cells at the front of invasion. The function of p16^{INK4A} seems to supersede in this context thus leading to low proliferation. Moreover, these tumor cells seem to govern the outcome of colorectal cancer independently of their proliferation.**

Histologically, many colorectal cancers (CRCs) display an infiltrative growth pattern at the front of tumor invasion. Here, a transition from the well-differentiated tubular and glandular organization to single isolated growing cells can be seen. The histological manifestation of this transition is known as "budding," which correlates very well with local metastasis and low survival.[1,2] The tumor cells at the edge of the front of invasion show characteristics of an epitheliomesenchymal transition (EMT), associated with the expression of vimentin as well as fibronectin.[3] Moreover, these tumor cells are often characterized by nuclear expression of β-catenin[3–5] and thus the expression of well-known β-catenin/TCF4 target genes.[6] These confer proliferation, angiogenesis, migration, invasion, as well as EMT and explain the observed morphological changes of the mesenchymal transition of tumor cells at the front of invasion on the molecular level. Thus, tumor budding and EMT do not just reflect a morphological pattern but rather a functional program with biological and clinical relevance. Although tumor cells at the front of invasion express the cell cycle promoting β-catenin/TCF4 target gene *CYCLIN D$_1$*,[5,7,8] they are characterized by low proliferation,[3,5,9] which significantly correlates with the expression of the cell-cycle inhibitor p16^{INK4A}.[5,9,10] In addition to regulating the cell cycle, p16^{INK4A} might induce migration and invasion of tumor cells in cooperation with laminin-5γ 2,[11] another β-catenin/TCF4 target gene.[12]

The transcriptional regulation of the *p16^{INK4A}*-gene in CRCs is unknown. In aberrant crypt foci (ACF), no associ-

Abbreviations used in this paper: ACF, aberrant crypt foci; APC, adenomatous polyposis coli; CBP, CREB-binding protein; CREB, cAMP response element-binding; CNAPI, chromosome condensation-related SMC-associated protein; NCAPD2, non-SMC condensin I complex; subunit D2; CRC, colorectal cancer; dn, dominant negative; ds, double stranded; EMSA, electromobility shift assay; EMT, epitheliomesenchymal transition; Ets, proto-oncogene E twenty six; GAPDH, glycerol-aldehyde-3-phosphate-dehydrogenase; GFP, green fluorescent protein; GST, glutathione-S-transferase; GST-TCF4, glutathione-S-transferase T-cell factor-4; HAT, histone-acetyl-transferase; HPRT, hypoxanthine-phospho-ribosyl-transferase; INK4, inhibitor of kinase 4; M, methylated DNA; MSP, methyl-specific PCR; MU, mutated; qRT-PCR, quantitative RT-PCR; RT, reverse transcriptase; TBE, TCF4-binding element; TFIIB, transcription factor II subunit B; U, unmethylated DNA; WT, wild-type; YWMAZ, tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein ξ-polypeptide.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2008.09.019

ation was found between activity of the Wnt signaling pathway and expression of p16INK4A.[10] Moreover, in endometrial carcinomas, the expression of both p16INK4A and p21Cip1/Waf1 is regulated via the action of β-catenin in the absence of TCF4.[13] But the mechanism for the regulation of *p16INK4A* might differ in CRCs, as this is already the case for p21Cip1/Waf1, which is down-regulated by β-catenin/TCF4 via up-regulation of the target gene *c-MYC*.[14]

Thus, we investigated the mechanism of transcriptional regulation of the expression of the *p16INK4A*-gene. Moreover, we analyzed if the p16INK4A expression in tumor cells at the infiltrative invasion front of CRCs also correlates with low survival rates.

Materials and Methods

Electrophoretic Mobility Shift Assay

Fifteen nanograms of unlabeled competitor oligonucleotides or water were incubated together with 1 μL of crudely purified recombinant GST-TCF4 (amino acid 265-496) or GST in binding buffer (10 mM Hepes, pH 7.9; 60 mM KCl; 1 mM EDTA; 1 mM DTT; 4% [w/v] Ficoll) in the presence of 12.5 ng/μL poly-(deoxyinosinic-deoxycytidylic) acid (Sigma–Aldrich, Taufkirchen, Germany) and 62.5 μg/mL bovine serum albumin (Sigma–Aldrich) in a total volume of 16 μL. After an incubation period of 5 minutes, 0.5 ng of ^{32}P end-labeled double-stranded oligonucleotides (Table 1, electrophoretic mobility shift assay [EMSA]) with a specific activity of 3×10^8 dpm/μg (Hartmann Analytik, Braunschweig, Germany) were added, incubated for another 20 minutes, and finally applied on a 0.5 × TBE 10% (w/v) polyacrylamide gel. The recombinant proteins, GST-TCF4 and GST, were produced according to the instructions given in GE Healthcare's (Freiburg, Germany) GST Protein brochure.

Chromatin Immune Precipitation

Chromatin immune precipitation (ChIP) was done using ChIP-It kits (Activ–Motif, Rixenart, Belgium) following essentially the instructions given in the user's manual. Chromatin was fragmented using an ultrasonic device (Branson, Schwäbish-Gmünd, Germany) to a

Table 1. Primers Used in the Study

EMSA		
16WT	TCTCCAAAAGGAATCCTTTGAACTAGGGTTTCTGACT	p16INK4A WT TBE
16MU	TCTCCAAAAGGAATCCTTTGCGCTAGGGTTTCTGACT	p16INK4A mutated TBE
myWT	CGCACCTTTGATTTCTGCACCTTTGATTTCT	c-myc WT TBE1[17]
ChIP		
GAPDH	GCGCCCCCGGTTTCT, CCTAGCCTCCCGGGTTTC	
c-myc	ACAGACGCCTCCCGCACGGG,CCACACCGAGAACGCACTGC	
p16INK4A	AGCCGTTTTACACGCAGGAG, CCACCGAGAAATCGAAATCAC	
CNAPI	ATGGTTGCCACTGGGGATCT, TGCCAAAGCCTAGGGGAAGA	
Site-directed mutagenesis		
Mutagenesis	CCAAAAGGAATCCTTTGCGCTAGGGTTTCTGACTTAGTG	
Sequencing	TGTCCCCCTACCCCCTCAAC	
siRNA		
β-catenin	CAGUUGUGGUUAAGCUCUUdTdT[23]	
GFP	AAGCUACCUGUUCCAUGGCCAdTT	
RT-PCR		
c-MYC	GTTGCGGAAACGACGAGAACAG, CCAAAGTCCAATTTGAGGCAGTTTAC	
GAPDH	AAGGGCATCCTGGGCTACAC, CCCCTCTTCAAGGGGTCTAC	
p14ARF	AGGGTTTTCGTGGTTCACATCC, CCATCATCATGACCTGGTCTTC	
p16INK4A	CACGCCCTAAGCGCACATTC, AAGCCAGTAACCCCCCTGAG	
qRT-PCR		
β-ACTIN	TTTGCGGATGTCCACGTCA (300 nM), GCCCTGAGGCACTCTTCCA (300 nM)	
β-CATENIN	CTTGGACTGAGACTGCTGATCTTG (900 nM), CACCAGAGTGAAAAGAACGATAGCTA (900 nM)	
c-MYC	GTGCTCCATGAGGAGACAC (300 nM), CCTCTTTTCCACAGAAACAACATC (300 nM)	
CYCLIN D1	TGCCCTCTGTGCCACAGAT (50 nM), CACCAGAGTGAAAAGAACGATAGCTA (900 nM)	
HPRT	TGACACTGGCAAAACAATGCA (300 nM), (CCAACGCACCGAATAGTTACG (300 nM)	
YWMAZ	ACTTTTGGTACATTGTGGCTTCAA (300 nM), CCGCCAGGACAAACCAGTAT (300 nM)	
MSP		
p16-M	TTATTAGAGGGTGGGGCGGATCGC GACCCCGAACCGCGACCGTAA	p16INK4A-methylated
p16-U	TTATTAGAGGGTGGGGTGGATTGT CAACCCCAAACCACAACCATAA	p16INK4A-unmethylated
p14-M	GTGTTAAAGGGCGGCGTAGC AAAACCCTCACTCGCGACGA	p14Arf-methylated
p14-U	TTTTTGGTGTTAAAGGGTGGTGTAGT CACAAAAACCCTCACTCACAACAA	p14Arf- unmethylated

EMSA, electromobility shift assay; ChIP, chromatin immune precipitation; MSP, methyl-specific PCR.

mean size of 300 bp. For precipitation, 1 μg of antibodies specific for β-catenin (clone 14; BD Transduction Laboratories, Heidelburg, Germany), TFIIB (Activ–Motif), or IgG (Sigma–Aldrich) as a control were used. For the subsequent detection of precipitated chromatin, PCRs were employed using primer pairs (Table 1, ChIP) specific for the promoter/enhancers of the *cMYC*-, *GAPDH*-, *p16*^{INK4A}- or the *CNAPI* genes. The TFIIB antibody and *CNAPI*-gene PCR readout were introduced as additional controls to obtain testable negative controls, which is not the case when using IgGs. PCR products were separated on 0.5 × TBE 3% (w/v) agarose gels.

DNA Clones

The luciferase gene reporter clone pGL2-p16 -3027 contains a 3017 bp fragment of the human *p16*^{INK4A} promoter/enhancer with respect to the translation start site of this gene. The functional proximal TBE (-708/-702) was mutated using QuikChange mutagenesis kits (Stratagene, Amsterdam, The Netherlands) together with appropriate primers (Table 1, site-directed mutagenesis). The success of mutagenesis was verified by sequencing using BigDye termination kits (Applied Biosystems, Darmstadt, Germany) together with a specific sequencing primer (Table 1, site-directed mutagenesis) following the user's manual.

Cell Lines, Cell Culture, and Transfections

The human embryonic kidney cell line 293T and the colorectal cell lines DLD-1 (APC: LOH, c.1417delC), HCT116 (β-catenin: p.S45del), LS174T (β-catenin: p.S45F), and SW480 cells (APC: LOH, c.4012C>T, p.R1338X) were purchased from the American Type Culture Collection (Manassas, VA). Mutations found in the APC or β-catenin genes of the colorectal tumor cell lines[15] are given in brackets. All cells were maintained in Dulbecco's Modified Eagle Medium with 10% (v/v) fetal bovine serum, 2 mM Glutamax I, and 50 μM 2-mercaptoethanol (Invitrogen, Karlsruhe, Germany) without antibiotics. For transient transfections, 5×10^4 293T, or 1.5×10^5 HCT116, or LS174T cells, respectively, were seeded in 12-cluster well plates. The next day, cells were transfected with 600 ng of luciferase reporter plasmids, 50 ng plasmid pRL-CMV (Promega, Heidelburg, Germany) as the transfection control, and 600 ng of pcDNA− or pcI-Neo−derived expression vectors encoding β-catenin, dnTCF4, Ets2, dnEts2, CBP, p300 in case of 293T cells, or 1200 ng in case of HCT116 or LS174T cells as indicated, using Superfect (Qiagen, Hilden, Germany). pcDNA3-Cat (Invitrogen) was added to fill up DNA to constant amounts of 1.25 μg in case of 293T cells or 2 μg when enrolling HCT116 or LS174T cells. After 16 to 28 hours, cells were harvested, lysed, and luciferase activity was determined, using Dual Light kits (Promega) following the user's manual. Results were normalized for transfection efficiency on the basis of the activity of Renilla luciferase. Transfection of siRNA cells was done as described previously.[16] Briefly, 3.9 μg double-stranded siRNA specific for *β-catenin* or *GFP* (Table 1, siRNA) as a control were incubated together with Oligofectamine (Invitrogen) following the user's instructions. About 48 hours after transfection, cells were harvested, and subsequently, RNA was isolated.

Adenovirus Transduction and Cell Sorting

Adenovirus encoding either *β-galactosidase* (AdEasy-βgal) or *dnTCF4* (AdEasy-dnTCF4) were propagated in 293 cells[17] and purified using the Adeno-X kit (Clontech, Heidelburg, Germany) following the user's manual. HCT116 cells were infected at an MOI of 25. Transduced cells were selected on the basis of their green fluorescence using a Cytomation MoFlo FACS (Ft Collins, CO) applying appropriate electronic gates for the sorting process, were subsequently washed, and used for the isolation of RNA.

RNA Isolation, Reverse Transcription, RT-PCR, qRT-PCR

Total RNA from cell lines was prepared employing RNeasy kits (Qiagen) following the user's instructions. One microgram RNA was reverse transcribed using 200 ng each oligo$(dT)_{15}$ and random hexamer primers in the presence (+) or absence (−) of 1 U of reverse transcriptase (Superscript II, Invitrogen) following the user's manuals. Thereby, the reactions without RT were utilized as negative controls. One microliter of the resulting cDNA was used in subsequent PCRs together with intron-spanning gene specific primer pairs (Table 1, RT-PCR). After electrophoresis intensity of PCR products as quantified by densitometry with the help of a UV-transilluminator (Eagle Eye II, Stratagene). For qRT-PCR, 1 μL of the RT reactions (+/−) was incubated together with 5 μL 2 × iQ-SYBR-Green (Biorad, Munich, Germany) in the presence of optimized amounts of intron-spanning primer pairs (Table 1, qRT-PCR) specific for the genes *β-ACTIN, β-CATENIN, c-MYC, CYCLIN D*$_1$, *HPRT, p16*^{INK4A}, or *YWMAZ* using a Prism 7700 Sequence Detection System (Applied Biosystems). All assays were run in triplicates. All data were normalized on the geometric mean of the values resulting from the qRT-PCRs specific for the 3 housekeeping genes *β-ACTIN*, *HPRT,* and *YWMAZ*.

Immunohistochemical Staining and Statistical Analysis

Eighty-two patients with CRC displaying an infiltrative front of invasion were enrolled in the study (Table 2). No metastases were detectable at the time of surgery. Moreover, only patients surviving the surgery for at least 6 months were selected to exclude surgery-associated effects as the cause of death. The patients did not show other malignancies or other diseases besides the CRCs. Immunohistochemical staining was performed as described previously.[5] Briefly, sections were stained after

Table 2. Clinicopathological Data and Statistical Results for the Patients Enrolled in This Study

Variable	Number of Cases	p16INK4A Score		P Value (χ^2 test)	P Value (Cox)
		Low	High		
Gender					
Male	43 (52.4%)	23	20	NS	NS
Female	39 (47.6%)	21	18	.974	.873
Age					
≤74	40 (48.8%)	22	18	NS	NS
>74	42 (52.1%)	22	20	.812	.012
T-stage					
T2	34 (41.5%)	19	15	NS	▸**.002**
T3	48 (58.5%)	25	23	.734	
N-stage					
N0	21 (25.6%)	15	6	NS	▸**.001**
N1	41 (50.0%)	18	23	.113	
N2	20 (24.4%)	11	9		
M-stage					
M0	82 (100%)	44	38	Ø	Ø
M1	0 (0%)				
Grade					
1	5 (6.1%)	1	4	NS	NS
2	72 (87.8%)	41	31	.214	.059
3	5 (6.1%)	2	3		
Adjuvant therapy					
No	23 (28.0%)	31	28	NS	NS
Yes	59 (72.0%)	13	10	.809	.197
R-stage					
0	82 (100%)	44	38	Ø	Ø
1	0 (0%)				
2	0 (0%)				
Survival (y)					
≤5	39 (47.6%)	12	27	▸**.002**	▸**.001**
>5–10	13 (15.9%)	11	2		
>10	30 (36.5%)	21	9		
Censored	0 (0%)				
p16INK4A	44 (53.7%)	Ø	Ø	Ø	▸**<.001**
	38 (46.3%)				

NOTE. Given is the number of cases (frequencies) as well as the numbers of p16INK4A low- or high-staining cases, respectively, within the variates. The χ^2 test was applied to correlate p16INK4A staining with the variates, the multivariate Cox regression (Cox) for the correlation of the variates with survival. Statistically significant data are shown in boldface and indicated by an arrowhead (▸).
NS, statistically not significant; Ø, statistics not applicable.

microwave-driven antigen retrieval in citrate buffer, pH 6.0 (Ventana, Illkirch, France) using a 1:25 dilution of a mouse monoclonal p16INK4A specific antibody (clone G174-405, Pierce, Ulm, Germany) and the subsequent application of Ventana's DAB staining system employing a semiautomated staining machine (Benchmark, Ventana) following the given recommendations. For the analysis of results, a labeling index was introduced. First, the intensity of immunohistochemical p16INK4A stainings was scored in 3 groups: absent or low (1), medium (2), and high (3). Second, the amount of p16INK4A-positive colorectal tumor cells or small cell clusters was counted in regions with maximum budding defined at 50× magnification. Buds were defined as structures of less than 5 tumor cells.[1,2] The amount of p16INK4A-positive tumor cells was counted in 10 viewing fields at 250× magnification and scored in 2 groups: up to 50 cells or clusters (1) and more than 50 cells or clusters (2).[2] The product of both parameters was calculated and was considered as low- (1−3) or high-labeling index (4, 6). For statistical analyses, the χ^2 test, multivariate Cox-regression, and Kaplan–Meier statistics together with log-rank regression were applied using SPSS version 16.0 software (Chicago, IL) (Table 2).

Results

The Promoter/Enhancer of the Human p16INK4A Gene Harbors a Functional TCF4 Binding Element

As nuclear expression of β-catenin correlates with the expression of p16INK4A in human CRCs in a highly significant manner,[5] we proposed that *p16INK4A* might be a target gene of β-catenin. Therefore, TBEs (WWCAAAG)[18] were expected to occur in the promoter/enhancer of the human *p16INK4A* gene. Two consensus TBEs were found at

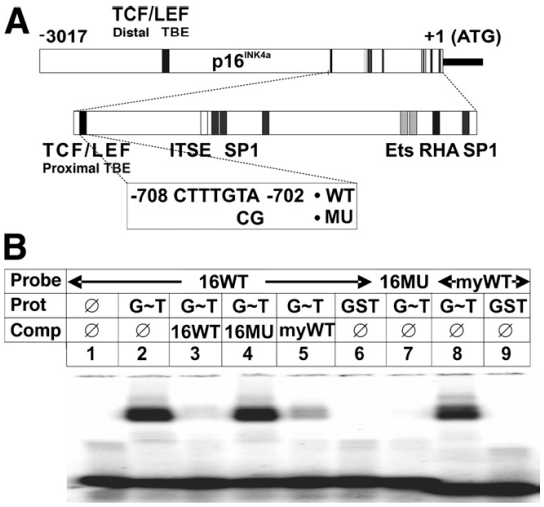

Figure 1. The proximal TCF binding element (TBE) of the human p16^{INK4A} promoter/enhancer binds recombinant TCF4 protein. (*A*) The promoter/enhancer region of the human p16^{INK4A} gene (accession no. AF022809). The start of translation (ATG) is 1. The human p16^{INK4A} promoter/enhancer harbors 2 consensus TBEs (WWCAAAG)[18] embedded in the context of a variety of other transcription factors known to be involved in the transcriptional regulation of the p16^{INK4A} gene: ITSE (INK4a transcription silence element),[29] SP1,[30] Ets,[21] RBRE (retinoblastoma-responsive element),[31] and RHA (RNA helicase A).[32] (*B*) EMSA. Radioactively labeled oligonucleotides containing the proximal TBE bind specifically to the DNA-binding domain of a recombinant GST-TCF4 (G~T) fusion protein (*lane 2*) but not when the TBE is mutated (*lane 7*). Binding is compatible using WT p16^{INK4A} (*lane 3*) or c-MYC (*lane 5*) but not MU p16^{INK4A} (*lane 4*) TBE-containing oligonucleotides. Binding does not depend on GST (*lanes 6 and 9*). Interaction with the radioactively labeled TBE2[19] of the c-MYC gene was provided as the positive control.

positions -702/-708 (proximal TBE) and -1980/-1986 (distal TBE) with respect to the translational start of the *p16^{INK4A}* gene (accession no. AF022809) (Figure 1*A*). Employing EMSAs, we investigated if recombinant GST-TCF4 fusion proteins bind radioactively labeled oligonucleotide probes containing either of the 2 TBEs. GST-TCF4 bound to the proximal (Figure 1*B*, *lane 2*) but not the distally located TBE (data not shown). Binding was competed when adding unlabeled oligonucleotides harboring WT TBE sequences of the human *p16^{INK4A}* (16WT) or the *c-myc* promoter/enhancer (myWT)[19] (Figure 1*B*, *lanes 3* and *5*) but not when mutated TBE-containing oligonucleotides of the *p16^{INK4A}* gene were used (Figure 1*B*, *lane 4*). Moreover, radioactively labeled oligonucleotides containing a mutated TBE of the *p16^{INK4A}* promoter/enhancer did not bind GST-TCF4 proteins (Figure 1*B*, *lane 7*). Binding was due to TCF4, as GST alone did not interact with labeled probes containing *p16^{INK4A}* WT TBE (Figure 1*B*, *lane 6*). Labeled oligonucleotides of the human *c-myc* gene containing the second TBE (TBE2)[19] were used as a control. Expectedly, they bound GST-TCF4 (Figure 1*B*, *lane 8*) but not GST (Figure 1*B*, *lane 8*). Taken together, the proximal TBE of the human *p16^{INK4A}* gene interacts specifically with the recombinant GST-TCF4 protein.

β-Catenin Interacts With the TBE of the Endogenous Human p16^{INK4A} Gene

Next, we analyzed if β-catenin interacts with the proximal TBE of the *p16^{INK4A}* gene in the context of native chromatin employing ChIP. As p16^{INK4A} is rarely expressed in cultured cell lines, and primers have to be selected carefully to exclude interference with expression from the *p14^{ARF}* locus,[20] we investigated both the methylation status of the promoter regions as well as the expression of the *p16^{INK4A}* and *p14^{ARF}* genes in the 4 cultivated colorectal tumor cell lines DLD-1, HCT116, LS174T, and SW480 (Supplementary Figure). As a result, the p16^{INK4A} expressing cell lines HCT116 and LS174T were selected for further functional tests. HCT116 cells were chosen for ChIP (Figure 2) due to their higher expression level of p16^{INK4A} compared with LS174T cells (data not shown). For precipitations, antibodies specific for β-catenin or RNA polymerase II (RNA pol II) as well as immunoglobulins (IgG) as negative controls were taken. Regions of the human *p16^{INK4A}* (Figure 2*A*) or *c-MYC* promoters (Figure 2*B*) harboring TBEs or the TATA box-containing sequence of the *glycerol-aldehyde-3-phosphate-dehydrogenase* (Figure 2*C*) were used for subsequent PCR-based readouts. A region of intron 25 of the chromosome condensation-related SMC-associated protein gene containing neither TATA box-containing sequences nor TBE sequences was used as a control for unspecific binding of chromatin to the employed antibodies (Figure 2*D*). β-catenin–specific antibodies

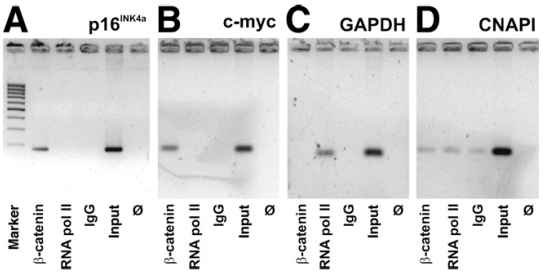

Figure 2. β-catenin binds to the proximal TBE of the human p16^{INK4A} gene in the context of native chromatin. ChIP using antibodies specific for β-catenin, RNA polymerase II, or immunoglobulins G (IgG) as a control. Human genomic DNA (input) was used as a positive control and water as the negative control (Ø). PCR primers covered sequences of the human p16^{INK4A}-gene containing the proximal TBE (*A*), the c-MYC gene containing TBE2[19] (*B*), or the GAPDH-gene harboring the TATA box (*C*). Intron 25 of the CNAPI-gene (*D*) is not expected to bind DNA and was thus used as a control for comparing the unspecific binding of DNA to the antibodies in use. β-catenin–specific antibodies precipitated DNA sequences containing TBEs of the p16^{INK4A}- and c-myc genes (*A* and *B*) but not GAPDH gene (*C*). The situation was expectedly found vice versa for an antibody specific for RNA polymerase II. The prepared chromatin did not bind unspecifically to immunoglobulins (IgG), and the antibodies in use did not differ in their unspecific binding to chromatin (*D*).

precipitated chromatin fragments containing TBEs of the *p16INK4A*- (Figure 2*A*, *lane β-catenin*) or *c-MYC*- (Figure 2*B*, *lane β-catenin*) but not TATA box-containing chromatin of the *GAPDH* promoter (Figure 2*C*). Vice versa, TATA box-containing chromatin of the *GAPDH*- (Figure 2*C*, *lane RNA pol II*) but neither TBE-containing chromatin of the *p16INK4A* (Figure 2*A*, *lane RNA pol II*) nor *c-MYC* promoter/enhancers (Figure 2*B*, *lane RNA pol II*) were precipitated by RNA polymerase II–specific antibodies, thus indicating the specificity of the β-catenin–driven ChIP. Additionally, IgGs did not precipitate chromatin at all (Figure 2*A*, *B*, *C*, and *D*, *lane IgG*). Moreover, all antibodies bound unspecifically comparable amounts of chromatin, which was indicated by identical PCR results specific for an intronic region of the *CNAPI* gene (Figure 2*D*, *lanes β-catenin, RNA pol II, and IgG*) as an indicator for unspecific binding of DNA to the antibodies. Taken together, these results demonstrate that β-catenin interacts specifically with the proximal TBE of the human *p16INK4A* promoter/enhancer in the context of native chromatin.

β-Catenin Drives the Expression of the Human p16INK4A Gene via the Proximal TBE

Next, we wanted to investigate if the proximal TBE of the human *p16INK4A* confers β-catenin/TCF responsiveness. Therefore, we used luciferase-reporter assays together with reporter plasmids regulated by fragments of the *p16INK4A* promoter/enhancer containing either WT or MU TBEs (Figure 1*A*). For the activation of the reporter plasmids, expression vectors encoding *β-catenin*, *Ets2* (Figure 3*A*, β-cat, ets), dominant-negative TCF4, dominant-negative Ets2 (Figure 3*B* and *C*) or the histone-acetyl-transferases (HAT) p300 or CBP, respectively (Figure 3*D*), were employed. Ets2 and HATs were taken because they are well-known activators of the transcription of the *p16INK4A* [21] or a variety of β-catenin target genes such as *CYCLIN D1*.[22] Both β-catenin as well as Ets2 transactivated WT *p16INK4A* promoter/enhancer reporters (Figure 3, *black bars*) in a dose-dependent manner when cotransfected into 293T cells (Figure 3*A*). In contrast, *p16INK4A* luciferase-reporter constructs containing a MU TBE (Figure 3, *stippled bars*) were transactivated as expected only by Ets2 (Figure 3*A*) but no longer by β-catenin (Figure 3*A*), as the TBE but not the Ets2 binding site was affected by the mutation. In combination, β-catenin and Ets2 stimulated WT but not MU *p16INK4A* reporters in an additive manner. In case of the MU TBE-containing *p16INK4A* reporter constructs, the activity was comparable with the value of the WT reporter when activating with Ets2 alone (Figure 3*A*). Comparably, the promoter/enhancer activity of WT but not MU *p16INK4A* reporters was down-regulated in HCT116 cells in a dose-dependent manner by dnTCF4-encoding expression constructs (Figure 3*B*). In combination, dnTCF4 and dnEts2 down-regulated the promoter activity of the WT *p16INK4A* reporter constructs additively in HCT116 cells (Figure 3*C*). Both HATs, p300 as well as CBP, stimulated transcription of WT p16INK4A reporter constructs in 293T cells already alone, which is in support with published data.[13] When added in combination with β-catenin–encoding expression vectors, transcriptional activity of WT *p16INK4A* reporters increased slightly synergistically (Figure 3*D*). Taken together, the proximal TBE-conferred β-catenin/TCF4–dependent transcriptional activity to the human *p16INK4A* promoter/enhancer could be increased further by action of Ets2 as well as HATs.

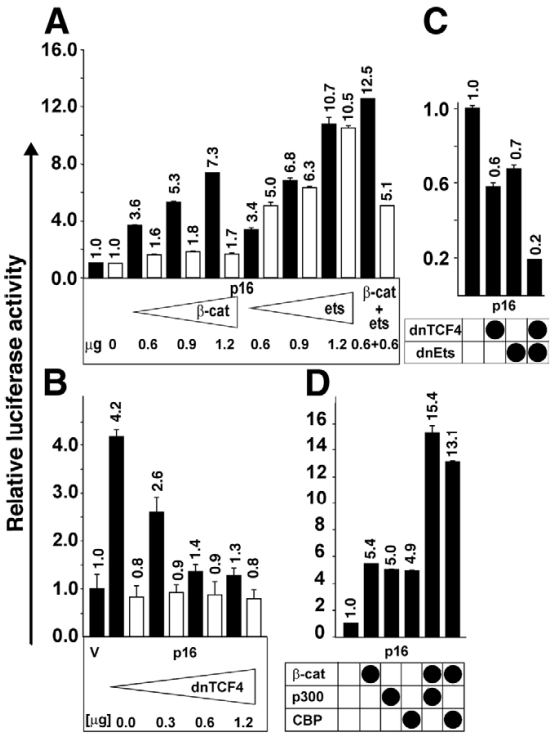

Figure 3. The proximal TBE of the human p16INK4A promoter/enhancer confers β-catenin/TCF-specific transactivation. (*A*) After transfection into 293 cells, luciferase reporter constructs containing the WT p16INK4A promoter/enhancer (*black bars*) were transactivated in a dose-dependent manner by a stabilized form of β-catenin (β-cat) as well as Ets2 (ets). Together, β-catenin and Ets2 (β-cat + ets) stimulated transactivation in an additive manner. Luciferase reporter constructs containing the MU p16INK4A promoter/enhancer (*stippled bars*) were expectedly transactivated only by Ets2 but no longer by β-catenin. When added together, the effect was thus comparable to the activity induced by Ets2 alone. (*B*) In HCT116 cells, dnTCF4 suppressed the activity of luciferase reporter constructs containing the p16INK4A promoter/enhancer containing a WT but not MU TBE sequence. (*C*) Moreover, dnTCF4 as well as dnEts2 suppressed the transcriptional activity of WT p16INK4A in HCT116 cells. (*D*) Two hundred ninety-3 cells displayed an activation of human p16INK4A promoter/enhancer luciferase reporter constructs by HATs, which was synergistically stimulated by β-catenin.

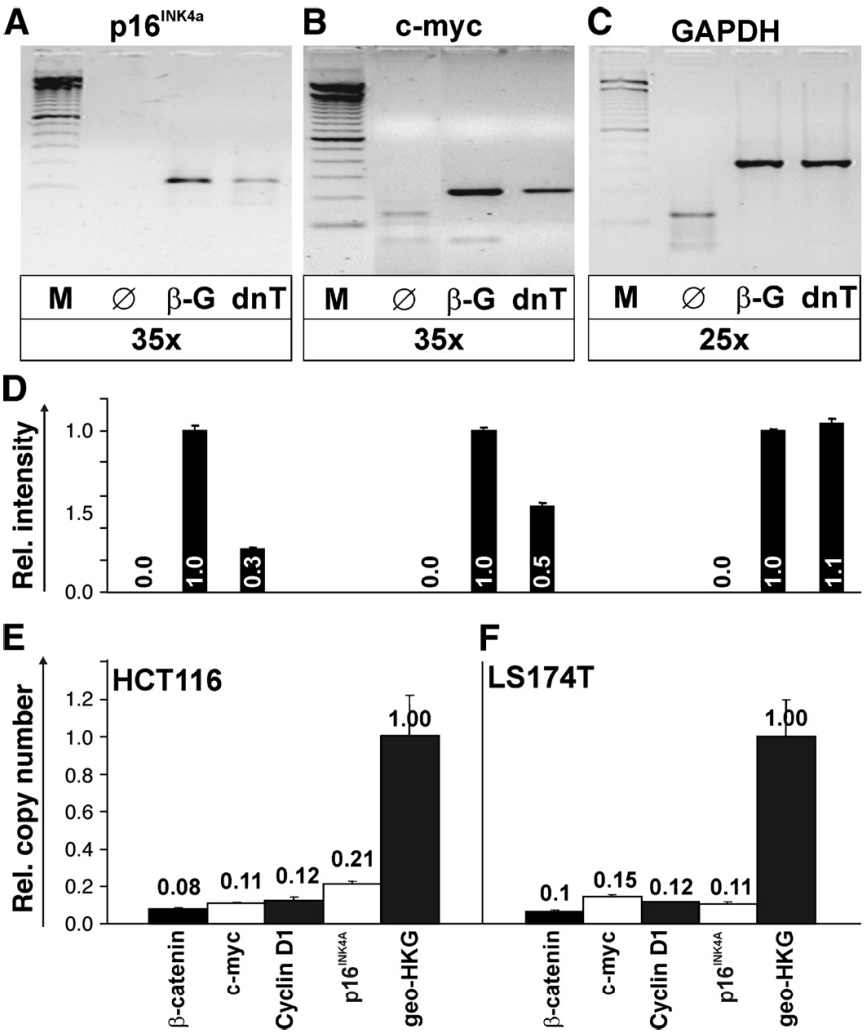

Figure 4. β-catenin/TCF regulates the endogenous expression of p16^{INK4A}. (*A* and *D*) dnTCF4 (dnT) in contrast to β-galactosidase (β-G) expressing adenovirus leads to the down-regulation of p16^{INK4A} (70%) and (*B* and *D*) c-myc mRNA levels in the human colorectal cell line HCT116. (*C* and *D*) In contrast, mRNA levels of the housekeeping gene GAPDH are unaffected by dnTCF4. (*D*) Densitometric analysis of the band intensities displayed in the gels. (M), 100-bp marker (Invitrogen); (∅), negative control using water instead of template. The number of cycles of the respective PCR is given below each gel photograph. (*E* and *F*) β-catenin–specific siRNA leads to the down-regulation of β-catenin-, c-MYC-, Cyclin D_1- and p16^{INK4A}-specific mRNA levels compared with the geometric mean of the 3 housekeeping genes HPRT, β-actin, and YWMAZ (geo-HKG) in the human colorectal tumor cell lines HCT116 (*E*) and LS174T (*F*).

The Activity of the Human p16^{INK4A} Gene Is Driven by β-Catenin/TCF

In the next set of experiments, we wanted to see if the activity of the endogenous *p16^{INK4A}* gene is regulated by β-catenin/TCF4. Therefore, cultivated human colorectal cells expressing p16^{INK4A} were transiently transduced applying adenoviruses (Figure 4*A–D*) or *β-catenin*–specific RNA interference (Figure 4*E* and *F*). Transient transduction of HCT116 cells using dnTCF4-expressing adenoviruses resulted in the reduction of *p16^{INK4A}*- (Figure 4*A*, *lane dnT* and *D*) and *c-MYC*- (Figure 4*B*, *lane dnT* and *D*) but not *GAPDH* mRNA levels (Figure 4*C*, *lane dnT* and *D*). Results were normalized on the respective amounts of PCR product levels of cells transduced with β-galactosidase–encoding adenoviruses (Figure 4*A–C*, *lanes β–G* and *D*). Next, we transiently transfected HCT116 as well as LS174T cells using siRNA specific for *β-catenin*-[23] or *GFP* RNA and analyzed changes in the expression levels applying qRT-PCR. All results were normalized on the geometric mean of the 3 housekeeping

genes *HPRT*, *β-ACTIN*, and *YWMAZ*. Application of *β-catenin*–specific siRNA resulted in the loss of *β-catenin*–specific mRNA (Figure 4*E* and *F*, *lane β-catenin*) and protein (data not shown) thus proving the experimental procedure. In parallel, mRNA levels of the β-catenin target genes *c-MYC*[17] and *CYCLIN* D_1[7,8,24] declined, as did the $p16^{INK4A}$-specific mRNA (Figure 4*E* and *F*, *lanes c-myc, cyclin* D_1, and $p16^{INK4A}$). Taken together, our results indicated that $p16^{INK4A}$ transcription is regulated by β-catenin/TCF4 in human CRC cell lines.

$p16^{INK4A}$ Expression at the Front of Invasion of Human CRC Is a Marker for Poor Survival

Finally, we wanted to investigate if the expression of $p16^{INK4A}$ correlates with low survival of patients. We expected such a result, as $p16^{INK4A}$ expression is a characteristic of the tumor cells in the budding zone, which has been shown already to be significantly correlated with low survival.[1,2] Therefore, 82 patients with CRCs (Table 2) that displayed budding at the infiltrative front of invasion in conjunction with tumor cells expressing β-catenin in the nucleus were immunohistochemically stained, employing $p16^{INK4A}$-specific antibodies. $p16^{INK4A}$ expression was found heterogeneously distributed (Figure 5*A*), which is in support with published data.[5,9,10] High expression levels of $p16^{INK4A}$ were seen especially in budding tumor cells located at the front of invasion (Figure 5*A* and *C*), whereas tumor cells found in central areas of the tumor displayed little-to-absent expression of $p16^{INK4A}$ (Figure 5*A* and *C*). Kaplan–Meier statistics and the log-rank test (Figure 5*D*) revealed a highly significant correlation of high $p16^{INK4A}$ expression with low survival ($P < .001$). $p16^{INK4A}$ expression neither correlated with gender, age, T- or N-stage, grading, nor adjuvant therapy (χ^2 test, Table 2). In a multivariate Cox-regression model, $p16^{INK4A}$ expression as well as T- and N-stages correlated significantly with lower survival rates (Table 2). Thus, expression of the cell cycle inhibitor $p16^{INK4A}$ at the infiltrative front of invasion of human CRC might be a prognostic marker for poor survival.

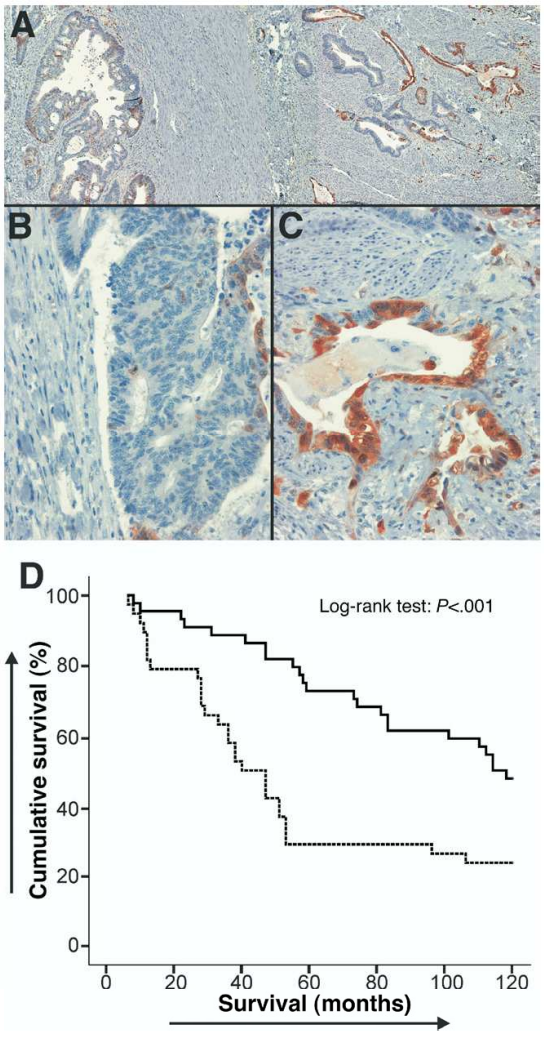

Figure 5. High expression of $p16^{INK4A}$ at the invasive front of CRC correlates with low survival rates. Representative (*A*) overview (50x) and high-power magnification (250x) of (*B*) central areas or (*C*) the invasive front of a typical CRC stained immunohistochemically using $p16^{INK4A}$-specific antibodies. (*D*) Kaplan–Meier curve and log-rank test assign high labeling index of $p16^{INK4A}$ to a lower survival rate in contrast to cases with a low labeling index.

Discussion

Tumor cells in the infiltrative front of invasion of CRCs displaying signs of EMT,[3] nuclear expression of β-catenin,[3,25] and low proliferation, correlated with the expression of $p16^{INK4A}$[3,5,9,10]. In this context, $p16^{INK4A}$ might play its well-known role of an inhibitor of the cell division cycle.[26] Alternatively, $p16^{INK4A}$ might induce—in conjunction with laminin-5γ2—migration and invasion of tumor cells, as has been demonstrated in a keratinocyte model of squamous cell carcinomas.[11] Interestingly, laminin-5γ2 is another β-catenin target gene,[12] and thus β-catenin might be an integrator of malignant transformation in this scenario.

Two conclusions might be drawn from our observations. First, $p16^{INK4A}$ is a target gene of β-catenin/TCF4 in CRCs. In contrast, in ACFs, $p16^{INK4A}$ expression was detected in the presence of intact APC, indicated by the immunohistochemical detection of full-length APC.[10] Thus, it was concluded that $p16^{INK4A}$ is not regulated via β-catenin/TCF4. But knowing that (1) most sporadic ACFs develop on the basis of a deregulated Ras/MAPK pathway[27] and that (2) Ras strongly transactivates the expression of $p16^{INK4A}$ [21] together with the fact that (3) most antibodies binding APC

are not specific for APC,[28] cast this conclusion into doubt. Additionally, in cell lines from endometrial carcinomas, *p16*^{INK4A} as well as *p21*$^{Cip1/Waf1}$ were both found to be regulated via β-catenin in a TCF4-independent manner.[13] Moreover, p16^{INK4A} was also transactivated by p21$^{Cip1/Waf1}$ even stronger than by β-catenin. But in CRCs, β-catenin/TCF4 leads to a robust down-regulation of p21$^{Cip1/Waf1}$ via up-regulation of the β-catenin/TCF4 target gene *c-MYC*.[14,17] Thus, the mechanisms of regulation transferred by β-catenin or β-catenin/TCF4 seem to differ from each other in colorectal and endometrial carcinomas and are thus not directly comparable.

Second, the role of proliferation might have been overestimated as a driving force for the malignant progression of CRCs. Interestingly, a minority of tumor cells defined by the expression of p16^{INK4A}, and therefore, probably low proliferation seems to be responsible for the correlation with low survival. These tumor cells are constituents of the budding zone, which was shown to be a good prognostic marker for low survival.[1,2] In such a sense, expression of p16^{INK4A} and the associated low proliferation[5,9,10] might be an attribute or characteristic of these tumor cells that differ from the rest of the tumor. Certainly, the clinical relevance of the p16^{INK4A} expression for survival presented here and in other studies is limited and should be re-evaluated in larger cohorts containing more clinical information and aspects than our small collection of patients.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.09.019.

References

1. Hase K, Shatney C, Johnson D, et al. Prognostic value of tumor "budding" in patients with colorectal cancer. Dis Colon Rectum 1993;36:627–635.
2. Ueno H, Murphy J, Jass JR, et al. Tumour "budding" as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology 2002;40:127–132.
3. Brabletz T, Jung A, Reu S, et al. Variable β-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci U S A 2001;98:10356–10361.
4. Brabletz T, Jung A, Spaderna S, et al. Opinion: migrating cancer stem cells–an integrated concept of malignant tumour progression. Nat Rev Cancer 2005;5:744–749.
5. Jung A, Schrauder M, Oswald U, et al. The invasion front of human colorectal adenocarcinomas shows co-localization of nuclear β-catenin, cyclin D1, and p16INK4A and is a region of low proliferation. Am J Pathol 2001;159:1613–1617.
6. Brabletz T, Jung A, Kirchner T. β-catenin and the morphogenesis of colorectal cancer. Virchows Arch 2002;441:1–11.
7. Shtutman M, Zhurinsky J, Simcha I, et al. The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 1999;96:5522–5527.
8. Tetsu O, McCormick F. β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 1999;398:422–426.
9. Palmqvist R, Rutegard JN, Bozoky B, et al. Human colorectal cancers with an intact p16/cyclin D1/pRb pathway have up-regulated p16 expression and decreased proliferation in small invasive tumor clusters. Am J Pathol 2000;157:1947–1953.
10. Dai CY, Furth EE, Mick R, et al. p16(INK4a) expression begins early in human colon neoplasia and correlates inversely with markers of cell proliferation. Gastroenterology 2000;119:929–942.
11. Natarajan E, Saeb M, Crum CP, et al. Co-expression of p16(INK4A) and laminin 5 γ2 by microinvasive and superficial squamous cell carcinomas in vivo and by migrating wound and senescent keratinocytes in culture. Am J Pathol 2003;163:477–491.
12. Hlubek F, Jung A, Kotzor N, et al. Expression of the invasion factor laminin γ2 in colorectal carcinomas is regulated by β-catenin. Cancer Res 2001;61:8089–8093.
13. Saegusa M, Hashimura M, Kuwata T, et al. Induction of p16INK4A mediated by β-catenin in a TCF4-independent manner: implications for alterations in p16INK4A and pRb expression during trans-differentiation of endometrial carcinoma cells. Int J Cancer 2006;119:2294–2303.
14. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–250.
15. Rowan AJ, Lamlum H, Ilyas M, et al. APC mutations in sporadic colorectal tumors: a mutational "hotspot" and interdependence of the "two hits." Proc Natl Acad Sci U S A 2000;97:3352–3357.
16. Hiendlmeyer E, Regus S, Wassermann S, et al. β-catenin up-regulates the expression of the urokinase plasminogen activator in human colorectal tumors. Cancer Res 2004;64:1209–1214.
17. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–1512.
18. Molenaar M, van de Wetering M, Oosterwegel M, et al. XTcf-3 transcription factor mediates β-catenin-induced axis formation in Xenopus embryos. Cell 1996;86:391–399.
19. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–1512.
20. Esteller M, Tortola S, Toyota M, et al. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. Cancer Res 2000;60:129–133.
21. Ohtani N, Zebedee Z, Huot TJ, et al. Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. Nature 2001;409:1067–1070.
22. Hecht A, Vleminckx K, Stemmler MP, et al. The p300/CBP acetyltransferases function as transcriptional coactivators of β-catenin in vertebrates. Embo J 2000;19:1839–1850.
23. Verma UN, Surabhi RM, Schmaltieg A, et al. Small Interfering RNAs directed against β-catenin inhibit the in vitro and in vivo growth of colon cancer cells. Clin Cancer Res 2003;9:1291–1300.
24. Sansom OJ, Reed KR, van de Wetering M, et al. Cyclin D1 is not an immediate target of β-catenin following Apc loss in the intestine. J Biol Chem 2005;280:28463–28467.
25. Brabletz T, Jung A, Hermann K, et al. Nuclear overexpression of the oncoprotein β-catenin in colorectal cancer is localized predominantly at the invasion front. Pathol Res Pract 1998;194:701–704.
26. Sherr CJ. Principles of tumor suppression. Cell 2004;116:235–246.
27. Takayama T, Ohi M, Hayashi T, et al. Analysis of K-ras, APC, and β-catenin in aberrant crypt foci in sporadic adenoma, cancer, and familial adenomatous polyposis. Gastroenterology 2001;121:599–611.
28. Brocardo M, Nathke IS, Henderson BR. Redefining the subcellular location and transport of APC: new insights using a panel of antibodies. EMBO Rep 2005;6:184–190.
29. Wang W, Wu J, Zhang Z, Tong T. Characterization of regulatory elements on the promoter region of p16(INK4a) that contribute to

overexpression of p16 in senescent fibroblasts. J Biol Chem 2001;276:48655–48661.
30. Pagliuca A, Gallo P, Lania L. Differential role for Sp1/Sp3 transcription factors in the regulation of the promoter activity of multiple cyclin-dependent kinase inhibitor genes. J Cell Biochem 2000;76:360–367.
31. Hara E, Smith R, Parry D, et al. Regulation of p16CDKN2 expression and its implications for cell immortalization and senescence. Mol Cell Biol 1996;16:859–867.
32. Myohanen S, Baylin SB. Sequence-specific DNA binding activity of RNA helicase A to the p16INK4a promoter. J Biol Chem 2001;276:1634–1642.

Received November 13, 2007. Accepted September 11, 2008.

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The authors disclose the following: the paper was supported by Wilhem-Sander-Stiftung Az.: 2004-111 to T.K. and A.J.

S.W. and S.K.S. contributed equally to this work.

The authors thank Dr Gordon Peters (UCL, London, UK) for kindly supporting the WT *p16INK4A*-luciferase reporter construct (pGL2-p16 -3027), Dr Bert Vogelstein (Johns Hopkins University, Baltimore) for the kind gifts of *GST-TCF4* (pGST-TCF4 [DBD]), *β-catenin* (pcI-Neo-β-catenin Δ45) and *dnTCF4*-encoding expression vectors (pcDNA/myc-ΔN-TCF4) as well as *dnTCF4*-encoding adenovirus and the adenoviral clone pAdEasy-Gal expressing *β-galactosidase*, Dr Craig Hauser (The Burnham Institute, La Jolla, CA) for donating *Ets2* (FNEts2) as well as *dnEts2* (FNEts2 DBD) encoding expression vectors, Dr Richard Eckner (Institute of Molecular Biology, Zurich, Switzerland) for supplying *p300* (pCMVβ-p300) and *CBP* (pCS2+/CBP-HA) expression clones.

3.6 Das β-CATENIN Zielgen FGF-2 ist ein Mediator von Stemness, EMT und Chemoresistenz in humanen kolorektalen Tumorzellen

The β-CATENIN Target Gene *FGF-2* is a Mediator of Stemness, EMT and Chemoresistance in Human Colorectal Tumor Cells

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Running Title

The β-CATENIN target FGF-2 mediates tumor stemness

Summary

In colorectal cancers mutations in the canonical WNT signaling pathway induce the stabilization of β-CATENIN which is the kingpin of colorectal carcinogenesis as it is involved in the regulation of the hallmarks of cancer namely proliferation, angiogenesis, survival, migration, invasion and evasion of apoptosis. Furthermore, β-CATENIN induces and maintains stemness and associated characteristics like epithelial-mesenchymal transition (EMT) or chemoresistance. Mechanistically, all of these functional properties are mediated by β-CATENIN target genes but those genes which are involved in the induction of stemness and accompanying traits remained elusive. Here, we show that FGF-2 (fibroblast growth factor-2) is transcriptionally up-regulated by β-CATENIN in colorectal cancer and is a mediator of stemness, EMT and chemoresistance in colorectal tumor cells.

Significance

Cancer stem cells (CSCs) are considered to be the driving force for many if not all tumors. Their progeny gives rise to the mass of the tumors whereas the CSCs themselves represent only a minor population. Importantly, they are responsible for the formation of metastases and are additionally highly resistant against chemotherapeutic agents. Therefore, unraveling the mechanisms that help to maintain pluripotent CSCs might be of great therapeutic value. Here, we present data that demonstrate a possible mechanism how β-CATENIN maintains CSCs by the transcriptional upregulation of the *FGF-2* gene. FGF-2 confers stemness and associated characteristics like EMT or chemoresistance. As a consequence, the FGFR/FGF-2 system emerges as an appealing target for therapeutically targeting the WNT-signaling pathway indirectly.

Introduction

The carcinogenesis of classical colorectal cancers (CRCs) proceeds via an adenoma carcinoma sequence which is driven by acquiring mutations in oncogenes (gain of function) and tumorsuppressor genes (loss of function). A sum of 60 to 80 mutations was found in various types of carcinomas, like the colorectum, pancreas, mamma, or others (Jones et al., 2008). In CRCs some definite driver genes are mutated with high frequency such that they comprise mountains when depicting the mutations as a landscape (Sjoblom et al., 2006). One of these mountain-genes is the tumorsuppressor gene *APC* (adenomatous polyposis coli) which is usually mutated at the very beginning of colorectal tumor development in the majority of cases (up to 80%). Therefore, *APC* is the gatekeeper of colorectal carcinogenesis (Kinzler and Vogelstein, 1996) but only when it is mutated in colorectal crypt stem cells (Barker et al., 2009). These altered stem cells develop into colorectal cancer stem cells (coCSCs) thereby gaining malignant potential. Although coCSCs comprise only the minor part of colorectal tumors, they are the driving force for the formation of metastases (Pang et al., 2010) and tumor associated death of patients (Horst et al., 2009). coCSCs are induced and maintained by high WNT activity (Vermeulen et al., 2010b) which is indicated by nuclear β-CATENIN and its transcriptional actions (Brabletz et al., 2005b). β-CATENIN is the transducer of the canonical WNT signaling pathway which regulates the stability of β-CATENIN via a multiprotein degradation complex containing APC as an integral part (Gregorieff and Clevers, 2005). Although *APC* is mutated, the subcellular localization of β-CATENIN still can be regulated, what is known as the β-CATENIN paradox (Fodde and Brabletz, 2007). Only when expressed in the cells` nuclei, β-CATENIN works in conjunction with DNA binding HMG (high mobility group) proteins of the LEF-1/TCF4 (lymphocyte enhancing factor-1/ T-cell factor 4) family and a variety of other proteins as a transcription factor (Gregorieff and Clevers, 2005). β-CATENIN target genes mediate the hallmarks of cancer (Hanahan and Weinberg, 2000) and moreover, β-CATENIN induces stemness of coCSCs (Vermeulen et al., 2010b) and EMT (epithelial-mesenchymal transition) (Brembeck et al., 2004), which are closely connected with each other if not the same (Mani et al., 2008). But it remains largely unknown, how β-CATENIN regulates stemness or EMT mechanistically. Such a regulator should ideally be a β-

CATENIN target gene and essentially involved in the maintenance of stemness and functions associated with stemness like EMT, resistance against chemotherapeutics besides others. A potential candidate for such a regulator are fibroblast growth factors (FGF). They are known to be important factors during the course of tumorigenesis and have been ascribed to many traits of CSCs, especially EMT and chemoresistance (Turner and Grose, 2010). Additionally, the strict dependency of (co)CSCs on FGF-2 when being cultured *in vitro* (Ricci-Vitiani et al., 2007; Vermeulen et al., 2010a) is another important support of this view. Here, we show evidence that *FGF-2* is a β-CATENIN target gene which confers stemness, expression of stem cell markers, EMT, and chemoresistance and is thus a possible mediator explaining how β-CATENIN regulates stemness and stemness associated functions in colorectal tumor cells.

Results

FGF-2 Is Expressed in Disseminated Tumor Cells at the Invasion Front and in Cultivated Cell Lines of Human CRC

Human CRCs displaying an invasion front with infiltrating tumor cells are a well suited model for the identification of β-CATENIN target genes (Brabletz et al., 2005b). Tumor cells at the invasion front are characterized by nuclear expression of β-CATENIN and β-CATENIN target genes, as well as EMT. Therefore, we investigated whether such cells express FGF-2 employing immunohistochemistry. We observed strong expression of FGF-2 in tumor cells with nuclear β-CATENIN, whereas those without staining for nuclear β-CATENIN displayed only background levels of FGF-2 (Figure 1 A). Furthermore, expression of FGF-2 was detected in cultivated colorectal tumor cell lines LoVo, DLD-1, HCT15 and HCT116 but not in SW480, LS174T, Caco2 or HT29 cells on the mRNA (Figure 1 B) and protein level (Figure 1 C). The latter was characterized by the appearance of various FGF-2 isoforms, which also included the secreted 18 kDa low molecular weight form (Figure 1 C) known to induce signaling via binding to its cognate receptors FGFR1-4 (Dvorak et al., 2006; Turner and Grose, 2010). Thus, we investigated if the cultivated colorectal cell lines also expressed mRNA specific for these FGFRs. Most of the tumor cell lines

expressed at least one of the FGFRs (Supplemental Figure 1) indicating that these tumor cells might possess an intact FGF-2/FGFR signaling system. As neither the mode of FGF-2 signaling nor the functional role of the different isoforms, which all had been linked to stemness (Thomas-Mudge et al., 2004; Yu et al., 2007), were within our scope, we did not follow these aspects. Taken together, we show that FGF-2 expression is commonly found in colorectal tumor cells and is associated with the nuclear expression β-CATENIN.

FGF-2 Expression is Regulated by β-CATENIN

The coexpression of FGF-2 and nuclear β-CATENIN in human CRCs implicated *FGF-2* to be a β-CATENIN target gene. To clarify this point, we first knocked-down the mRNA of *β-CATENIN* specifically using RNA-interference (RNAi) in the *FGF-2* expressing cell lines LoVo, HCT15 and DLD-1 (Figure 1 B, C). The knock-down led to a strong reduction of *β-CATENIN* mRNA levels (86% to 93%), which was accompanied by the down-regulation of the well known β-CATENIN target gene *c-MYC* (He et al., 1998) to residual amounts of 14 to 38% (Figure 2 A), thus approving the success of the knock-down. Importantly, *FGF-2* mRNA amounts were also down-regulated in all three cell lines to levels between 14 and 66% (Figure 2 A). Second, the existence of TCF4 binding elements (TBE, consensus sequence: WTCAAAG (Hatzis et al., 2008)) in the promoter/enhancer regions of β-CATENIN target genes is a prerequisite for the transcriptional activation of genes via β-CATENIN as they are the binding sites for TCF4/β-CATENIN complexes. The promoter/enhancer of the *FGF-2* gene (accession no.: S81809) contains a single consensus TBE at position –387 (TTCAAAG) upstream of the transcription start (Figure 2 B). Binding of TCF4 to this site was analyzed with the help of EMSAs (electric mobility shift assay) and ChIPs (chromatin immune precipitation). Recombinant GST-TCF4 fusion protein bound radioactively labeled oligonucleotide probes when containing the *FGF-2* TBE in its WT (Figure 2 C, lane 2) but not mutated (CGCAAAG, lane 7) form. Binding of GST-TCF4 was competed by unlabeled WT *FGF-2* (WT, lane 3) or *c-MYC* ((He et al., 1998), MWT, lane 5) but not mutated *FGF-2* probes (MUT, lane 4). Binding was specific for TCF4 as GST alone did not bind the WT *FGF-2* probe (lane 6). Next, we

analyzed the binding of TCF4 to a region of the *FGF-2* gene containing the promoter/enhancer in the context of native chromatin employing ChIPs. LoVo cells were selected for ChIPs as they expressed the highest amounts of *FGF-2* mRNA in our panel of cultivated colorectal cell lines (Figure 1 B). Antibodies specific for TCF4 (α-TCF4) but neither immunoglobulins (IgG) nor antibodies specific for RNA-Polymerase II (α-PolII) could precipitate DNA fragments containing the TBE of the human *FGF-2* promoter (Figure 2 D). The detection of a TATA box-containing sequence of the *glycerol-aldehyde-3-phosphate-dehydrogenase* (*GAPDH*) gene made sure that the α-PolII specific antibodies worked in general. Unexpectedly, α-TCF4 also slightly precipitated these TATA-box containing regions of the *GAPDH* gene (Figure 2 D) which might be due to the fact that a consensus TBE was found at -423 bp upstream of the TATA-box (accession no. AY340484). Third, we investigated whether β-CATENIN/TCF4 conferred functional activity to the *FGF-2* gene employing *FGF-2* promoter/enhancer-*luciferase* gene reporter assays. The *luciferase* gene was driven by fragments of the human *FGF-2* gene (-1.800 / +314) containing either the WT TBE or the mutant form (Figure 2 B, MUT, CGCAAAG) that did not bind TCF4 in EMSAs (Figure 2 C). These reporter plasmids were transiently transfected into LoVo or HCT15 cells together with increasing amounts of a plasmid encoding dominant negative (dn) TCF4, which is an inhibitor of β-CATENIN/TCF4 transcriptional activity (Tang et al., 1998). This resulted in a dose-dependent decline in the activity of the WT- but not MUT *FGF-2* reporter constructs which did not respond at all (Figure 2 E). These results were comparable with data obtained when using Super-TOPflash or Super-FOPflash as a positive control system for the function of the experimental setting (Supplemental Figure 2). Taken together, we present several lines of evidence indicating that *FGF-2* is a direct target gene of β-CATENIN.

Loss of FGF-2 Ameliorates the Transformed Phenotype of Cultivated Colorectal Tumor Cells

Now we wanted to learn about the effects of FGF-2 on the transformed phenotype and initiation of tumor growth. Both are well known functional characteristics of CSCs and had been shown to depend on FGF2 (Turner and Grose, 2010). Thus, FGF-2 expression was stably knocked down in colorectal LoVo cells, which were chosen

due to their high FGF-2 expression level. Two independent clones each expressing FGF-2 specific (sh FGF-2#1 and #2) or enhanced Green Fluorescent Protein specific shRNA (sh eGFP#1 and #2), respectively, were selected from a screen using five different FGF-2 or eGFP specific shRNA encoding lentiviruses for either target. This RNAi approach led to strong reduction of FGF-2 expression in sh FGF-2#1 and #2 clones on mRNA- (12 and 11% respectively) and protein levels compared to sh eGFP#1 and #2 clones (Figures 3 A and B). The knockdown did neither alter proliferation, measured as the change in cell numbers over time (Figure 3 C), nor apoptosis indicated by the absence of cleaved Caspase 3 (Figure 3 D). However, the loss of FGF-2 led to a substantial decrease in the transformation capacity (Figure 3 E) when investigating anchorage independent growth of sh FGF-2 and sh eGFP clones in methylcellulose medium. To exclude cell line specific or off target effects, the same set of experiments was repeated with the cell lines HCT15 and HCT116. Here, bulk cultures grown for 14 days under puromycin selection after lentiviral transduction with two different sh FGF-2 encoding lentiviruses were employed instead of stable clones yielding comparable results (Supplemental Figure 3). Finally, we tested the tumorigenic potential of the different LoVo clones. Therefore, $2 \cdot 10^6$ parental, sh FGF-2 or sh eGFP LoVo cells were injected subcutaneously (s.c.) into nude mice. Parental (6/6) and sh eGFP cells (5/6) developed tumors of 2 cm^3 size after 4 to 8 weeks whereas sh FGF-2 cells hardly initiated tumor growth (1/8). Moreover, this single tumor was smaller (~0.6 cm^3) and needed 10 to 12 weeks for outgrowth (Figure 3 F). Of note, this effect is unlikely to be based on differences in proliferation or apoptosis as both were not affected in sh FGF-2 LoVo tumor cells. In summary, our results demonstrated that FGF-2 is an essential component for the maintenance of a malignant phenotype reflected by anchorage independent growth and tumor initiation in colorectal tumor cells but does not seem to influence proliferation or anti-apoptosis.

FGF-2 Contributes to the Mesenchymal Phenotype of LoVo Cells

Stemness and EMT are closely related if not the same (Mani et al., 2008). Both are made responsible for malignancy (Pang et al., 2010; Thiery, 2002) and are induced by β-CATENIN (Brembeck et al., 2004; Vermeulen et al., 2010b). Thus, we wanted to

know if FGF-2 might play a crucial role in the maintenance of EMT, especially as a connection of FGF-2 and EMT had been shown in nasopharyngeal carcinomas and other cellular systems before (Billottet et al., 2004; Chen et al., 1994; Strutz et al., 2002). When inspecting the growth patterns of LoVo cell clones in closer detail, we found that parental LoVo cells and LoVo sh eGFP clones contained patches of fibroblast like looking cells, which was reminiscent of a mesenchymal differentiation (Figure 4 A, lower panel). In contrast, sh FGF-2 clones were mostly flattened, enlarged, and seldom showed a fibroblast like morphology (Figure 4 A, upper panel). On the molecular level, sh FGF-2 clones expressed significantly higher mRNA amounts of the epithelial marker molecule *E-CADHERIN* (4.2 to 6.3 times) but at the same time less mRNA of the mesenchymal marker genes *VIMENTIN* (11 to 26%) or *FIBRONECTIN* (28 to 31%), respectively, when compared to sh eGFP clones (Figure 4 B). Moreover, we were keen to see if FGF-2 also effected the expression of ZEB-1 (zinc finger E-box binding), the master regulator of EMT in colorectal cancer cells (Spaderna et al., 2006). Indeed, loss of FGF-2 expression was related with the downregulation of ZEB-1 on the mRNA (11 to 18%) (Figure 4 C) and protein level (Figure 4 D). As expected, this effect was accompanied by higher protein levels of E-CADHERIN (Figure 4 D and E) which is a direct target of ZEB-1 mediated transcriptional repression. However, this discovery could not be extended to the cell lines HCT15 and HCT116 (data not shown), which might be explained by the fact that both cell lines expressed naturally low amounts of *FGF-2* mRNA (Figure 1 B; 10 to 20%) compared to LoVo cells. Moreover, they were characterized by an already high epithelial differentiation indicated by high levels of *E-CADHERIN* mRNA expression (9.5 or 2.4 fold sh eGFP HCT15 or -HCT116 cells, respectively) but low levels of *VIMENTIN* as well as *ZEB-1* (Supplemental Figure 4 A) compared to sh eGFP LoVo cells. As the induction of EMT is frequently paralleled by an increase in migration and invasion, we analyzed the impact of FGF-2 on the invasive properties of LoVo sh FGF-2 clones employing Matrigel® coated Boyden-chambers. The knockdown of FGF-2 did not alter the invasive properties of sh FGF-2 clones (Supplemental Figure 4 B). As the knock-down of β-CATENIN affects both, EMT as well as migration and invasion, our results indicated that FGF-2 relays only the EMT effect of β-CATENIN but neither migration nor invasion for which other β-CATENIN target genes have been made responsible (Brabletz et al., 2005b).

FGF-2 Contributes to a Cancer Stem Cell Phenotype in LoVo Cells

Stemness of coCSCs is indicated by the expression of surface markers like CD133 (O'Brien et al., 2007; Ricci-Vitiani et al., 2007), CD44 or CD166 (Dalerba et al., 2007), as well as the export of the Hoechst 33342 dye (Sussman et al., 2007) which are therefore commonly employed for the isolation and enrichment of coCSCs. First, we found that LoVo sh FGF-2 clones expressed reduced levels of *CD44* and *CD166* mRNA compared to sh eGFP clones (Figure 5 A). Second, we enriched side population (SP) cells from parental LoVo cells employing FACS together with Hoechst 33342 dye (Figure 5 B). Expectedly, the SP of LoVo cells (1.36%) expressed significant higher amounts of the coCSC markers *CD166* and *CD133* when compared to the non-SP fraction (Figure 5 B). Interestingly, SP LoVo cells also expressed higher mRNA amounts of *FGF-2* than non-SP cells (Figure 5 B) indicating that FGF-2 expression might be an attribute of tumor cells with signs of stemness. Third, we were interested to follow the effect of FGF-2 loss on the SP. LoVo sh FGF-2 clones contained a significant smaller SP (0.097%) compared to the sh eGFP clones (0.58%) or parental cells (0.76%) (Figure 5 C). Fourth, we showed previously that a mixture of conditioned medium from bone marrow stromal cells exposed to hypoxia mixed with conditioned medium from “injured cells” induced the migration of SP cells through Matrigel® coated Boyden chambers resulting in mSP (migratory SP) cells. These cells displayed enhanced characteristics of stemness (Das et al., 2008). Using this approach, LoVo sh FGF-2 clones contained only a small fraction of mSP cells (8%) compared to sh eGFP clones (54.5%) or parental LoVo cells (61.5%) (Figure 5 D). Taken together, this panoply of experimental data assigned FGF-2 a fundamental and mechanistic role in maintaining stemness in colorectal tumor cells.

FGF-2 Confers Resistance against 5-FU

Another well known functional trait of (co)CSCs is their resistance against chemotherapeutic drugs like 5-Fluorouracil (5-FU) (Gupta et al., 2009; Todaro et al., 2007; Tsuchida et al., 2008). As a consequence, chemotherapeutic treatment over time enriches for CSCs which is paralleled by an increase of features of stemness as well as EMT (Creighton et al., 2009). First, we examined if 5-FU treatment (two

weeks, 10 µg/ml) led to changes in the expression of FGF-2 and CSC markers in LoVo cells. Surviving cells displayed a mesenchymal morphology (Figure 5 E) and expressed higher mRNA levels of *VIMENTIN* (data not shown), thus indicating EMT, as well as the coCSC markers *CD44* (3.06 fold) and *CD166* (2.26 fold). Interestingly, *FGF-2* mRNA levels became also elevated (2.56 fold, Figure 5 F). Second, we increased the amount of 5-FU over a two days' period and followed the expression levels of *CD44*, *CD166*, and *FGF-2*. The expression of all genes increased in parallel with the concentration of 5-FU in a dose dependent manner (Supplemental Figure 5 A). Incubation for a longer period of 7 days did not change the expression levels any more (Supplemental Figure 5 B). These experiments were repeated using HCT15 cells which yielded comparable results (Supplemental Figure 5 B). Third, we were interested to see if the effects of chemotherapy were reversible and if surviving cells could give rise to a normal, epithelially differentiated population again. As LoVo cells harbor a WT p53 gene, the chemotherapy surviving cells showed an arrest of the cell cycle (data not shown) which was reflected by the upregulation of the cell cycle inhibitor and p53 target gene $p21^{CIP1/WAF1}$ (Supplemental Figure 5 C, upper panel). Retraction of 5-FU treatment for two weeks resulted in a complete reversion of the expression of the CSC markers *CD44* and *CD166* (Figure 5 H), the cell cycle inhibitor $p21^{Cip1/WAF1}$ (Supplemental Figure 5 C), and the morphology of the cells (Figure 5 G). In contrast, the expression level of *FGF-2* remained unaltered after this period of time (Figure 5 H). Finally, we were keen to investigate the role of FGF-2 as a driving force for 5-FU resistance. Therefore, we employed our LoVo clone system by comparing the viability of sh FGF-2- to sh eGFP clones after exposition to increasing amounts of 5-FU. LoVo shFGF-2 clones proved to be less resistant (Figure 5 I, IC50 between 100 and 333µM) than sh eGFP clones (Figure 5 I, IC50 higher than 666µM 5-FU). In summary, we showed that a chemotherapeutic challenge increased attributes of stemness in colorectal tumor cells together with an increase in FGF-2 expression. Vice versa, FGF-2 is an essential mediator for chemoresistance and again stemness.

Stemness and Transformation of Weakly FGF-2 Expressing Caco2 cells also Depend on FGF-2

As our experiments supported the view that endogenous FGF-2 expression might be a β-CATENIN regulated mechanism of stemness in CRC cell lines, we wondered wherefore some colorectal cell lines with an activated WNT/β-CATENIN pathway should be virtually negative for FGF-2 expression (Figure 1B). Thus, we aimed to repeat the experimental procedure with FGF-2 negative Caco2 cells. We found an SP-fraction of only 0.14% in size (Figure 6 A) indicating that the amounts of coCSCs represent in this cell line only $^1/_{10}$ to $^1/_5$ of that found in Lovo cells (Figure 5 B and C). In general, analogy to our findings in LoVo cells was observed in Caco2 cells. Caco2 SP-cells expressed higher mRNA levels of the coCSC markers *CD44*, *CD166* (Dalerba et al., 2007) and *CD133* (O'Brien et al., 2007; Ricci-Vitiani et al., 2007) compared to parental and non-SP cells. The small size of the fraction of cells with traits of coCSCs might explain why the bulk culture of Caco2 cells remained negative in the detection of FGF-2 (Figure 1 B, C). Expectedly, expression of *FGF-2* became detectable in the SP-fraction of Caco2 cells enriched for coCSCs (Figure 6 B). Next, the tumor initiation capacity of Caco2 SP-cells was tested. Therefore, $1 \cdot 10^5$ cells of either Caco2 SP-, non-SP- or parental cells were injected s.c. into nude mice. Tumors were only initiated with Caco2 SP cells within 6 – 8 weeks (Table 1). To pin down the importance of FGF-2, the undetectable amounts of FGF-2 were knocked down in Caco2 cells comparably to LoVo cells. Interestingly, the results were completely analogous to LoVo cells as the knockdown of FGF-2 had no obvious effect on apoptosis (Figure 6 C) or proliferation (Figure 6 D), but substantially decreased anchorage independent growth in methylcellulose (Figure 6 E).

Moreover, the capacity of Caco2 sh FGF-2 (sh FGF-2 C) clones to initiate tumors after xenografting s.c. into immunocompromised mice was significantly affected. Only mice being injected with $2 \cdot 10^6$ Caco2 sh eGFP (sh eGFP C) cells developed visible and palpable tumors after an observation time of 8 weeks (8 out of 8 animals), while mice being injected with Caco2 sh FGF2 cells did not show formation of tumors in this period (data not shown). However, because tumor initiating potency might be underestimated when monitoring only for 8 weeks (Quintana et al., 2008), we waited up to twelve weeks and reanalyzed the tumor incidence in the mice. We found that all

animals displayed visible tumors but that those deriving from Caco2 sh FGF2 cells were significantly smaller than the tumors from the control cells (Figure 6 F). Since we did not observe changes in proliferation or apoptosis, these results collectively suggest that Caco2 sh FGF2 clones contained less cells with the ability for tumor initiation. Thus, even in colorectal cell lines expressing only trace amounts of FGF-2 characteristics of stemness and transformation are lost when knocking down FGF-2 expression.

Discussion

We present evidence that *FGF-2* is a β-CATENIN target gene in CRCs and is upregulated in colorectal tumor cells displaying nuclear expression of β-CATENIN. Moreover, our data indicate that FGF-2 has an essential role in the maintenance of stemness and EMT which are functionally tightly coupled characteristics (Mani et al., 2008). This also applies for the observed resistance against chemotherapy which is also a reminiscence of stemness (Gupta et al., 2009; Todaro et al., 2007). As a transcription factor β-CATENIN exerts its effects via expression of its target genes what has been shown for most if not all of the functions resembling the hallmarks of cancer (Brabletz et al., 2005a; Hanahan and Weinberg, 2000). Stemness together with its associated EMT and chemoresistance might also be considered as a more recently found hallmark of cancer. But for this hallmark no β-CATENIN target genes have been described up to know. This missing link might be filled in nicely by FGF-2 (Figure 7A).

The addition of the β-CATENIN target gene *FGF-2* now helps to better understand the process of colorectal carcinogenesis. For doing so we start with the migrating cancer stem cell concept (Brabletz et al., 2005b) because this conception integrates first of all the histological adenoma-carcinoma sequence (Muto et al., 1975), second the accumulation of mutations in oncogenes (gain of function) and tumorsuppressor genes (loss of function) of the multistep carcinogenesis model (Kinzler and Vogelstein, 1996) and finally the more recent CSC-model (Pardal et al., 2003). Normal adult stem cells are located at the bottom of intestinal crypts where pericryptal lying myofibroblasts are thought to secrete Wnt ligands and by this generate a niche for the intestinal stem cells (Gregorieff and Clevers, 2005). It is

unknown if EGF (*epidermal growth factor*) and FGF-2 which are both essential for the maintenance of CSCs *in vitro* are also needed for the survival of adult stem cells. Moreover, it is also not known if both factors are made by the pericryptal myofibroblasts or the stem cells themselves as a result of paracrine WNT stimulation. The latter possibility not only applies for FGF2 but also for EGF which might be a β-CATENIN target gene as well, since it contains a TBE in its promoter/enhancer region, too (accession no NT_016354.19, position -499) (Figure 7 B). With this in mind it might become clear why the initiation of colorectal tumorigenesis works already when the WNT-signaling pathway is deregulated for example only by mutations in the APC tumorsuppressor gene (Barker et al., 2009) which is another manifestation of the gatekeeper function of APC (Kinzler and Vogelstein, 1996). As a consequence, adult stem cells are transformed into coCSCs. With a β-CATENIN mediated autocrine expression of FGF-2 and maybe EGF coCSCs now become independent from their niche and are licensed to leave this defined region (Figure 7 B) while developing adenomas and carcinomas as a consequence of the actions of other β-CATENIN target genes.

Second, our results suggest wherefore colorectal tumor cells at the invasive front of CRCs display EMT. We demonstrated that FGF-2 is an essential regulator of ZEB-1, which is known to be the master switch factor for the induction of EMT in colorectal tumor cells (Spaderna et al., 2006). This is in line with the observation that the epithelial marker E-CADHERIN is up- but the mesenchymal markers VIMENTIN as well as FIBRONECTIN are down-regulated in the absence of FGF-2. Interestingly, FGF-2 does not seem to be involved in the regulation of invasion or migration. Here, other β-CATENIN target genes are known to be involved, like MMP-7 (matrix metalloproteinase-7), UPA (urokinase plasminogen activator), laminin-5γ2 or MT-MMP1 (membrane type matrix metalloproteinase 1) (Brabletz et al., 2005b) (Figure 7 A).

Third, aggressiveness of CRCs is correlated with the number of disseminated cells which are characterized by nuclear β-CATENIN at the invasive margin of the tumors (Brabletz et al., 2005b; Ueno et al., 2002) and also show reasonable expression of FGF-2. Our data are thus in support with others that anti-FGF-2 targeted therapies might be a reasonable strategy to attack these potential coCSCs (Turner and Grose,

2010). Therefore, by targeting the activity of the β-CATENIN target gene *FGF-2* it might become possible to treat CRCs with a deregulated WNT/β-CATENIN signalling pathway and coCSCs with expression of FGF-2 and nuclear β-CATENIN indirectly. By this, WNT-signaling pathway specific treatment becomes possible which is known to be a challenging operation (Barker and Clevers, 2006).

Material and Methods

All experiments were done at least twice in replicates. When using kits it was done according to the respective user`s manuals. Oligonucleotides used in this study are given in Supplemental Table 1.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was done as described previously (Wassermann et al., 2009). Briefly, fifteen nanograms unlabeled competitor oligonucleotides or water were mixed together with 1 μL of crudely purified recombinant GST-TCF4 (amino acid 265-496) or GST in binding buffer (10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 4% (w/v) Ficoll), poly-(deoxyinosinicdeoxycytidylic) acid (Sigma–Aldrich, final concentration: 12.5 ng/μL) and bovine serum albumin (Sigma– Aldrich, final concentration: 62.5 μg/mL) in a total volume of 16 μL. After 5 minutes incubation, 0.5 ng of ^{32}P end-labeled double stranded oligonucleotides (Hartmann Analytik, Braunschweig, Germany; specific activity: $3 \cdot 10^8$ dpm/μg) were added, incubated for 20 minutes, and subsequently applied on a 0.5xTBE 10% (w/v) polyacrylamide gel. Recombinant GST-TCF4 and GST were generated according to the instructions given in GE Healthcare's GST Protein brochure.

Chromatin Immune Precipitation (ChIP)

Chromatin was fragmented to a mean size of 300 bp using an ultrasonic device (Heinemann). For chromatin immune precipitations (ChIP) precipitation, ChIP-It Express kits (Active–Motif) were used together with 3 μg of antibodies specific for TCF4 (clone 6H5-3, Upstate Biotechnology), or Polymerase II (Active–Motif) and IgG (Active-Motif) as controls. *FGF-2* and *GAPDH* promoter/enhancer specific PCRs were employed to detect precipitation of chromatin. Positive and negative controls for the PCR were input chromatin or water, respectively. PCR products were separated on 0.5xTBE 3% (w/v) agarose gels.

Plasmids

The TBE in the FGF-2 promoter/enhancer in the luciferase gene reporter clone pGL2-(-1798/+296) FGF-2Luc (Moffett et al., 1998) was mutated using QuikChange mutagenesis kits (Stratagene) together with appropriate primers. The success of mutagenesis was verified by sequencing using BigDye termination kits (Applied Biosystems) together with a specific sequencing primer.

Cell lines, cell culture and transfections

The human colorectal tumor cell lines Caco2, DLD1, HCT15, HCT116, HT29, LoVo, LS174T, and SW480 were purchased from LGL Promochem GmbH, Wesel, Germany. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 7.5% (v/v) fetal bovine serum (Biochrom) and 50 µM 2-mercaptoethanol (Invitrogen) without antibiotics. For transient transfections, $5 \cdot 10^4$ LoVo or HCT15 cells still suspended were seeded in duplicates in 96-well plates and transfected with 30 ng of luciferase reporter plasmids, 10 ng pRL-CMV (Promega) as the transfection control and a pcDNA derived expression vector encoding dnTCF4 in increasing amounts ranging from 0 to 60 ng using Fugene6 (Roche). pcDNA3-Cat (Invitrogen) was used to fill up DNA to constant amounts of 100 ng. After 48 to 72 h, cells were harvested, lysed, and luciferase activity was determined using Dual Light kits (Promega) in an OrionII microplate luminometer (Berthold). Results were normalized for transfection efficiency on the basis of the activity of Renilla luciferase. Transfection of siRNA was done as described previously (Beiter et al., 2005). Briefly, cells were seeded at 20% confluence in 12-cluster well plates and transiently transfected 24 h later using 100 pmol of specific siRNA specific together with 2 µl Oligofectamine (Invitrogen). About 72 h after transfection, cells were harvested and RNA was isolated.

RNA Isolation, Reverse Transcription, RT-PCR, qRT-PCR

Total RNA from cell lines was prepared employing RNeasy kits (Qiagen). 1 µg RNA was reverse transcribed using QuantiTect Reverse Transcription kit (Qiagen) in the presence (+) or absence (-) of reverse transcriptase. 1 µl of the resulting cDNA was

used in subsequent PCRs together with intron-spanning, specific primer pairs. For RT-qPCR, 1 μg of the RT reactions (+/-) was incubated in the presence of optimized amounts of specific primer pairs together with 5 µL 2 x iQ-SYBRGreen (Biorad) or 5 µl 2xLightCycler® 480 Probes Master and the corresponding human universal probe (100 nm, Roche), respectively. Analyses were done using a LightCycler®480 device (Roche) at least three times in duplicates. All data were normalized on *β-ACTIN* or the geometric mean of the expression values of the 3 reference genes *β-ACTIN*, *RPL13A (*Ribosomal Protein L13a), and *YWHAZ (*Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein, ξ-Polypeptide*)*.

Protein Isolation and Western Blotting

20µg of protein lysates (Maniatis, triple lysis buffer) supplemented with 0.7 mM PMSF and 1x Complete protease inhibitor (Roche) were used for PAGE (polyacrylamide gel electrophoresis). After Blotting onto a PVDF membranes (Millipore), anti-FGF-2 (Sigma, clone FB-8 1:1,000), anti-E-Cadherin (Invitrogen, clone 4A2C7, 1:1,000), anti-ZEB-1 (Sigma, clone HPA027524, 1:1,000) or anti-cleaved Caspase 3 (Cell Signaling, clone ASP175, 1:1.000) and after stripping anti-β-Actin specific antibodies (Sigma, clone Ac15, 1:3,000) were added. Signals were visualized with HRP-conjugated secondary antibodies (Pierce, 1:20,000) and subsequent incubation with ECL solution (Millipore).

Lentiviral transduction

Mission® lentiviral particles (Sigma) encoding shRNA specific for *enhanced green fluorescent protein* (SHC005V) as the control or the human *FGF-2* (SHCLNV) were used for transduction of Caco2, HCT15, HCT116, or LoVo cells at an MOI of 5 and selected for stable expression applying puromycin (Calbiochem; Caco2, Lovo, HCT116: 2.5 µg/ml, HCT15: 8µg/ml), respectively. For Caco2 and LoVo cells two stably *FGF-2* specific shRNA expressing cell clones each with maximal loss of FGF-2 were selected for experimental work whereas polyclonal bulk cultures were used when working with HCT15 and HCT116 cells. The behavior of clones stably

transduced with the control shRNA specific for eGFP or the corresponding bulk cultures largely reflected that of parental cell lines.

Immunohistochemical Staining and Immunofluorescence

For immunohistochemical staining (Wassermann et al., 2009) 2μm histological sections were incubated in citrate buffer, pH 6.0 (Ventana) heated in a microwave three times at 600 W, applying thereafter mouse monoclonal FGF-2 (1:20, Sigma, clone AC15) or β-CATENIN (1: 40, Transduction Laboratories, clone 14) specific antibodies. Development was done applying of APAP (DAKO). For immunofluorescence, 10^4 cells were seeded in wells of culture-slides (BD Biosciences). After attachment, washing with PBS, fixing in 2 % (v/v) formalin and blocking with 10% (v/v) FCS in PBS anti-E-Cadherin specific antibody (1:200, Invitrogen, clone 4A2C7) was incubated over night at 4°C in 2% (v/v) FCS, 0.1% (w/v) Saponin in PBS. After washing with PBS anti-mouse Alexa-Flour 488 (Invitrogen, 1:400) was added under the same conditions for 2 h, washed again and covered with mounting medium containing DAPI (Vector laboratories) and finally analyzed using a fluorescence microscope (Zeiss).

Functional assays

Migration of cells was analyzed using ibidi chambers (IBIDI, Germany), invasion by usage of matrigel® coated transwells, apoptosis by measuring cleaved CASPASE-3, and anchorage independent growth by outgrowth of cells in methylcellulose essentially as described previously (Horst et al., 2009).

For generation of growth curves, 50,000 cells were seeded in 6-well plates and the resulting cell numbers were evaluated by counting individual wells every 24 hours after trypsination. For chemotherapy assays, cells were incubated with 5-FU (Sigma) dissolved in DMSO (Sigma) or in the presence of 0.1 % DMSO as a control. For determining cell viability, cells were seeded in 96-well culture plates (10^4 cells/well), allowed to adhere overnight and treated with 5-FU (Sigma) at different concentrations for 48 h. Then, 0.1 mg of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide, Sigma) was added to each well and incubated at 37° C for further 4 h. The medium was removed, and 100 µl DMSO (Sigma) were added to each well. The plates were shaken on a microplate shaker to dissolve the blue MTT formazan. Absorbances at 490 nm were read using a microplate reader. Inhibition of cell growth was calculated from the equation: inhibition (%) = (1 – A*t*/A*c*) x 100%, where A*t* and A*c* represent absorbances of treated and control cultures, respectively.

FACS analyses

For the analysis and isolation of SP cells $2 \cdot 10^6$ tumor cells cultured in 10 % serum containing medium were incubated with Hoechst 33342 (4.5µg/ml/10^6 cells) in 5% FCS containing medium either alone or in combination with 50 µM verapamil (Sigma Chemical, USA). The Hoechst treated cells were incubated at 37° C for 45 min, counterstained with 1 µg/ml propidium iodide (PI) for labeling dead cells and cellular debris, and analyzed using a MoFlo FACS (Dako-Cytomation) as described (Das et al., 2008). Settings: 488 nm solid state laser (Lyt 200.s, iCyt), and multiline high UV output laser (Coherent 300-C). Laser power: 200 mW for the 488 nm laser, and 50 mW for the multiline laser. Filters: Emissions signals from Hoechst and PI were first separated using a 510 SP dichroic mirror. PI was detected using 670/40 (band pass). Hoechst fluorescence was detected with a 450/65 filter (Hoechst blue) and a 630/30 optical filter (Hoechst red). PI-positive dead cells were excluded from the analysis. Gating: for each sample to be analyzed, a verapamil treated control was used to define the upper limit of SP (side population) / MP (main population) boundary.

Bone marrow stromal cell derived "injured cell" conditioned medium driven Matrigel® invasion assay

This invasion assay was performed as described previously (Das et al., 2008). Briefly, Conditioned medium was prepared by treating primary human bone marrow derived stromal cells with 0.5 mM H_20_2 in 5ml serum free media and exposition to extreme hypoxia (0.5 % O_2) for 20 h and 4 h of normoxia. 8 µm pore sized polyvinyl membrane based chambers (Corning Inc., Life Science, Lowell, MA) were coated with 100 µl of ice-cold Matrigel® (7.5 mg/ml; BD Bioscience), and incubated at 37°C

for 4 h. 10^4 cells (following trypsin neutralization) were added to the upper chamber, and the lower chamber was filled with injured conditioned medium. The chamber was incubated at 37°C for 8-24hrs, and invading cells were counted after crystal violet staining.

In vivo tumorigenicity assays

Tumorigenicity of the SP and non-SP cells was measured by injecting viable cells subcutaneously into female nude mice (BALB/c, nude/nude). Tumorigenicity was measured by tumor incidence (number of tumors/number of injection) and latency (time to form palpable tumor of > 0.04 cc). Briefly, viable SP and non-SP cells mixed with matrigel (50 µl of cell suspension in 50 µl of matrigel) were injected subcutaneously into both flanks of female nude mice (Balb/c, nude/nude). Resulting tumors were surgically removed and tumor size was measured with a caliper using the formula $4/3\pi abc$, where a, b and c are the perpendicular semiaxes of the ellipsoid.

Acknowledgements

We thank Dr Michal Stachowiak (University of Buffalo, Buffalo, USA) for giving us his pGL-FGF-2 (-1.800 / +314) reporter plasmid, Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, U.S.A.) for kindly supplying pdnTCF4 DNA and pGST-TCF4 expression plamids, and Dr. Randy Moon for supplying Super-TOPflash and Super-FOPflash plasmids. Furthermore, we thank Angela Neumann, Sabine Sagebiel as well as Pooja Dalvi (animal facility of the Hospital for Sick Children, Toronto, Canada) for their expert technical support. This work was supported by grants of the Wilhelm-Sander Stiftung (Az.: 2004.111) to TK and AJ and by the Canadian Cancer Society, Hospital for Sick Children Research Foundation to BD.

Authors` contributions

SKS and BD performed all experiments and analyzed the data together with AJ. LK, RBM and MT were partly involved in the experiments. SKS and AJ planned and coordinated the whole study and wrote together with TK the manuscript. All authors approved the study, finally.

The authors declare no conflict of interests.

References

Barker, N., and Clevers, H. (2006). Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov *5*, 997-1014.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608-611.

Beiter, K., Hiendlmeyer, E., Brabletz, T., Hlubek, F., Haynl, A., Knoll, C., Kirchner, T., and Jung, A. (2005). beta-Catenin regulates the expression of tenascin-C in human colorectal tumors. Oncogene *24*, 8200-8204.

Billottet, C., Elkhatib, N., Thiery, J. P., and Jouanneau, J. (2004). Targets of fibroblast growth factor 1 (FGF-1) and FGF-2 signaling involved in the invasive and tumorigenic behavior of carcinoma cells. Mol Biol Cell *15*, 4725-4734.

Brabletz, T., Hlubek, F., Spaderna, S., Schmalhofer, O., Hiendlmeyer, E., Jung, A., and Kirchner, T. (2005a). Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs *179*, 56-65.

Brabletz, T., Jung, A., Spaderna, S., Hlubek, F., and Kirchner, T. (2005b). Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer *5*, 744-749.

Brembeck, F. H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M., and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. Genes Dev *18*, 2225-2230.

Chen, J. K., Shen, L. S., and Chao, H. H. (1994). Correlation of transformation from epithelial to mesenchymal-like morphology and endogenous bFGF levels in human nasopharyngeal carcinoma cells. J Cell Physiol *160*, 401-408.

Creighton, C. J., Li, X., Landis, M., Dixon, J. M., Neumeister, V. M., Sjolund, A., Rimm, D. L., Wong, H., Rodriguez, A., Herschkowitz, J. I., *et al.* (2009). Residual breast cancers after conventional therapy display mesenchymal as well as tumor-initiating features. Proc Natl Acad Sci U S A *106*, 13820-13825.

Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W., Hoey, T., Gurney, A., Huang, E. H., Simeone, D. M., *et al.* (2007). Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A *104*, 10158-10163.

Das, B., Tsuchida, R., Malkin, D., Koren, G., Baruchel, S., and Yeger, H. (2008). Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. Stem Cells *26*, 1818-1830.

Dvorak, P., Dvorakova, D., and Hampl, A. (2006). Fibroblast growth factor signaling in embryonic and cancer stem cells. FEBS Lett *580*, 2869-2874.

Fodde, R., and Brabletz, T. (2007). Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol *19*, 150-158.

Gregorieff, A., and Clevers, H. (2005). Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev *19*, 877-890.

Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A., and Lander, E. S. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell *138*, 645-659.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Hatzis, P., van der Flier, L. G., van Driel, M. A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I. J., Koster, J., Santo, E. E., Welboren, W., *et al.* (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol Cell Biol *28*, 2732-2744.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

Horst, D., Scheel, S. K., Liebmann, S., Neumann, J., Maatz, S., Kirchner, T., and Jung, A. (2009). The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. J Pathol *219*, 427-434.

Jones, S., Chen, W. D., Parmigiani, G., Diehl, F., Beerenwinkel, N., Antal, T., Traulsen, A., Nowak, M. A., Siegel, C., Velculescu, V. E., *et al.* (2008). Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A *105*, 4283-4288.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M., *et al.* (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133*, 704-715.

Moffett, J., Kratz, E., Myers, J., Stachowiak, E. K., Florkiewicz, R. Z., and Stachowiak, M. K. (1998). Transcriptional regulation of fibroblast growth factor-2 expression in human astrocytes: implications for cell plasticity. Mol Biol Cell *9*, 2269-2285.

Muto, T., Bussey, H. J., and Morson, B. C. (1975). The evolution of cancer of the colon and rectum. Cancer *36*, 2251-2270.

O'Brien, C. A., Pollett, A., Gallinger, S., and Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*, 106-110.

Pang, R., Law, W. L., Chu, A. C., Poon, J. T., Lam, C. S., Chow, A. K., Ng, L., Cheung, L. W., Lan, X. R., Lan, H. Y., *et al.* (2010). A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell *6*, 603-615.

Pardal, R., Clarke, M. F., and Morrison, S. J. (2003). Applying the principles of stem-cell biology to cancer. Nat Rev Cancer *3*, 895-902.

Quintana, E., Shackleton, M., Sabel, M. S., Fullen, D. R., Johnson, T. M., and Morrison, S. J. (2008). Efficient tumour formation by single human melanoma cells. Nature *456*, 593-598.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. Nature *445*, 111-115.

Sjoblom, T., Jones, S., Wood, L. D., Parsons, D. W., Lin, J., Barber, T. D., Mandelker, D., Leary, R. J., Ptak, J., Silliman, N., *et al.* (2006). The consensus coding sequences of human breast and colorectal cancers. Science *314*, 268-274.

Spaderna, S., Schmalhofer, O., Hlubek, F., Berx, G., Eger, A., Merkel, S., Jung, A., Kirchner, T., and Brabletz, T. (2006). A Transient, EMT-Linked Loss of Basement Membranes Indicates Metastasis and Poor Survival in Colorectal Cancer. Gastroenterology *131*, 830-840.

Strutz, F., Zeisberg, M., Ziyadeh, F. N., Yang, C. Q., Kalluri, R., Muller, G. A., and Neilson, E. G. (2002). Role of basic fibroblast growth factor-2 in epithelial-mesenchymal transformation. Kidney Int *61*, 1714-1728.

Sussman, R. T., Ricci, M. S., Hart, L. S., Sun, S. Y., and El-Deiry, W. S. (2007). Chemotherapy-resistant side-population of colon cancer cells has a higher sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4. Cancer Biol Ther *6*, 1490-1495.

Tang, R., Cheng, A. J., Wang, J. Y., and Wang, T. C. (1998). Close correlation between telomerase expression and adenomatous polyp progression in multistep colorectal carcinogenesis. Cancer Res *58*, 4052-4054.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer *2*, 442-454.

Thomas-Mudge, R. J., Okada-Ban, M., Vandenbroucke, F., Vincent-Salomon, A., Girault, J. M., Thiery, J. P., and Jouanneau, J. (2004). Nuclear FGF-2 facilitates cell survival in vitro and during establishment of metastases. Oncogene *23*, 4771-4779.

Todaro, M., Alea, M. P., Di Stefano, A. B., Cammareri, P., Vermeulen, L., Iovino, F., Tripodo, C., Russo, A., Gulotta, G., Medema, J. P., and Stassi, G. (2007). Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell *1*, 389-402.

Tsuchida, R., Das, B., Yeger, H., Koren, G., Shibuya, M., Thorner, P. S., Baruchel, S., and Malkin, D. (2008). Cisplatin treatment increases survival and expansion of a highly tumorigenic side-population fraction by upregulating VEGF/Flt1 autocrine signaling. Oncogene *27*, 3923-3934.

Turner, N., and Grose, R. (2010). Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer *10*, 116-129.

Ueno, H., Murphy, J., Jass, J. R., Mochizuki, H., and Talbot, I. C. (2002). Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology *40*, 127-132.

Vermeulen, L., De Sousa, E. M. F., van der Heijden, M., Cameron, K., de Jong, J. H., Borovski, T., Tuynman, J. B., Todaro, M., Merz, C., Rodermond, H., *et al.* (2010a). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol *12*, 468-476.

Vermeulen, L., De Sousa, E. M. F., van der Heijden, M., Cameron, K., de Jong, J. H., Borovski, T., Tuynman, J. B., Todaro, M., Merz, C., Rodermond, H., *et al.* (2010b). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol *12*, 468-476.

Wassermann, S., Scheel, S. K., Hiendlmeyer, E., Palmqvist, R., Horst, D., Hlubek, F., Haynl, A., Kriegl, L., Reu, S., Merkel, S., *et al.* (2009). p16INK4a is a beta-catenin target gene and indicates low survival in human colorectal tumors. Gastroenterology *136*, 196-205 e192.

Yu, P. J., Ferrari, G., Galloway, A. C., Mignatti, P., and Pintucci, G. (2007). Basic fibroblast growth factor (FGF-2): the high molecular weight forms come of age. J Cell Biochem *100*, 1100-1108.

Figures

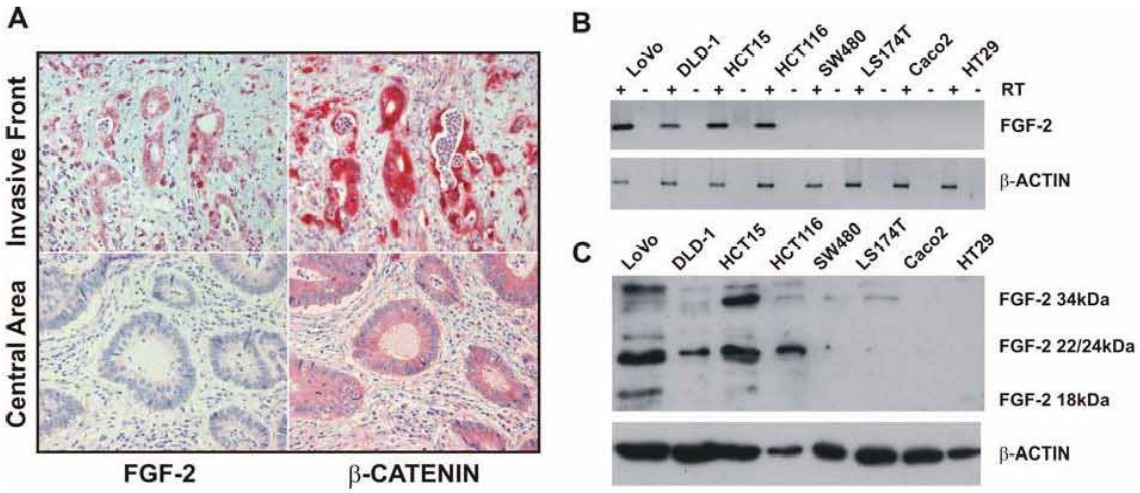


Figure 1. FGF-2 is expressed in CRCs and colorectal tumor cells

(A) Disseminated tumor cells and small clusters of tumor cells at the invasive front of CRCs showed coexpression of FGF-2 and nuclear β-CATENIN (upper panel) when inspecting comparable regions of the same tumors on serial sections. In contrast, FGF-2 expression was found at background levels or even absent in tumor cells from without nuclear expression of β-CATENIN which are mostly found in central areas of the same tumors (lower panel).

(B) Cultivated colorectal tumor cell lines expressed FGF-2 mRNA and (C) protein. The expression levels of the reference gene β-ACTIN were used in both cases for comparison.

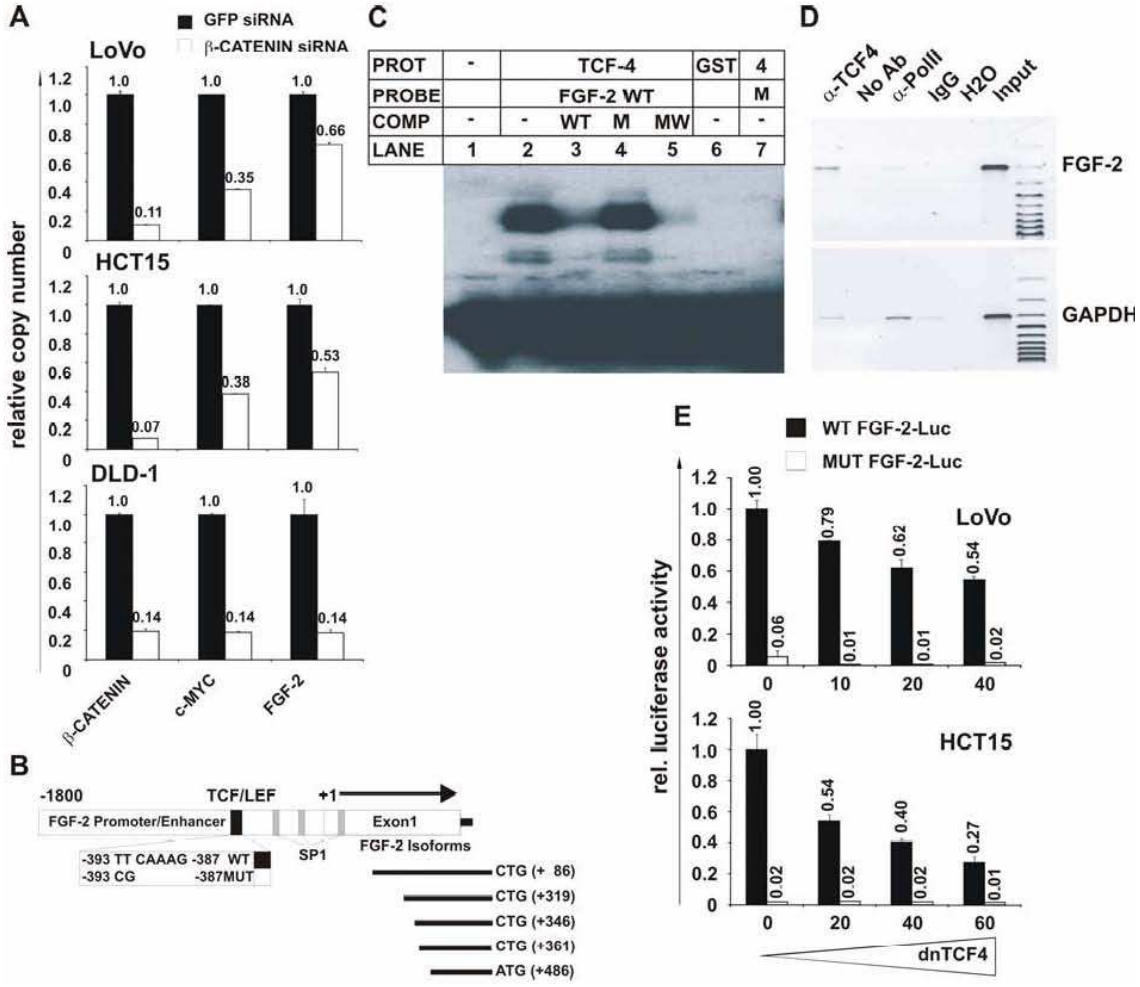


Figure 2. FGF-2 expression in CRCs is regulated by β-CATENIN/TCF4

(A) siRNA mediated knockdown of β-CATENIN in LoVo-, HCT15- and HCT116 cells led to a decline in the mRNA expression levels of *β-CATENIN*, *c-MYC*, and *FGF-2*. All levels were related on the expression levels of GFP (green fluorescent protein) specific siRNA transfected cell lines.

(B) Scheme of the *FGF-2* promoter/enhancer containing SP1 binding sites and a consensus TCF4 binding element (TBE) located at -387 with respect to the transcription start (+1). The positions and sequence of the start codons of the five FGF-2 isoforms as well as the sequence of the WT and mutated (MUT) TBE are given. Figure not drawn to scale.

(C) Electric Mobility shift assay (EMSA) showing the specific binding of recombinant GST-TCF4 (Glutathion-S-Transferase-T-cell -factor 4) fusion protein to the WT (lane

2) but not the MUT (lane 7) TBE of the human *FGF-2* promoter/enhancer . Binding was competed by addition of unlabelled WT *FGF-2* (lane 3) or *c-MYC* TBEs (second TBE of the *c-MYC* gene, lane 5) but not by MUT *FGF-2* TBE (lane 4).

(D) ChIP (chromatine immune precipitation) experiments demonstrated that TCF4 specifically bound to fragments of the human *FGF-2* promoter/enhancer containing the TBE in the context of native chromatin from cultivated Lovo cells.

(E) Human *FGF-2* promoter/enhancer luciferase reporter gene expression demonstrated that dnTCF-4 suppressed the transcriptional activity of such reporter construct in LoVo and HCT15 cells in a dose dependent manner. The same construct showed loss of activity when the TBE is mutated. All results are shown as mean ± SD from at least triplicates from a representative experiment.

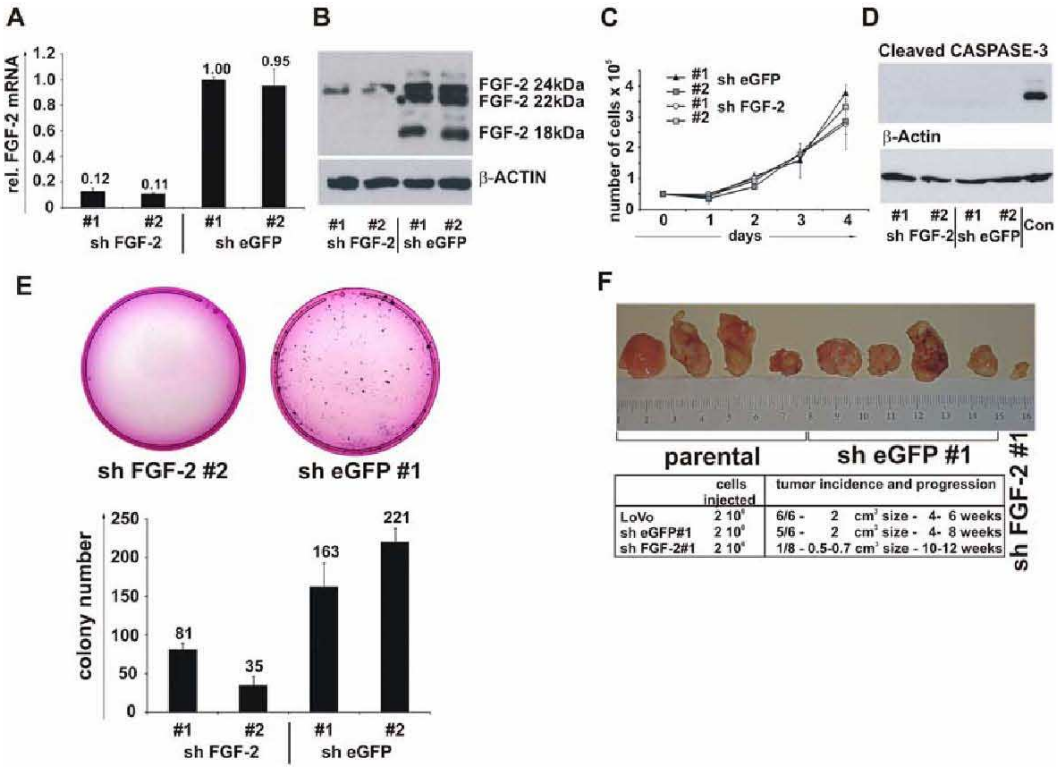


Figure 3. Stable knockdown of FGF-2 leads to a less transformed phenotype in colorectal tumor cells

(A) Stable knockdown of *FGF-2* mRNA expression in LoVo cells when using shRNA specific for FGF-2 (sh FGF-2) but not eGFP (sh eGFP).

(B) This effect was also seen on the protein level.

(C) Stable knockdown of FGF-2 did neither alter the proliferation of sh FGF-2 Lovo cells (D) nor apoptosis. To exclude a systematic error in the procedure, cytochrome c treated Jurkat cell protein extracts (Cell Signaling) were used as controls.

(E) Anchorage independent growth in methylcellulose medium was strongly impaired in sh FGF-2 LoVo cells compared to control cells.

(F) 2,000,000 s.c. injected tumor cells (LoVo, sh eGFP, sh FGF-2) were monitored for tumor initiation. sh FGF-2 cells almost completely lost their tumorigenic capabilities as less and in a single case a smaller tumor developed over a longer periode of time (2 to 3 fold). Surgically removed tumors are shown.

All values are shown as mean ± SD from at least duplicates or triplicates in one representative experiment.

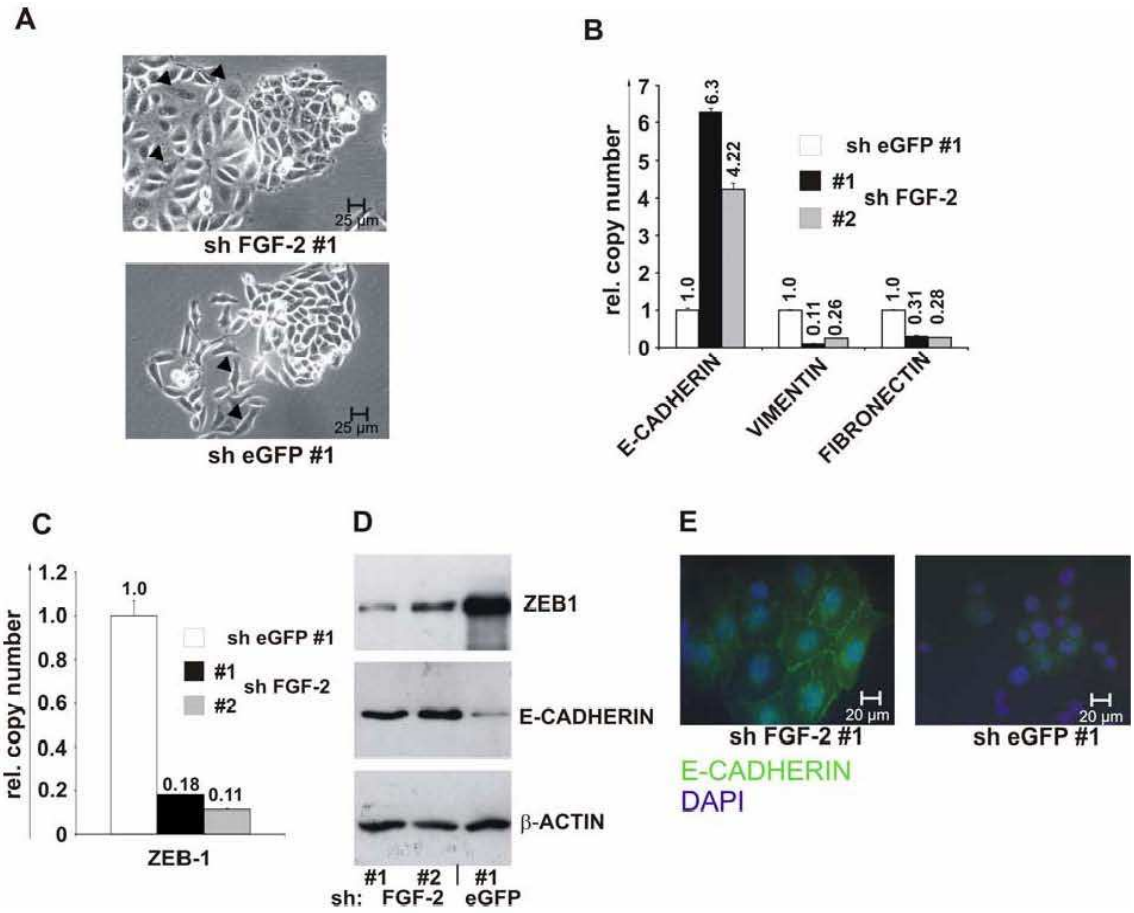


Figure 4. Loss of FGF-2 expression results in a gain of epithelial characteristics of tumor cells

(A) sh FGF-2 LoVo cells displayed a large, flat morphology (arrowheads, upper panel). In contrast, control LoVo cells showed patches of cells a mesenchymal morphology of fibroblasts (arrowheads, lower panel).

(B) sh FGF-2 LoVo cells were characterized by higher expression of mRNAs for the epithelial marker *E-CADHERIN* and lower expression of the mesenchymal markers *VIMENTIN*, *FIBRONECTIN* applying qRT-PCR. Values are given as mean ± SD from duplicates of one representative experiment.

(C) Expression of the EMT master switch factor ZEB-1 was strongly reduced in sh FGF-2 LoVo cells on the mRNA and (D) protein level. This was paralleled by higher protein levels of the ZEB-1 target E-CADHERIN as shown by Western Blotting (D) and immunofluorescence (E). Values are given as mean ± SD from duplicates of one representative experiment.

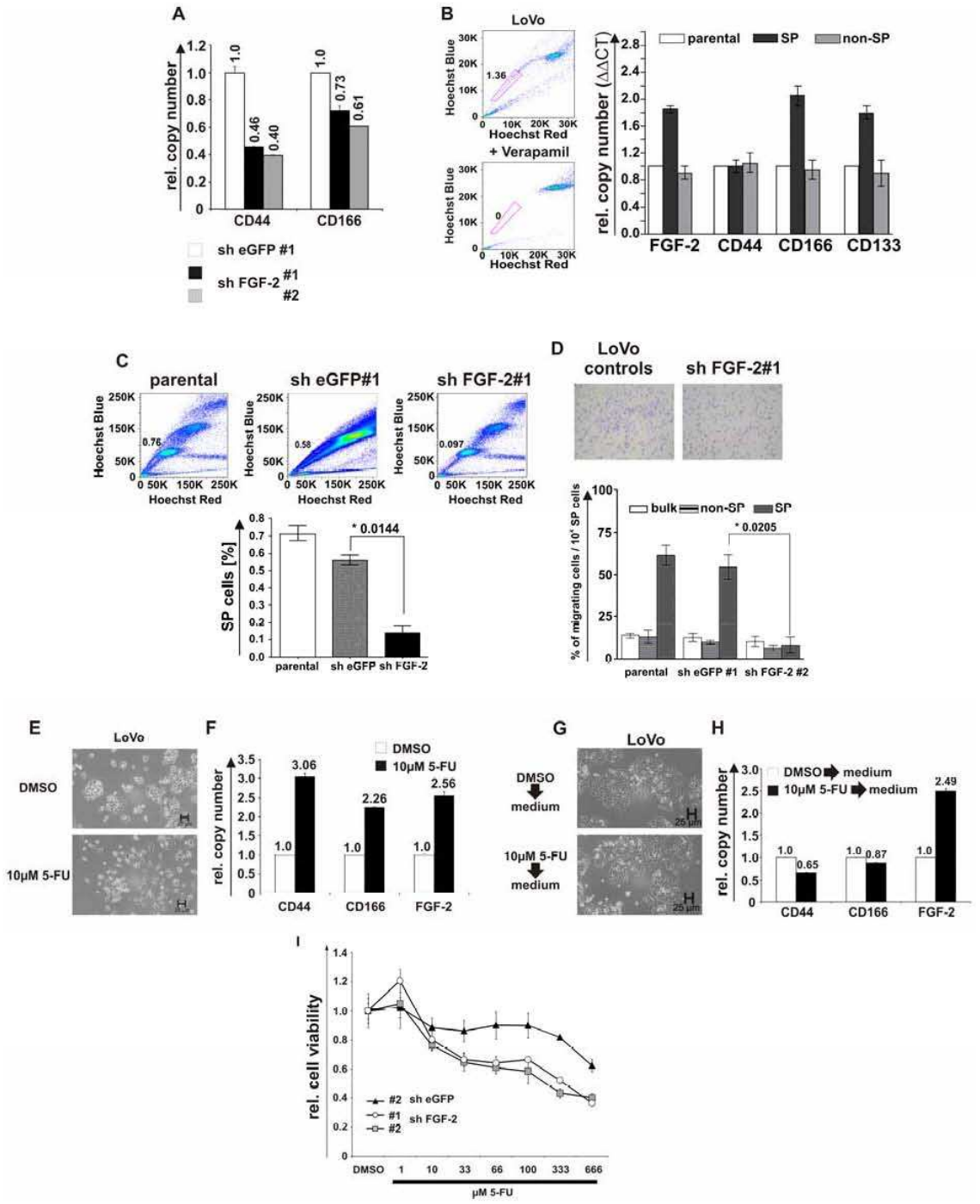


Figure 5. sh FGF-2 LoVo cells show loss of cancer stem cell like characteristics

(A) sh FGF-2 cells showed lower mRNA expression of the CSC markers *CD44* and *CD166* determined by qRT-PCR.

(B) Side population (SP) LoVo cells which were defined by the addition of the inhibitor Verapamil were characterized by higher mRNA expression levels of the CSC markers *CD166* and *CD133*, as well as *FGF-2*.
(C) The SP of sh FGF-2 LoVo cells was significantly smaller compared to control LoVo cells.
(D) The migratory behavior towards injury conditioned medium (hypoxia 0.5%) of SP sh FGF-2 cells is significantly reduced compared to SP cells from sh eGFP or LoVo cells.
(E) After two weeks incubation with 10 µM 5-FU LoVo cells displayed a mesenchymal morphology (F) with expression of higher mRNA levels of the CSC markers *CD44* and *CD166*, as well as *FGF-2*.
(G) LoVo cells surviving 5-FU treatment gave rise to epithelially re-differentiated progeny (H) with normalized CSC marker expression.
(I) sh FGF-2 LoVo cells showed reduced survival in response to increasing concentration of 5-FU compared to the controls. All results are shown as mean ± SD from duplicates (F, I) or triplicates of one representative experiment.

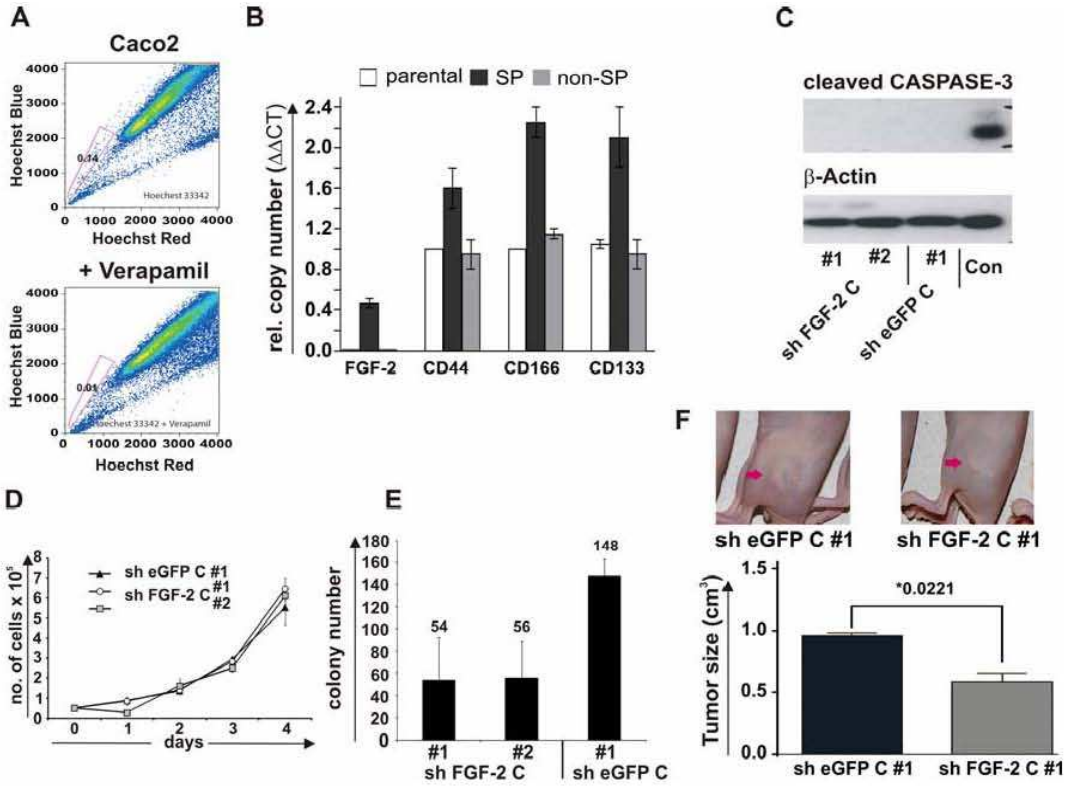


Figure 6. Caco2 cells contain a small CSC like subpopulation correlating with FGF-2 expression

(A) Caco2 cells contained a small fraction of SP cells

(B) which displayed higher mRNA expression levels of the CSC markers *CD44*, *CD166* and *CD133* as well as detectable levels of *FGF-2* (calculated upon the CT value of LoVo parental FGF-2 expression).

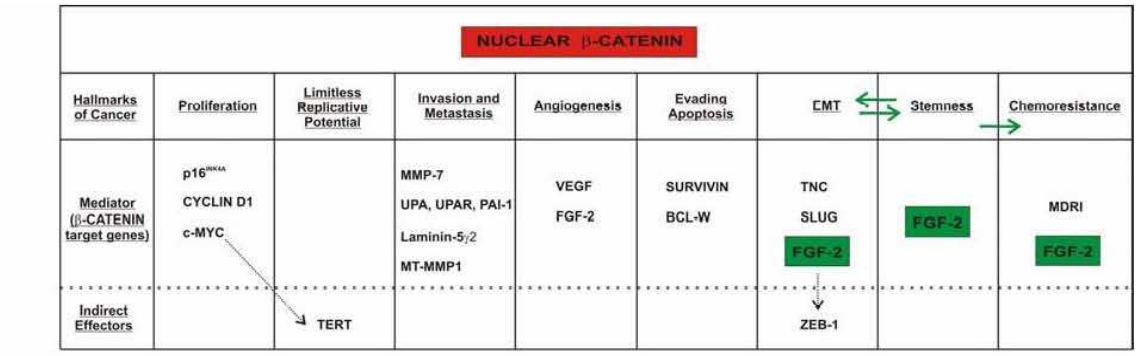
(C) Stable knockdown of *FGF-2* (sh FGF-2) expression in Caco2 cells did neither alter apoptosis (D) nor proliferation compared to control cells sh eGFP.

(E) sh FGF-2 Caco2 cells displayed a loss of transformation capacities measured as anchorage independent colonies formation in methylcellulose and

(F) a reduced tumor growth after s.c. injection into nude mice. A representative injection area of an immunocompromized mouse with (Caco2 sh eGF) or without (Caco2 sh FGF-2) tumor is depicted.

All results are shown as mean ± SD from duplicates or triplicates and tumor size as mean ± SD from eight animals each of one representative experiment each.

A



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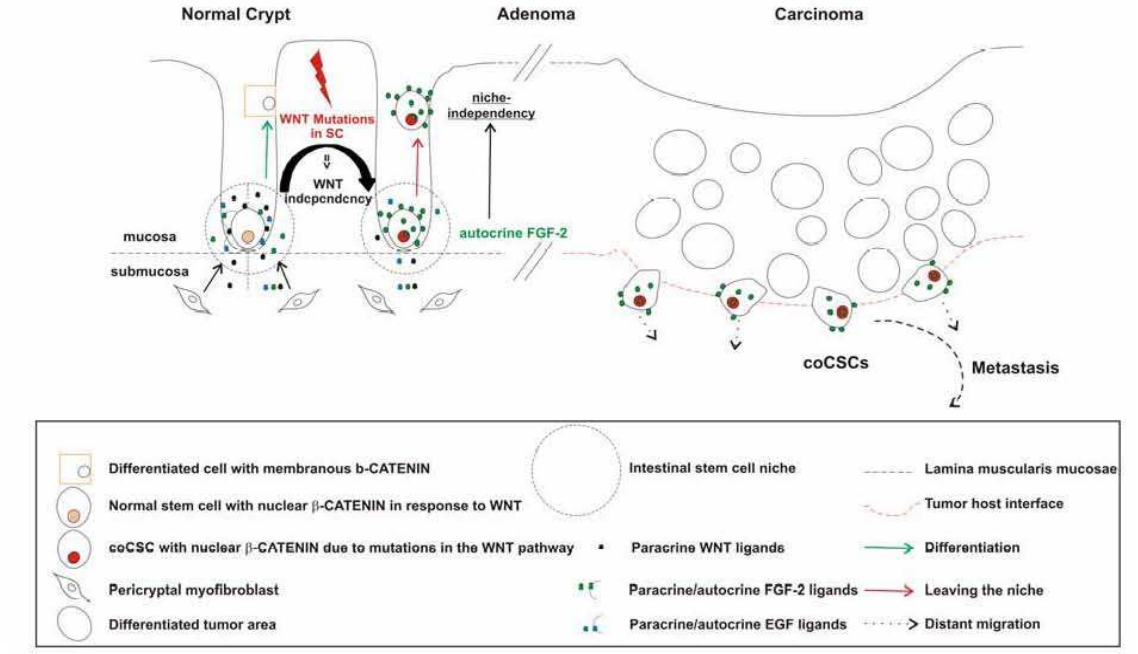


Figure 7. Comprehensive model of colorectal carcinogenesis: integration of 1) the adenoma-carcinoma sequence, 2) the multistep carcinogenesis and 3) the CSC model and 4) the involvement of β-CATENIN in EMT and maintenance of stemness via regulation of FGF-2

(A) Nuclear β-CATENIN is the driving force in the development of CRCs as it transcriptionally upregulates functional mediators for all essential hallmarks of cancer. These work either directly or indirectly when they are involved in transcriptional regulation. Stemness and its associated traits might also be seen as a new hallmark of cancer. Here, nuclear β-CATENIN is also centrally involved by the transcriptional upregulation of *FGF-2*. Therefore, developing anti-FGF-2 therapies

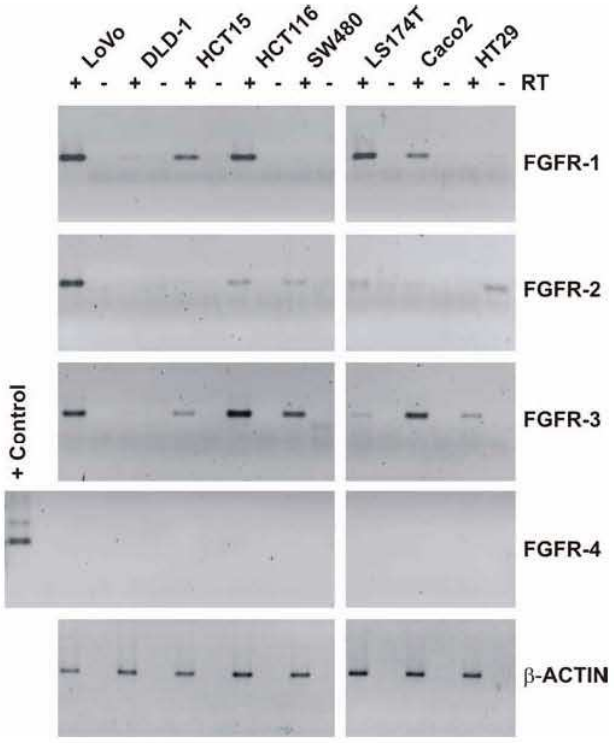
might prove useful in the treatment of colorectal cancer. (B) Adult intestinal stem cells (SCs) are restricted to a defined stem cell niche by myofibroblast-dependent paracrine activity of the WNT pathway and nuclear β-CATENIN. Furthermore, autocrine or paracrine signaling of FGF-2 and EGF might also be implicated in the generation of the niche. The adult SCs give rise to differentiated progeny, where inactivity of the WNT pathway is indicated by membranous localization of β-CATENIN. By mutations in components of the WNT pathway, SCs transform into colorectal cancer stem cells (coCSCs) as long as β-CATENIN remains in the nucleus. These coCSCs gain the trait of niche independency as a result of deregulated β-CATENIN transcriptional activity with consequent autocrine FGF-2 expression playing an essential role. In addition to other β-CATENIN regulated mediators of malignancy (A), FGF-2 expression drives via the adenoma-carcinoma sequence the formation of metastases.

Tables

Table 1 Caco2 SP cells initiate tumors

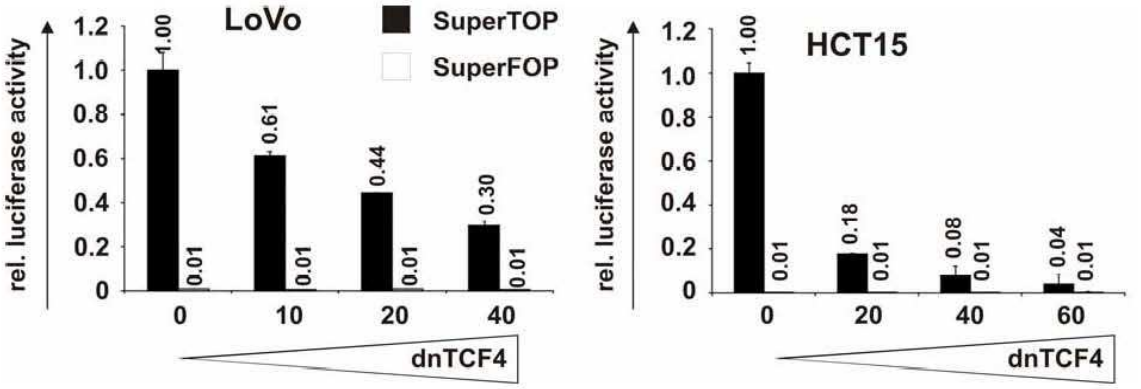
no of injected Caco2 cells		tumor incidence and latency (from injection to 0.04 cm^3 size)	tumor progression (from 0.04 to 1 cm^3 size)
parental	100,000	0/4	Ø
SP	100,000	2/2 (6 to 8 weeks)	3 to 5 weeks
non-SP	100,000	0/4	Ø

Supplemental Data



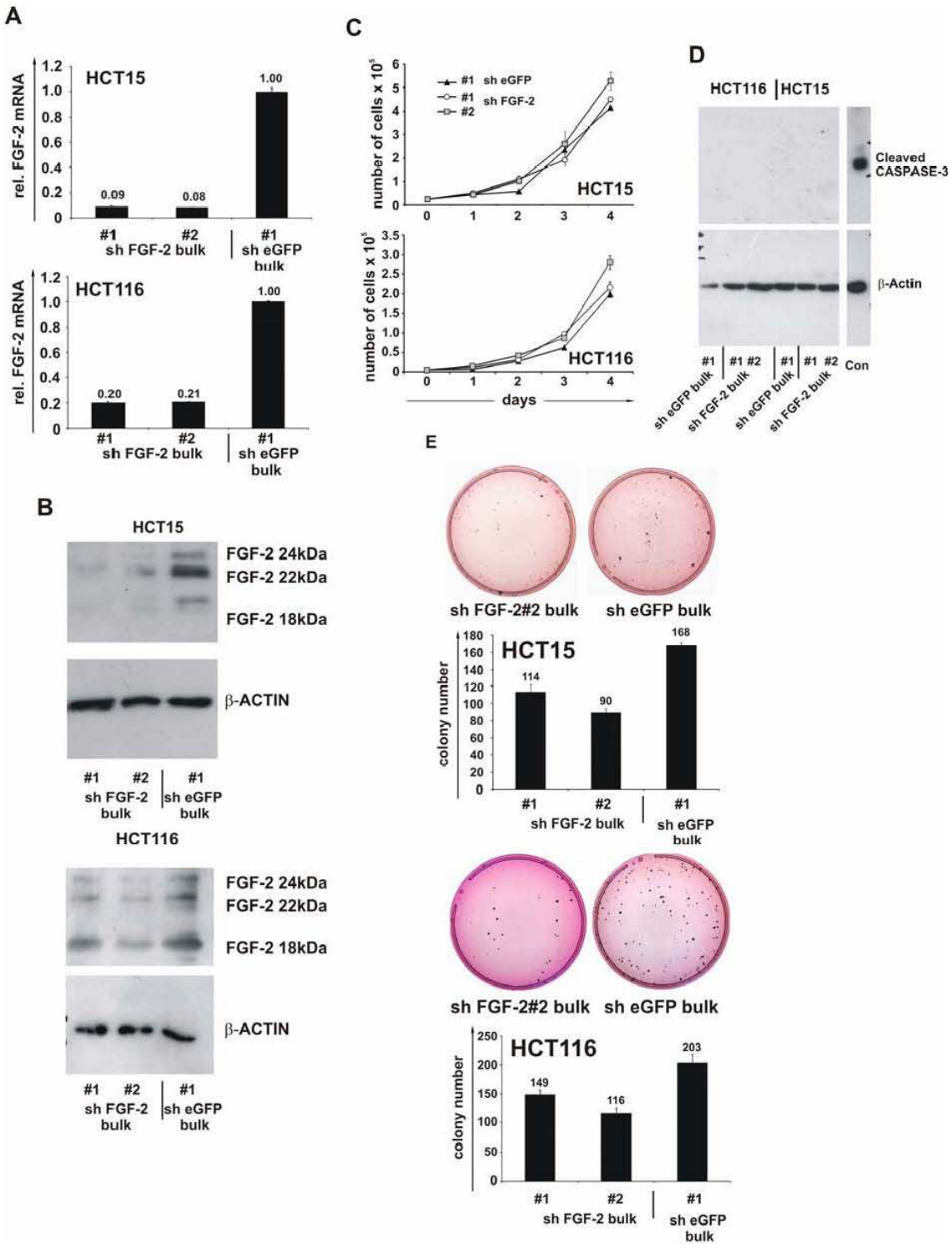
Supplemental Figure 1. Colorectal tumor cell lines show expression of various FGF receptors

FGF receptor (1 – 4) expression was analyzed in some cultivated colorectal tumor cell lines applying specific RT-PCRs. All cell lines were negative for expression of FGFR-4. A positive control for FGFR-4 was used to indicate functionality of the PCR.



Supplemental Figure 2. SuperTOPflash activity as a control for β-CATENIN/TCF4 dependent activity in LoVo and HCT15 cells

dnTCF-4 suppressed the transcriptional activity of SuperTOPflash activity in LoVo and HCT15 cells in a dose dependent manner. SuperFOPflash was completely inactive in this setting. Results are shown as mean ± SD from triplicates in one representative experiment.



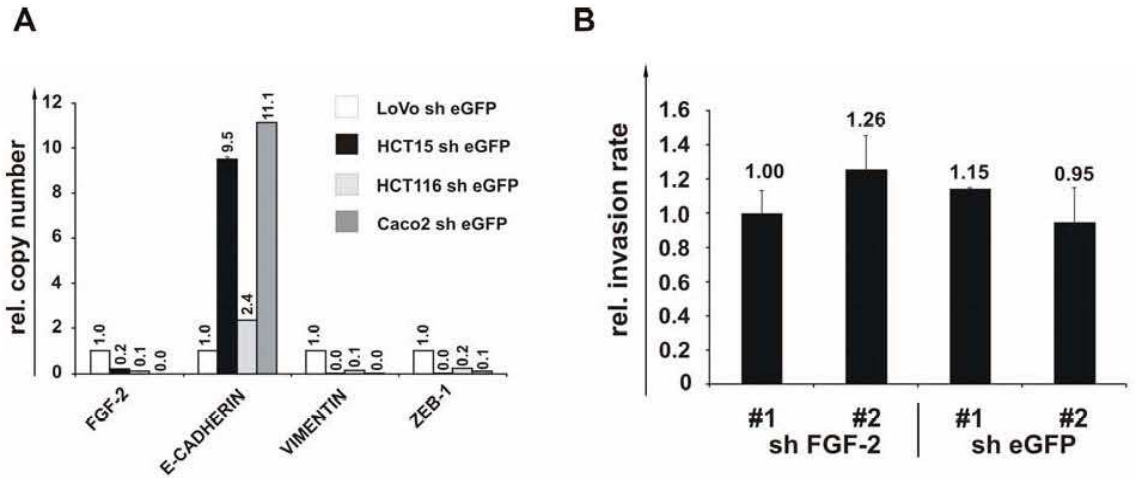
Supplemental Figure 3. Stable knockdown of FGF-2 in HCT15 and HCT116 leads to a less transformed phenotype

(A and B) shRNA mediated knockdown of FGF-2 in HCT15 and HCT116 cells shown on the mRNA and the protein level. Expression levels of bulk cultures with stable integration of one of two (#1 and #2) shRNA specific for FGF-2 and eGFP as a control are shown.

(C and D) Stable knockdown of FGF-2 did not alter proliferative or apoptotic behavior in HCT15 and HCT116 cells.

(E) Anchorage independent growth in methylcellulose medium was impaired in HCT15 and HCT116 cells with a stable knockdown of FGF-2 expression compared to control cells.

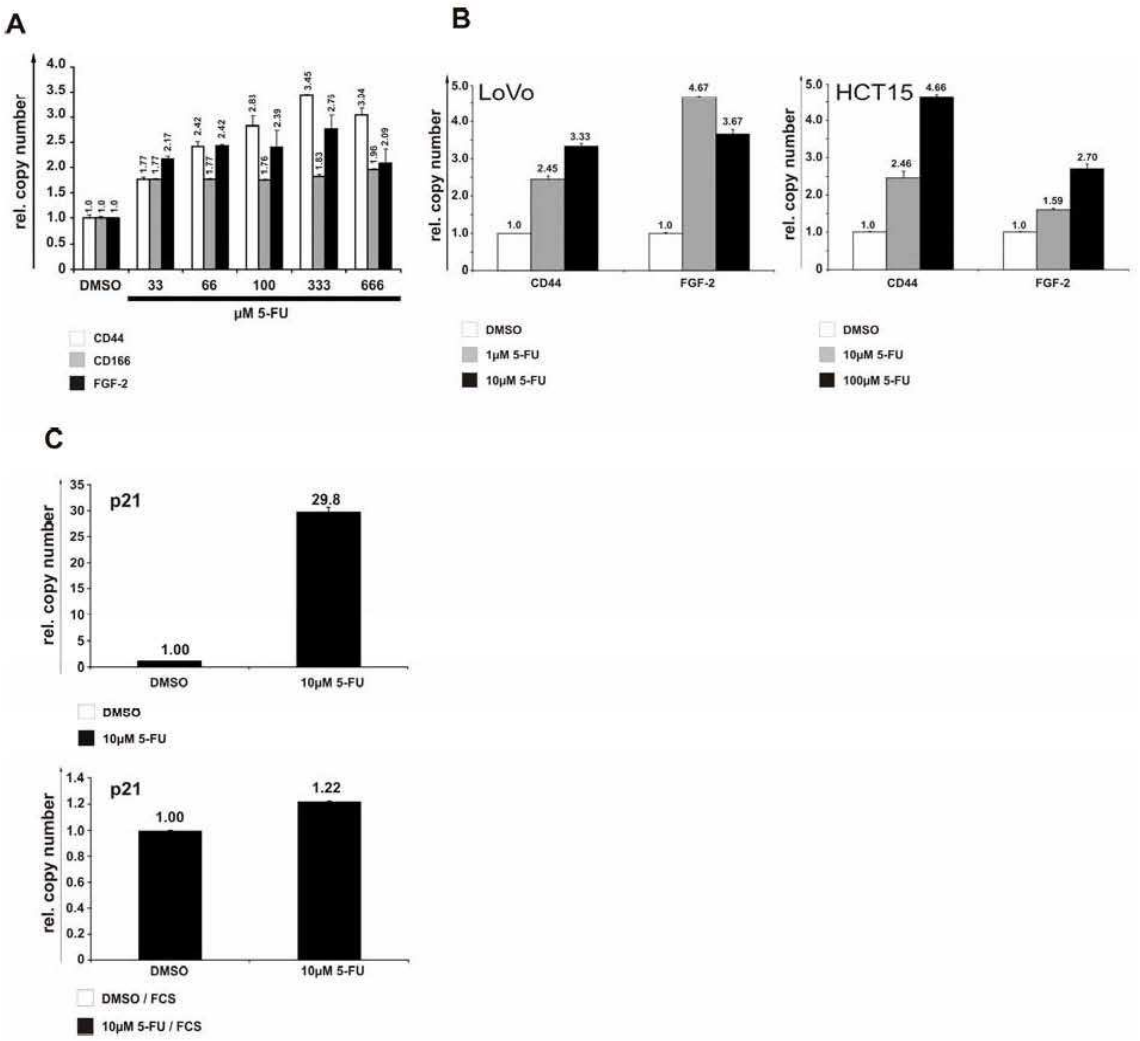
All results are shown as mean ± SD from duplicates or triplicates in one representative experiment.



Supplemental Figure 4. EMT properties of LoVo, HCT15, HCT116 and Caco2 cells

(A) sh FGF-2 LoVo cells did not show impaired invasive behavior towards FCS containing medium through Boyden chambers covered with matrigel.

(B) LoVo cells showed a much higher level of mesenchymal characteristics compared to HCT15, HCT116 and Caco2 cells which was paralleled by the FGF-2 mRNA expression levels. mRNA values are shown as mean ± SD from duplicates in one representative experiment.



Supplemental Figure 5. Treatment of colorectal tumor cell lines with 5-FU leads to a higher CSC like phenotype partly depending on FGF-2

(A) Two days incubation of LoVo cells with high concentrations of 5-FU led to higher mRNA levels of CD44, CD166 and FGF-2.

(B) Seven days incubation of LoVo and HCT15 cells with high 5-FU led to higher mRNA levels of CD44, CD166 and FGF-2.

(C) Two weeks incubation of LoVo cells with 10µM 5-FU led to cell cycle arrest by strong upregulation of p21 mRNA. After two additional weeks in normal culture medium containing 7.5 % FCS the p21 levels dropped back down to control levels.

All Results are shown as mean ± SD from triplicates in one representative experiment.

Supplemental Table 1 Oligonucleotides Used in this Study

EMSA (Electric Mobility Shift Assay)	
FGF-2 WT	GGTGATTTAGAGATTTTCAAAGCCTGCTCTGACACAG
FGF-2 MUT	GGTGATTTAGAGATTCGCAAAGCCTGCTCTGACACAG
c-MYC WT	CGCACCTTTGATTTCTGCACCTTTGATTTCT
TBEs are underlined. The *c-MYC* WT oligonucleotide comprises the second TBE of the human *c-MYC* promoter/enhancer duplicated (He et al., 1998).	

ChIP (Chromatin Immune Precipitation)		
FGF-2	GGCCTCTTTCTCTCCTTTTGTTG CCCAAACCACGTTTAGGCTTT	
GAPDH	TGTTTCATCCAAGCGTGTAAGG TGGCGACGCAAAAGAAGAT	

Site Directed Mutagenesis	
mutagenesis	CTTTTGGTGATTTAGAGATTCGCAAAGCCTGCTCTGACACAGAC
sequencing	CCCAAACCACGTTTAGGCTTT
The mutated TBE is underlined.	

siRNA (small interfering RNA)	
β-CATENIN	CAGUUGUGGUUAAGCUCUUdTdT (Verma et al., 2003)
GFP	AAGCUACCUGUUCCAUGGCCAdTT
GFP – green fluorescent protein	

RT-PCR (reverse transcription-PCR)	
β-ACTIN	TTGCGGATGTCCACGTCA, GCCCTGAGGCACTCTTCCA
FGF-2	CCGACGGCCGAGTTGAC, TGCTTGAAGTTGTAGCTTGATGTGA
FGFR-1	GTCACAGCCACACTCTGCAC, CAGGAAGGACTCCACTTCCA
FGFR-2	TGCATGGTTGACAGTTCTGC, CCCTATGCAGTAAATGGCTATCTC
FGFR-3	TCCTCGGGAGATGACGAA, CAGCAGCTTCTTGTCCATCC
FGFR-4	GGGCCTCCAGTCTTGTCC, GGCTACTGTCAGCTCCTGCT
FGFR – fibroblast growth factor receptor	

qRT-PCR (quantitative reverse transcription-PCR)		conc [nM]	UPL
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	300	
	TTGAGGACCTCTGTGTATTTGTCAA	300	
YWHAZ	CTTTTGGTACATTGTGGCTTCAA	300	
	CCGCCAGGACAAACCAGTAT	300	
β-ACTIN	TTGCGGATGTCCACGTCA	900	
	GCCCTGAGGCACTCTTCCA	900	
β-CATENIN	CTTGGAATGAGACTGCTGATCTTG	900	
	CACCAGAGTGAAAAGAACGATAGCTA	900	
c-MYC	CACCACCAGCAGCGACTCT	50	
	GCCTGCCTCTTTTCCACAGA	300	
FGF-2	CCGACGGCCGAGTTGAC	300	
	TGCTTGAAGTTGTAGCTTGATGTGA	300	
p21	TCGAAAACGGCGGCAGACC	300	
	CGGCGTTTGGAGTGGTAGAAATCT	300	
ZEB-1	GGGAGGAGCAGTGAAAGAGA ,	300	UP #3
	TTTCTTGCCCTTCCTTTCTG	300	
VIMENTIN	TACAGGAAGCTGCTGGAAGG ,	900	UP #13
	ACCAGAGGGAGTGAATCCAG	600	
E-CADHERIN	CCCGGGACAACGTTTATTAC ,	300	UP #35
	GCTGGCTCAAGTCAAAGTCC	600	
FIBRONECTIN	CTTTGGTGCAGCACAACTTC	900	UP #15
	TCCTCCTCGAGTCTGAACCA	900	
CD44	GGTCCCATACCACTCATGGA	300	UP #39
	TCCTTATAGGACCAGAGGTTGTG	900	
CD166	GGCAGTGGAAGCGTCATAA	300	UP #6
	CATTCTCTTCAGGGGAAATGA	900	
CD133	TCCACAGAAATTTACCTACATTGG	300	UP #83
	CAGCAGAGAGCAGATGACCA	300	

RPL13A - ribosomal protein L13a, UP – Universal Probe, UPL – Universal Probe Library (Roche), YWHAZ - tyrosine 3-mono-oxygenase/tryptophane 5-mono-oxygenase activation protein-ξ-polypeptide

shRNA

eGFP	CCGGTACAACAGCCACAACGTCTATCTC*GAGATAGACGTTGTGGCTGTTGTA*
FGF-2 #1	CCGGCTATCAAAGGAGTGTGTGCTACTC*GAGTAGCACACACTCCTTTGATAG*
FGF-2 #2	CCGGGTTACGGAATGAGTGTTTCTTTCTC*GAGAAAGAAACACTCATCCGTAAC*
eGFP – enhanced green fluorescent protein, the first half of the complementary part of the shRNAs is underlined and the second in italics.	

4 DISKUSSION

Dickdarmkrebs ist eine der am besten untersuchten Tumorarten und viele molekulare Veränderungen während der Entwicklung dieser Krankheit von lokal wachsenden Polypen bis hin zum vollinvasiven Karzinom und der Metastase sind hier mittlerweile sehr gut verstanden. Die überwiegende Mehrzahl aller kolorektalen Karzinome zeigt eine aberrante Aktivierung des WNT Signalwegs und eine daraus folgende Stabilisierung und Akkumulation von β-CATENIN. Bei nukleärer Lokalisation ist dieses Protein als Teil eines transkriptionell aktivierenden Komplexes sowohl an der Entstehung als auch an der Progression humaner KRK essentiell beteiligt und somit das zentrale Effektormolekül der kolorektalen Tumorigenese. Aufgrund dieser Sonderstellung β-CATENINs im KRK sind die hier vorgestellten Untersuchungen, die sich funktionell und mechanistisch mit seiner Regulation sowie der Auswirkungen von Defekten hierin auseinandersetzen, von grundlegender Bedeutung für das weitere Verständnis der kolorektalen Tumorentstehung.

4.1 Stabilisierung von β-CATENIN und WNT Aktivität in KRK

WTX Mutationen in humanen MSI-H KRK

Für die Stabilisierung von β-CATENIN kommen im KRK je nach Subtyp verschiedene Mechanismen zur Geltung. So weisen CIN KRK fast ausschließlich Mutationen im *APC* Tumorsuppressorgen auf, welches in dieser Form des KRK den *gatekeeper* der kolorektalen Karzinogenese darstellt (Kinzler and Vogelstein, 1996). Im Gegensatz hierzu sind MSI-H KRK durch frühe Mutationen im *BRAF* - oder im *KRAS* - Onkogen charakterisiert (Ogino and Goel, 2008). Defekte im WNT Signalweg treten in diesem Tumortyp erst in späteren Stadien oft als direkte Folge der Mikrosatelliteninstabilität auf und betreffen neben *APC* auch das *AXIN2* Gen oder *β-CATENIN* selber (Liu et al., 2000). Ich konnte nahelegen, dass zumindest in einem kleinen Anteil hochgradig mikrosatelliteninstabiler KRK des Tumorstadiums UICCIV außerdem noch Leseraster - Mutationen im *WTX* Gen (Wilms Tumor gene on the X-

Chromosome) als weiterer Mechanismus der β-CATENIN Stabilisierung dienen könnten (Scheel et al., 2010c). WTX ist eine erst kürzlich beschriebene neue Komponente des β-CATENIN Degradationskomplexes. Es interagiert direkt mit β-CATENIN und APC (Grohmann et al., 2007; Major et al., 2007) und weist in 7-30% aller Wilms Tumore Mutationen auf, welche entsprechend deregulierte WNT Signalweg-Aktivierung nach sich ziehen (Perotti et al., 2008; Rivera et al., 2007). Aufgrund seiner Lage auf dem X-Chromosom handelt es sich bei *WTX* um ein One-Hit-Tumorsuppressorgen, so dass von einer erhöhten Penetranz in Männern im Vergleich zu Frauen auszugehen ist. Entsprechend fand ich in dieser rein deskriptiven Studie auch nur einen männlichen Patienten aus einer Gruppe von 41 MSI-H Fällen (2,5%), der sowohl genomisch als erwartungsgemäß auch auf transkriptioneller Ebene eine Leseraster-Mutation in einem T_6-Mikrosatelliten aufwies. Da sich dieser Mikrosatellit in der N-terminal kodierenden Region befindet, ist von einem funktionellen Verlust des WTX Proteins und somit von einer Driver Mutation auszugehen, insbesondere da sich die Interaktionsdomänen mit APC und β-CATENIN jenseits des Mutationslokus befinden. Im Gegensatz dazu fand ich zwar insgesamt zwei weibliche Patientinnen mit einer genomischen *WTX* Leseraster-Mutation, die allerdings beide wildtypische WTX mRNA exprimierten. Dies deutet darauf hin, dass das mutierte Gen in den Tumoren dieser Patientinnen auf dem inaktivierten X-Chromosom lokalisiert war. Dieser Befund deckt sich mit Erkenntnissen aus Wilms Tumoren, in denen *WTX* Mutationen ebenfalls auf dem inaktivierten X-Chromosom gefunden wurden (Perotti et al., 2008). Dennoch ist zu vermuten, dass auch in einem geringen Anteil weiblicher Patientinnen ein funktioneller Verlust von WTX in MSI-H KRK aufzufinden sein könnte. Zusammenfassend konnte ich in diesem Projekt zeigen, dass das *WTX* Gen zumindest in einer kleinen Gruppe von MSI-H KRK eine Rolle in der Stabilisierung von β-CATENIN und somit der kolorektalen Karzinogenese spielen könnte.

Verlust der Expression des β-CATENIN Zielgens DKK4 in humanen KRK

Trotz der diversen Mutationsstrategien, durch die es zur Stabilisierung von β-CATENIN in KRK kommt, scheinen kolorektale Tumorzellen auch weiterhin auf extrazelluläre WNTs als Liganden in parakriner oder autokriner Weise reagieren und

somit ihre Signalwegaktivität noch verstärken zu können (Bafico et al., 2004; He et al., 2005). Dies ist unter anderem darin begründet, dass selbst mutiertes APC immer noch eine funktionelle Restaktivität in der Regulation der β-CATENIN Stabilität wahrnehmen kann (Spirio et al., 1998), und Tumorzellen von daher solche zusätzlichen Stimulationen nutzen um die daraus folgende Inhibition zu umgehen. Mit diesem Hintergrund erklärt sich auch, warum KRK häufig die Expression von WNT Inhibitoren wie SFRPs (Secreted frizzled related proteins) oder DKK1 - 3 (Dickkopf) mittels epigenetischer Mechanismen (DNA Methylierung) inaktiviert haben (Aguilera et al., 2006; Sato et al., 2007; Suzuki et al., 2004). In diesem Zusammenhang wurden die Expression und funktionellen Auswirkungen des beschriebenen β-CATENIN Zielgens *DKK4* in kolorektalen Tumoren und kultivierten kolorektalen Zelllinien untersucht (Baehs et al., 2009). Es konnte gezeigt werden, dass DKK4 Überexpression in kolorektalen Tumorzellen tatsächlich einen potenten negativen *feedback loop* darstellt, der eine stark hemmende Wirkung auf das Wachstum und die Aggressivität der Zellen hatte. Dies könnte physiologisch einen wichtigen Mechanismus in der Feinregulierung der WNT Aktivität in den Zellen der Darmkrypte darstellen. Ein Großteil der untersuchten Tumore und Zelllinien zeigte jedoch eine starke Selektion gegen die Expression von DKK4. Folglich ließ sich auch die Aktivität von β-CATENIN/TCF4 durch Stimulation mit WNTs erheblich steigern. Dies bringt für die Tumorzellen natürlich einen Wachstumsvorteil mit sich und begünstigt auch ihr aggressives Verhalten. Die Hemmung der DKK4 Expression lässt sich hierbei wie für die anderen DKKs oder SFRPs ebenfalls durch epigenetische Mechanismen erklären, wobei hier allerdings nicht DNA Methylierung sondern Histon Modifikationen im Mittelpunkt stehen. Somit konnte in dieser Arbeit gezeigt werden, dass es sich bei *DKK4* zwar einerseits tatsächlich um ein β-CATENIN Zielgen handelt, dieses aber andererseits viele Charakteristika eines Tumorsuppressorgens aufweist und daher seine Expression in KRK häufig unterdrückt ist.

4.2 Kolorektale Krebsstammzellmarker und ihre funktionelle Bedeutung für die maligne Progression

Weitere Zielgene von nukleärem β-CATENIN sind interessanterweise zelluläre Oberflächenmoleküle wie vermutlich CD133 (Katoh and Katoh, 2007) oder CD44 und CD166 (Hatzis et al., 2008; Wielenga et al., 1999) mit deren Hilfe es gelang, Zellen mit tumorinitiierenden Eigenschaften direkt aus kolorektalen Tumoren zu isolieren (Dalerba et al., 2007; O'Brien et al., 2007; Ricci-Vitiani et al., 2007; Vermeulen et al., 2008). Man spricht bei diesen Zellen auch von kolorektalen Krebs- oder Tumorstammzellen (koKSZ) und von den Molekülen entsprechend als Tumorstammzellmarkern. Denn experimentell sind so z. B. CD133 positive Zellen in der Lage, bereits in geringer Anzahl Tumore nach subkutaner Injektion in immundefiziente Mäuse zu bilden, während CD133 negative Zellen diese Eigenschaft nicht aufweisen (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). Darüber hinaus behalten diese Zellen auch bei *in vitro* Kultivierung als sogenannte Kolosphären ihre Fähigkeit zur Tumorinitiation bei und sind durch erhöhte Chemotherapie-Resistenz gekennzeichnet (Todaro et al., 2007). Jedoch wurde in einer dieser Studien kalkuliert, dass lediglich eine von 262 solcher CD133 positiven Zellen tatsächlich eine koKSZ darstellt (O'Brien et al., 2007). Dies bedeutet, dass CD133 nicht absolut spezifisch für Tumorstammzellen des Kolorektums ist, sondern nur in der Lage diese anzureichern. Passenderweise gilt dies auch für normale Stammzellen des Kolorektums. So konnte in eleganten Experimenten mit transgenen Mäusen gezeigt werden, dass sich Tumorstammzellen zwar im Dünndarm sehr wohl auf ebenfalls CD133 positive Kryptenstammzellen zurückführen lassen, hierüber im Dickdarm allerdings noch Unklarheit herrscht (Shmelkov et al., 2008; Snippert et al., 2009; Zhu et al., 2009). Im Tumorstammzellkonzept geht man davon aus, dass solche, durch Tumorstammzellmarker gekennzeichneten Zellen, die treibende Kraft hinter der Progression und der Metastasierung und dadurch schließlich dem Tod der Patienten darstellen (Brabletz et al., 2005b; Clarke et al., 2006; Reya et al., 2001). In Unterstützung dieser Hypothese korreliert hohe Expression von CD133 in humanen KRK mit einer signifikant schlechteren Überlebensprognose (Horst et al., 2008) und ist indikativ für eine signifikant erhöhte Resistenz der Tumore gegen eine 5-FU

basierte chemotherapeutische Behandlung (Ong et al., 2010). Es konnte gezeigt werden, dass CD133 Expression auch hoch assoziativ mit dem Auftreten von Fernmetastasen verknüpft ist (Horst et al., 2009c). Allerdings scheint das Vorhandensein von CD133 im Gegensatz zu z. B. CD44 für die tumorinitiierenden Fähigkeiten der koKSZ keine funktionelle Rolle zu spielen (Du et al., 2008). Ich untersuchte daher, ob CD133 zumindest an der Vermittlung anderer klassischer Hallmarks der Tumorprogression wie erhöhter Migration und Invasion, Proliferation sowie Transformation beteiligt ist oder ob es sich lediglich um ein reines Markermolekül handelt (Horst et al., 2009c). Da ich keinen Zugriff auf primäre koKSZ hatte, arbeitete ich mit CD133 positiven kultivierten Tumorzelllinien. In diesen lassen sich ebenfalls Subfraktionen an Zellen finden, die durch KSZ Eigenschaften charakterisiert sind (Dallas et al., 2009; Yeung et al., 2010). Mit Hilfe von siRNA konnte die Expression von CD133 mRNA und Protein sehr effizient inhibiert werden. Trotz dieses knockdowns zeigten die Zellen im Vergleich zu kontrollbehandelten Zellen keinerlei Veränderungen in ihrem Verhalten hinsichtlich Migration, Invasion, Proliferation oder Transformation. Diese Daten unterstützen daher Studien, die nahelegen, dass CD133 lediglich ein Marker für koKSZ in Verbindung mit schlechterer Überlebensprognose und Metastasierung ist, ihnen jedoch keine für diesen Phänotypen notwendige Funktionalität vermittelt (Du et al., 2008). Anders verhält es sich diesbezüglich mit dem jüngst veröffentlichten koKSZ Marker CD26, der auch kausal eine wichtige Rolle während der Metastasierung kolorektaler Tumore innehat (Pang et al., 2010). Allerdings wurde in einer kürzlich erschienenen Publikation die Rolle von CD133 an sich im Zusammenhang mit koKSZ kontrovers diskutiert (Kemper et al., 2010). Hier wurde gezeigt, dass Differenzierung von koKSZ mit dem Verlust einer ganz bestimmten, glykosylierten Variante von CD133 korreliert ist, während die generelle Menge an CD133 mRNA und Protein hierbei unverändert blieb. Dieses spezielle, glykosylierte Epitop von CD133 wird spezifisch durch den Antikörper AC133 detektiert. Diese Arbeit lässt somit sicher Zweifel aufkommen was die Bedeutung der veränderten mRNA Mengen in unserer Studie angeht. Den Nachweis der Protein-Expression führten wir jedoch mit einem Antikörper durch, der sowohl im Western Blot als auch in der Immunhistochemie das gleiche Färbeverhalten wie AC133 aufgezeigt hatte (Horst et al., 2008). Dies deutet darauf hin, dass in unseren Experimenten auch das für koKSZ spezifische CD133 Epitop

vom knockdown betroffen war und CD133 somit tatsächlich keine funktionelle Rolle für die Progression kolorektaler Tumore spielt. Unterstützt wird diese Annahme weiterhin durch die Daten von Du et al., die keinen Verlust der Tumorinitiationsfähigkeit von koKSZ nach durch AC133 überprüften knockdown von CD133 zeigen konnten (Du et al., 2008).

4.3 Identifizierung von β-CATENIN Zielgenen mit funktioneller Relevanz für kolorektale Krebsstammzellen

Der Befund, dass es sich bei CD133 lediglich um ein Markermolekül für koKSZ handelt ist insofern erstaunlich, da es sich hierbei vermutlich um ein weiteres Zielgen von nukleärem β-CATENIN handelt (Katoh and Katoh, 2007). Unterstützung findet diese Vermutung in der Tatsache, dass sich die Expressionsprofile dieser beiden Proteine in KRK teilweise überlagern und sie zusammen eine Markerkombination für eine schlechte Überlebensprognose für Patienten mit kolorektalen Tumorfrühstadien darstellen (Horst et al., 2009a). Im Gegensatz zu *CD133* ist ein Großteil bekannter β-CATENIN Zielgene in KRK essentiell an der Vermittlung von Krebs-Hallmarks beteiligt (Brabletz et al., 2005a; Hanahan and Weinberg, 2000), was die außerordentliche Rolle von β-CATENIN in der kolorektalen Tumorentstehung eindrucksvoll herausstreicht. Neben der transkriptionellen Aktivierung solcher Zielgene die an der Vermittlung der klassischen *hallmarks of cancer*, wie insbesondere Migration und Invasion, beteiligt sind, spielt nukleäres β-CATENIN auch eine wichtige Rolle bei der Induktion von EMT (Brembeck et al., 2004) und *stemness* (Vermeulen et al., 2010). Diese stehen miteinander in engem funktionellen Zusammenhang (Brembeck et al., 2004; Mani et al., 2008; Wellner et al., 2009). Von daher eignet sich das KRK als idealer Modeltumor für das Konzept der migrierenden Krebsstammzellen (mKSZ), deren Biologie sich durch die regulierte Lokalisation und Aktivität lediglich eines Moleküls erklären lassen könnte (Brabletz et al., 2005b) (Abbildung 10). Somit stellte sich die Frage, ob β-CATENIN neben *CD44* (Du et al., 2008; Wielenga et al., 1999), *MDR1* (Yamada et al., 2000) oder *SURVIVIN* (Zhang et

al., 2001a) noch weitere Zielgene hochreguliert, die auch für koKSZ von direkter funktioneller Relevanz sein könnten.

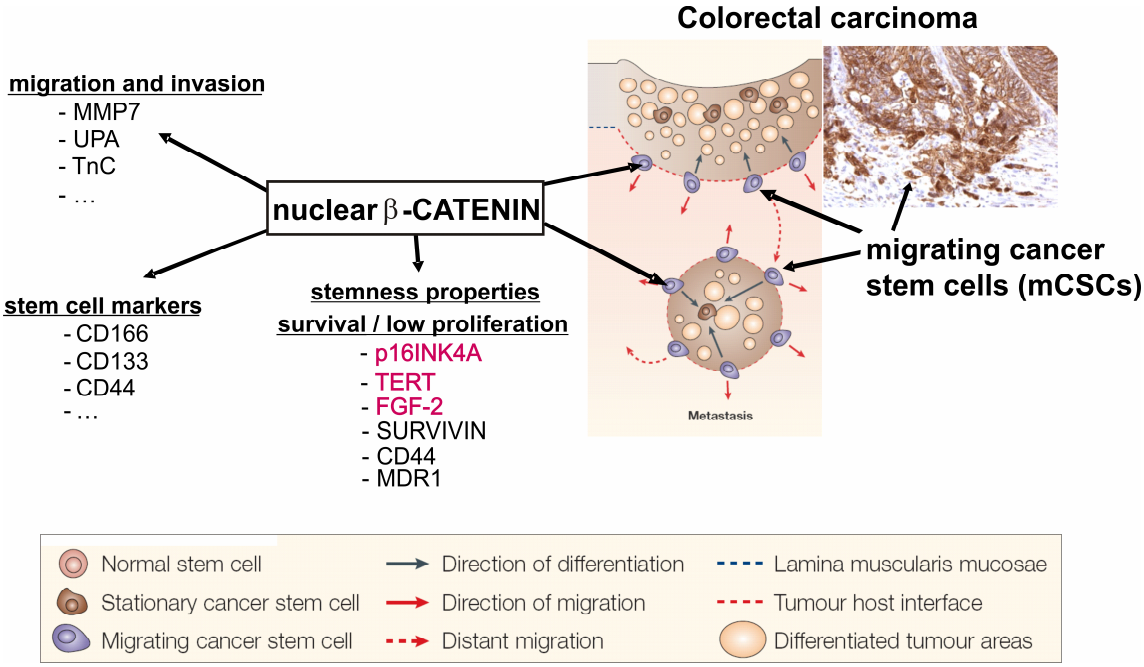


Abbildung 10: Die Rolle von β-CATENIN im Konzept der migrierenden Krebsstammzellen

Die transkriptionelle Aktivität nukleären β-CATENINs ist die molekulare Voraussetzung für den Phänotyp der migrierenden Krebsstammzellen (mKSZ) und somit der Metastasierung: an der Invasionsfront induziert nukleäres β-CATENIN EMT sowie die Aktivierung von Zielgenen die Migrations- und Invasionseigenschaften vermitteln. Gleichzeitig werden durch β-CATENIN spezifische Markermoleküle für koKSZ sowie funktionell relevante Gene für ihre Stammzelleigenschaften transaktiviert. In dieser Doktorarbeit neu beschriebene Zielgene sind in rot dargestellt. In den Metastasen findet eine Redifferenzierung einhergehend mit mesenchymaler-epithelialer Transition (MET) statt; *modifiziert nach (Brabletz et al., 2005b)*

β-CATENIN reguliert die Langlebigkeit von kolorektalen Tumorzellen mit Tumorstammzelleigenschaften

Im Tumorstammzellkonzept geht man davon aus, dass sich Tumorstammzellen bezüglich vieler Eigenschaften ähnlich wie normale adulte Stammzellen verhalten, was z. B. ihre Fähigkeit zur Multipotenz, Selbsterneuerung durch asymmetrische Zellteilung oder Langlebigkeit anbelangt (Clarke et al., 2006). In anderen funktionellen Eigenschaften jedoch könnten sich KSZ von ihren normalen

Gegenstücken unterscheiden. So wird z. B. vermutet, dass koKSZ durch eine geringe Proliferation gekennzeichnet sind, während für die vermutlichen kolorektalen Stammzellen gezeigt werden konnte, dass diese durch aktiven Zellzyklus gekennzeichnet sind (Barker et al., 2007). Nach dem Konzept der mKSZ sind koKSZ weiterhin durch mesenchymale Differenzierung mit hoher Migrations- und Invasionsfähigkeit charakterisiert (Brabletz et al., 2005b), wodurch sie sich ebenfalls von ihren normalen Gegenstücken unterscheiden. Genau hierin könnte auch die Ursache für die fehlende Proliferation dieser Zellen an der Invasionsfront begründet sein (Brabletz et al., 2001), da eine hohe Teilungsrate im Widerspruch stehen würde zu ihren Wandereigenschaften. Zusätzlich wirkt geringe Proliferation auch dem Alterungsprozess der Zellen entgegen, der mit jeder Zellteilung beschleunigt würde. Von daher wurde untersucht, wie der Zellzyklus in den Tumorzellen mit nukleärem β-CATENIN an der Invasionsfront kolorektaler Karzinome inhibiert wird. Dabei fokussierten wir uns auf den Zellzyklusinhibitor und Tumorsuppressor $p16^{INK4A}$, der in diesen Zellen ebenfalls exprimiert wird (Jung et al., 2001). Interessanterweise wurde herausgefunden, dass es sich bei *$p16^{INK4A}$* um ein durch β-CATENIN/TCF4 reguliertes Zielgen handelt (Wassermann et al., 2009). Dieses induziert in kolorektalen Tumorzellen Zellzyklusarrest und seine Expression kann dabei sogar die proliferationssteigernde Wirkung der β-CATENIN Zielgene *cMYC und CYCLIN D1* unterdrücken. Weiterhin wurde festgestellt, dass gerade diese $p16^{INK4A}$ - Expression und damit wohl auch die geringe Zellteilung dieser vermeintlichen koKSZ an der Invasionsfront statistisch signifikant mit einem schlechteren Überleben der Patienten korreliert ist. Demzufolge könnte der Einfluss hohen Tumorwachstums für die Progression kolorektaler Tumore in der Vergangenheit überschätzt worden sein. Im Gegenteil ist insbesondere das Vorliegen von nur wenigen Tumorstammzellen von entscheidender Bedeutung, welche β-CATENIN/$p16^{INK4A}$ – vermittelt eine geringe Teilungsrate aufweisen.

Zusätzlich zur geringen Zellteilungsrate kommt als weiterer Mechanismus der Langlebigkeit von Stammzellen und Tumorstammzellen die Expression der Telomerase Reverse Transkriptase Komponente (TERT) zur Geltung, die eine essentielle Komponente des Telomerasekomplexes darstellt (Blackburn, 2000). Ähnlich wie SURVIVIN spielt TERT ebenfalls eine wichtige Rolle in der Biologie von Stammzellen und nimmt auch eine wichtige Funktion als einer ihrer funktionellen

Marker wahr (Melton and Cowan, 2004). Passend zum Konzept der koKSZ korreliert in KRK hohe Expression von TERT ebenfalls mit einer schlechten Überlebensprognose für die Patienten (Tatsumoto et al., 2000) und seine Expression scheint analog zu $p16^{INK4A}$ mit der nukleären β-CATENINs und geringer Proliferation übereinzustimmen (Kolquist et al., 1998). Es gelang mir in einem Projekt *TERT* als neues direktes Zielgen von β-CATENIN in den Zellen der Invasionsfront von KRK zu identifizieren (Scheel et al., 2010b). Dieser Befund wurde experimentell dadurch erschwert, dass *TERT* gleichzeitig ein Zielgen von cMYC ist (Kyo et al., 2008; Takakura et al., 1999). Folglich könnte seine Regulation lediglich ein indirekter β-CATENIN Effekt sein, der über die Expression des Zielgens *cMYC* (He et al., 1998) gesteuert wird. Jedoch gelang es mir nach β-CATENIN spezifischer RNA Interferenz (RNAi) ein experimentelles Zeitfenster zu finden, in dem bei verringertem β-CATENIN- und gleichem cMYC Proteinlevel die mRNA Menge von TERT stark reduziert war, so dass eine direkte Regulation durch β-CATENIN als sehr wahrscheinlich anzunehmen ist. Durch diese Beobachtungen konnte die fundamentale Bedeutung von β-CATENIN für die kolorektale Karzinogenese und die Vermittlung der Tumorstammzelleigenschaft Langlebigkeit weiter bestätigt werden. Überdies hinaus helfen diese Daten zu verstehen, warum die Telomere kolorektaler Tumorzellen generell kürzer sind als ihre normalen Gegenstücke, obwohl sich nur die Tumorzellen durch TERT Aktivität auszeichnen (Hastie et al., 1990), wenn man annimmt, dass nur die wenigen koKSZ diese Aktivität besitzen.

β-CATENIN reguliert die Aufrechterhaltung von Tumorstammzelleigenschaften in kolorektalen Tumorzellen

Die herausragende Rolle von β-CATENIN für die kolorektale Karzinogenese und das Konzept der koKSZ besonders zu untermauern, gelang mir in der Aufklärung eines fundamentalen aber vorher ungeklärten Mechanismus (Scheel et al., 2010a): welche funktionellen Besonderheiten ermöglichen es Tumorstammzellen und insbesondere koKSZ außerhalb einer definierten und fixierten Stammzellnische zu überleben, also ihre *stemness* aufrechtzuerhalten? Für normale Stammzellen des Dickdarms geht man davon aus, dass deren Nische durch die Aktivität des WNT Signalwegs induziert und fixiert wird (Gregorieff and Clevers, 2005). Vermutlich

stammen die WNT Liganden hierbei von stromalen, intestinalen Myofibroblasten, die somit in parakriner Weise auf die Stammzellen wirken (van de Wetering et al., 2002). Über andere Regulationswege konnten diese Zellen jüngst als verantwortliche Kandidaten für die nukleäre Translokation von β-CATENIN in KRK ermittelt werden (Vermeulen et al., 2010). Sie stellen demnach eine im mKSZ Konzept postulierte stromale Komponente für die Induktion des koKSZ Phänotypen dar. Durch die Identifizierung des Zielgens FGF-2 gelangen mir funktionelle Einblicke, wie allein diese Kernlokalisation β-CATENINs ausreichend für die Aufrechterhaltung und eventuell auch Induktion der *stemness* in KRK ist. Bei FGF-2 handelt es sich um ein Protein, welches exogen hinzugefügt einen essentiellen Faktor darstellt für die *in vitro* Kultivierung von (embryonalen) Stammzellen und auch Tumorstammzellen, wie z. B. aus dem Dickdarm (Dvorak et al., 2005; Ricci-Vitiani et al., 2007; Todaro et al., 2007). Bei Abwesenheit von FGF-2 gehen die Zellen rasch in die Differenzierung und verlieren dabei ihre Multipotenz. Weiterhin ist Vorhandensein von Fgf-2 auch nötig für das Überleben intestinaler Stammzellen nach klassischen Antitumortherapien wie Bestrahlung (Houchen et al., 1999). Zusätzlich konnte zumindest in embryonalen und leukämischen Stammzellen eine bedeutende Funktion von endogen exprimiertem und autokrin wirkendem FGF-2 für die Aufrechterhaltung der entsprechenden Eigenschaften aufgezeigt werden (Dvorak et al., 2006; Eiselleova et al., 2009). Meine Daten bestätigen diesen Zusammenhang nun auch im KRK und bieten somit eine Erklärung, wie koKSZ durch nukleäres β-CATENIN Unabhängigkeit von normalerweise nötigen Nischenbedingungen erlangen können. So ist FGF-2 im KRK der Mediator für die Vermittlung eines transformierten Phänotypen, verstärkt mesenchymale Eigenschaften, die wiederum funktionell in engem Zusammenhang mit *stemness* stehen und schließlich wird es auch in den vermeintlichen Tumorstammzellen mit nukleärem β-CATENIN an der Invasionsfront kolorektaler Tumore exprimiert. Umgekehrt führte ein shRNA vermittelter *knockdown* von FGF-2 in kultivierten kolorektalen Tumorzelllinien zu einem Verlust der beschriebenen Fähigkeiten. Dies traf als unumstößliche Bestätigung der Befunde sogar für die zunächst als FGF-2 negativ eingestufte Zelllinie Caco2 zu. Denn auch diese enthält eine sehr kleine Fraktion an Zellen mit Tumorstammzellähnlichen Eigenschaften, die erst nach Anreicherung ebenfalls durch eine signifikante FGF-2 Expression charakterisiert ist. Ferner konnte ich in dieser Arbeit auch Hinweise darauf gewinnen,

dass endogene FGF-2 Expression weiterhin eine wichtige Rolle in der beschriebenen Chemoresistenz von Tumorzellen und isolierten koKSZ spielen könnte (Song et al., 2000; Todaro et al., 2007). Somit könnte FGF-2 eine potenzielle therapeutische Zielstruktur für die Behandlung kolorektaler Tumore darstellen, die u. a. im Mausmodell auch schon erfolgversprechende Resultate lieferte (Sukhthankar et al., 2008). Die Wirksamkeit verschiedener FGF spezifischer Medikamente wird derzeit in einer Reihe klinischer Studien eingehend untersucht (Turner and Grose, 2010).

Im Gegensatz zu den beschrieben Manuskripten über $p16^{INK4A}$ und TERT korreliert die Expression von FGF-2 zumindest auf Plasmaebene jedoch nicht mit einer schlechteren Prognose für die Patienten (Akbulut et al., 2002) und dient allenfalls als Surrogatmarker für das Auftreten von Metastasen (George et al., 2002). Allerdings wurde in diesen Arbeiten nicht berücksichtigt, dass es zum einen auch intrazellulär wirksame FGF-2 Isoformen gibt und zum anderen auch im Falle autokrinen Signalings der Tumorzellen keine Änderungen in den FGF-2 Plasmalevels auftreten werden, da das Protein oft direkt wieder auf den Zellen bindet und somit kein Nachweis im Blut möglich sein wird. Die Korrelation von immunhistochemisch nachgewiesener FGF-2 Expression in KRK mit dem weiteren Krankheitsverlauf der Patienten ist somit eine noch ausstehende Untersuchung, die weitere wichtige Auskünfte über die Bedeutung von FGF-2 in der kolorektalen Tumorprogression geben kann.

4.4 Ausblick

Trotz der enormen Erkenntnisse, die man im Zusammenhang mit β-CATENIN und seiner prominenten Rolle in der kolorektalen Karzinogenese bisher gewinnen konnte, bleiben weiterhin einige wichtige und grundlegende Fragen unbeantwortet. So ist z. B. die Notwendigkeit weiterer Mutationen wie in *KRAS*, *TP53*, *SMAD4* oder anderen Genen (Kinzler and Vogelstein, 1996) schwer nachvollziehbar, wenn man sich die Beteiligung von β-CATENIN an allen tumorrelevanten Prozessen (Hanahan and Weinberg, 2000) sowie EMT und *stemness* vor Augen führt. Weiterhin bleibt unklar, welcher Mechanismus dafür Verantwortung trägt, dass nukleäres β-CATENIN

und assoziierte EMT fast ausschließlich in den Zellen der Invasionsfront auftreten. Die Erkenntnis, dass in den Metastasen der Umkehrprozess der MET beobachten werden kann (Brabletz et al., 2001), spricht natürlich für einen entscheidenden Einfluss des umgebenden stromalen Gewebes in diesem Prozess. Allerdings konnten effektiv beteiligte Zellen oder gar deren molekulare Wirkung auf die Lokalisation von β-CATENIN bisher nur ansatzweise aufgeklärt werden (Brembeck et al., 2004; Vermeulen et al., 2010). Vieles deutet in diesem Zusammenhang auf eine Beteiligung der Serin-Threonin-Kinase AKT hin (Fang et al., 2007; Vermeulen et al., 2010), die im Zentrum verschiedenster zellulärer Signalwege steht und die über ILK (INTEGRIN LINKED KINASE) auch durch Zell-Matrix Kontakte aktiviert werden kann (Marotta et al., 2003; Tan et al., 2001). Zusätzlich existieren Daten, dass zwischen membranständigem und nukleärem β-CATENIN molekulare Unterschiede bestehen (Gottardi and Gumbiner, 2004). Die Regulation dieser Isoformen oder posttranslationalen Modifikationen stellt somit einen zusätzlichen, weiter aufzuklärenden Mechanismus in der Aktivität des WNT Signalwegs dar. In diesem Kontext stellt sich weiterhin die Frage, welche zusätzliche Stimulation nötig ist um das Zweiphasenmodell der β-CATENIN Zielgenaktivierung (s. S. 22) zu erklären. Gelänge eine Aufklärung dieser Mechanismen, wäre die Entwicklung einer zielgerichteten Therapie denkbar, die z. B. den nukleären Transport von β-CATENIN oder dessen Aktivität inhibiert und somit unter Umständen für eine Reduktion der Fraktion der koKSZ zu verwenden wäre (Takahashi-Yanaga and Kahn, 2010).

Nach dieser Idee unterscheiden sich die einzelnen Tumorzellen schließlich lediglich durch ihre Lokalisation innerhalb eines Tumors voneinander. Transiente *stemness* resultiert hierbei plastisch aus den Einflüssen der zellulären Umgebung, wie zum Beispiel benachbarter Myofibroblasten, die durch parakrine HGF-Produktion ebenfalls auf die intrazelluläre β-CATENIN Lokalisation und somit den koKSZ-Phänotypen erheblichen Einfluss nehmen (Vermeulen et al., 2010). Dies entspricht somit dem Konzept der extrinsisch induzierten KSZ (Rosen and Jordan, 2009) (Abbildung 11B). Bisher konnten Tumorstammzellen des Kolorektums mit Hilfe der Markermoleküle und β-CATENIN Zielgene CD133, CD44 und CD166 isoliert und charakterisiert werden. Allerdings scheint es mit Hilfe dieser Marker lediglich möglich, die Tumorstammzellfraktion anzureichern. Eine eindeutige Identifikation wie z. B. für normale intestinale Stammzellen mit Lgr5 (Barker et al., 2007) scheint hierdurch

jedoch noch nicht möglich zu sein. Geht man davon aus, dass sich koKSZ direkt auf solche normale Kryptenstammzellen zurückführen lassen und ihre Nachkommen ähnlich schnell differenzieren (Barker et al., 2009), ist zu erwarten, dass sich die Tumorstammzellenthaltende Fraktion mit Hilfe z. B. einer LGR5 basierten Anreicherung nochmals deutlich enger eingrenzen lassen wird. Dieser Vermutung liegt das Modell der intrinsischen *stemness* zugrunde, das davon ausgeht, dass *stemness* eine festgelegte Eigenschaft einer kleinen Subpopulation an Tumorzellen ist (Abbildung 11A).

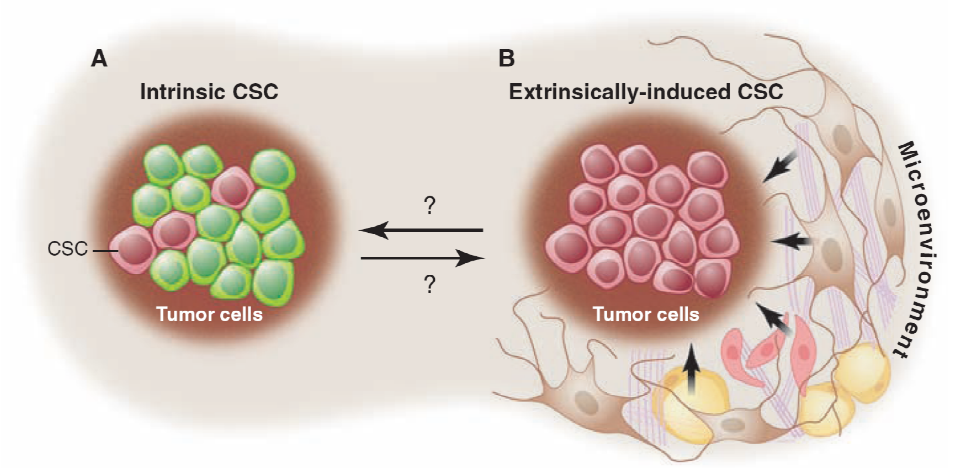


Abbildung 11: Tumorstammzellmodelle

Das intrinsische Tumorstammzellmodell (A) geht davon aus, dass spezifische Subpopulationen innerhalb eines Tumors durch die funktionellen Eigenschaften von KSZ charakterisiert sind. Das extrinsische Modell (B) hingegen vertritt die Ansicht, dass alle Zellen innerhalb eines Tumors funktionell identisch sind und lediglich durch die Aktivität ihrer zellulären Umgebung in ihrem Verhalten moduliert werden. In KRK ist ein paralleles Auftreten beider Erscheinungsformen denkbar; *(Rosen and Jordan, 2009).*

Bisher existieren allerdings nur gut gängige Antikörper gegen die anderen Marker, deren immunhistochemische Expressionsprofile in humanen KRK interessanterweise nur teilweise mit nukleärem β-CATENIN und vermeintlichen koKSZ überlappen. So findet sich z. B. CD133 Expression eher in epithelial differenzierten, zentralen Tumorarealen ohne nukleäre Expression von β-CATENIN und auch eine Koexpression der Markerkombination CD44 und CD166 kann kaum detektiert werden (Horst et al., 2008; Horst et al., 2009b; Horst et al., 2009c). Diese Befunde eröffnen somit Spielraum für weitere Modelle: es könnte sich demnach eventuell um

unterschiedliche Pools von KSZ handeln, die sich nach Anreicherung jedoch ähnlich verhalten, was sich unter Umständen auf einen gemeinsamen, funktionellen Mechanismus zurückführen lässt. Alternativ könnten diese verschiedenen Pools auch funktionell unterschiedlich sein und je nachdem z. B. entweder an der Bildung von Primärtumoren oder Metastasen beteiligt sein (Dieter et al., 2010; LaBarge, 2010).

Weiterhin werden durch diese Daten gleichzeitig sowohl das intrinsische als auch das extrinsische Modell der *stemness* unterstützt (Abbildung 11) und ein paralleles Auftreten dieser verschiedenen Zellfraktionen innerhalb eines Tumors scheint eine plausible Erklärung für die beobachteten Diskrepanzen darzustellen. Alternativ besteht die Möglichkeit, dass es sich bei diesen Zellen nur um die direkten Nachkommen mit residualer Funktionalität von tatsächlichen Tumorstammzellen handelt. Diese verfügen weiterhin über die Expression der Marker, während durch nukleäres β-CATENIN gekennzeichnete Stammzellen bereits weiter in das Nachbargewebe invadiert sind. Passenderweise herrscht diesbezüglich auch noch Unklarheit, ob sich Tumorstammzellen durch Umprogrammieren auch von bereits höher differenzierten Tumorzellen ableiten lassen. All diese Möglichkeiten tragen dazu bei zu verstehen, warum die KSZ Frequenzen zwischen Tumoren verschiedener Patienten teils erheblich voneinander abweichen können (Rosen and Jordan, 2009). Darüber hinaus gilt es den Befund einzuordnen, dass ein gewisser Anteil an KRK (insbesondere MSI-H) vom klassischen histologischen Erscheinungsbild mit Tumorzentrum und Invasionsfront abweicht. Dies gilt vornehmlich für KRK des medullären Typs (Jass, 2007; Lanza et al., 1999) und könnte bedeuten, dass hier andere Mechanismen für den Tumorstammzellphänotyp verantwortlich sind oder gar, dass diese Tumore nach einer ganz anderen Strategie in die Progression gehen und Tumorstammzellen hierbei keine Rolle spielen.

Die in dieser Arbeit präsentierten Daten sprechen jedoch klar für die Existenz und die funktionelle Rolle von Tumorstammzellen. Insofern stellt sich für die neu entdeckten β-CATENIN Zielgene *p16*^{INK4A}, *TERT* und *FGF-2* die Frage, ob ihre Beteiligung an der Vermittlung dieses Phänotyps von universeller Natur ist und ihre Aktivierung somit auch in Tumoren anderer Organe nachweisbar sein sollte. Da sich Tumore der meisten Organe jedoch nicht auf eine Hyperaktivität des WNT

Signalwegs zurückführen lassen, wäre hier gegebenenfalls die Aufklärung der entsprechenden Mechanismen von sehr interessantem Wert.

Eine zunehmend wichtige Rolle spielen diesbezüglich die sog. miRNAs (micro RNAs). Hierbei handelt es sich um kleine, nichtkodierende RNA-Moleküle mit komplexer Regulation, die eine hohe Komplementarität und somit Bindungsfähigkeit für eine große Anzahl an Zielgen-mRNAs aufweisen. Nach Bindung in der 3` UTR (*untranslated region*) der mRNAs werden diese entweder der Degradation zugeführt oder können nicht mehr als Vorlage für die Protein-Translation verwendet werden (Bartel, 2004). Abhängig von ihren jeweiligen Zielgen-mRNAs können miRNAs somit sowohl tumorsupportive als auch tumorsuppressive Aufgaben wahrnehmen und stellen dadurch einen wichtigen Regulationsmechanismus für die zelluläre Homöostase dar (Esquela-Kerscher and Slack, 2006; Faber et al., 2009). Weiterhin sind sie auch direkt an der Regulation von EMT (Burk et al., 2008; Gregory et al., 2008; Martin-Berenjeno and Vanhaesebroeck, 2009; Park et al., 2008) und *stemness* (Shimono et al., 2009; Wellner et al., 2009) beteiligt und können z. B. über die in vielen Tumoren deregulierten Proteine MYC und RAS aktiviert werden (Ma et al., 2010; Shimono et al., 2009; Wellner et al., 2009). Folglich könnte dies in Tumorarten mit intaktem WNT Signalweg einen Mechanismus für das Auftreten von Tumorstammzellen darstellen. Umgekehrt ist über die Beteiligung von miRNAs an der kolorektalen Karzinogenese gerade im Zusammenhang mit β-CATENIN Aktivität bisher nur sehr wenig bekannt und bleibt somit ein attraktiver Gegenstand in der künftigen molekularen Erforschung dieser Krankheit.

Abschließend lässt sich festhalten, dass das Tumorstammzellmodel attraktive Erklärungsmöglichkeiten für viele bekannte Eigenheiten von Krebserkrankungen, wie z. B. der Therapieresistenz oder der Tumorheterogenität bietet und der Eingang der Konsequenzen des Modells in den klinischen Alltag in absehbarer Zeit erfolgen könnte. Mit sicheren Markern könnten diese Zellen demnach detektiert und im Wissen ihrer funktionellen Besonderheiten in Kombination mit gegen die übrige Tumormasse gerichteten Therapeutika spezifisch attackiert werden. Genährt wird diese Hoffnung bereits von ersten Studien z. B. am KRK (Gupta et al., 2009). Hier konnten bereits KSZ-selektiv wirksame Stoffe identifiziert werden, während Nicht-KSZ wie erwartet ein sehr gutes Ansprechen auf herkömmliche Chemotherapeutika

wie 5-FU aufzeigten. Die Weiterverfolgung dieses Gedankens der Kombitherapie eröffnet somit Hoffnung einem großen Ziel der medizinischen Forschung ein wichtiges Stück näher kommen zu können: der Verbesserung der Lebensumstände sowie der Prognose der betroffenen Patienten.

5 LITERATURVERZEICHNIS

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J *16*, 3797-3804.

Aguilera, O., Fraga, M. F., Ballestar, E., Paz, M. F., Herranz, M., Espada, J., Garcia, J. M., Munoz, A., Esteller, M., and Gonzalez-Sancho, J. M. (2006). Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. Oncogene *25*, 4116-4121.

Akbulut, H., Altuntas, F., Akbulut, K. G., Ozturk, G., Cindoruk, M., Unal, E., and Icli, F. (2002). Prognostic role of serum vascular endothelial growth factor, basic fibroblast growth factor and nitric oxide in patients with colorectal carcinoma. Cytokine *20*, 184-190.

Akiyoshi, T., Nakamura, M., Koga, K., Nakashima, H., Yao, T., Tsuneyoshi, M., Tanaka, M., and Katano, M. (2006). Gli1, downregulated in colorectal cancers, inhibits proliferation of colon cancer cells involving Wnt signalling activation. Gut *55*, 991-999.

Albuquerque, C., Breukel, C., van der Luijt, R., Fidalgo, P., Lage, P., Slors, F. J., Leitao, C. N., Fodde, R., and Smits, R. (2002). The 'just-right' signaling model: APC somatic mutations are selected based on a specific level of activation of the beta-catenin signaling cascade. Hum Mol Genet *11*, 1549-1560.

Baehs, S., Herbst, A., Thieme, S. E., Perschl, C., Behrens, A., Scheel, S., Jung, A., Brabletz, T., Goke, B., Blum, H., and Kolligs, F. T. (2009). Dickkopf-4 is frequently down-regulated and inhibits growth of colorectal cancer cells. Cancer Lett *276*, 152-159.

Bafico, A., Liu, G., Goldin, L., Harris, V., and Aaronson, S. A. (2004). An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells. Cancer Cell *6*, 497-506.

Bao, S., Wu, Q., McLendon, R. E., Hao, Y., Shi, Q., Hjelmeland, A. B., Dewhirst, M. W., Bigner, D. D., and Rich, J. N. (2006). Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature *444*, 756-760.

Barker, N., and Clevers, H. (2001). Tumor environment: a potent driving force in colorectal cancer? Trends Mol Med *7*, 535-537.

Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M., and Clevers, H. (2001). The chromatin remodelling factor Brg-1 interacts with beta-catenin to promote target gene activation. Embo J *20*, 4935-4943.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J., and Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. Nature *457*, 608-611.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P. J., and Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature *449*, 1003-1007.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281-297.

Batzler, W. U., Hentschel, S., Husmann, G., Kaatsch, P., Katalinic, A., Kieschke, J., Kraywinkel, K., Meyer, M., Stabenow, R., Stegmaier, C.*, et al.* (2008). Krebs in

Deutschland 2003 - 2004. Häufigkeiten in Trends. 6. überarbeitete Auflage Robert Koch-Institut (Hrsg.) und die Gesellschaft der epidemiologischen Krebsregister in Deutschland eV (Hrsg) Berlin.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. Nature *382*, 638-642.

Beiter, K., Hiendlmeyer, E., Brabletz, T., Hlubek, F., Haynl, A., Knoll, C., Kirchner, T., and Jung, A. (2005). beta-Catenin regulates the expression of tenascin-C in human colorectal tumors. Oncogene *24*, 8200-8204.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311-320.

Bissell, M. J., and Labarge, M. A. (2005). Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment? Cancer Cell *7*, 17-23.

Blackburn, E. H. (2000). Telomere states and cell fates. Nature *408*, 53-56.

Boon, E. M., van der Neut, R., van de Wetering, M., Clevers, H., and Pals, S. T. (2002). Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. Cancer Res *62*, 5126-5128.

Brabletz, S., Schmalhofer, O., and Brabletz, T. (2009). Gastrointestinal stem cells in development and cancer. J Pathol *217*, 307-317.

Brabletz, T., Herrmann, K., Jung, A., Faller, G., and Kirchner, T. (2000). Expression of nuclear beta-catenin and c-myc is correlated with tumor size but not with proliferative activity of colorectal adenomas. Am J Pathol *156*, 865-870.

Brabletz, T., Hlubek, F., Spaderna, S., Schmalhofer, O., Hiendlmeyer, E., Jung, A., and Kirchner, T. (2005a). Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs *179*, 56-65.

Brabletz, T., Jung, A., Dag, S., Hlubek, F., and Kirchner, T. (1999). beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol *155*, 1033-1038.

Brabletz, T., Jung, A., Hermann, K., Gunther, K., Hohenberger, W., and Kirchner, T. (1998). Nuclear overexpression of the oncoprotein beta-catenin in colorectal cancer is localized predominantly at the invasion front. Pathol Res Pract *194*, 701-704.

Brabletz, T., Jung, A., Reu, S., Porzner, M., Hlubek, F., Kunz-Schughart, L. A., Knuechel, R., and Kirchner, T. (2001). Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. Proc Natl Acad Sci U S A *98*, 10356-10361.

Brabletz, T., Jung, A., Spaderna, S., Hlubek, F., and Kirchner, T. (2005b). Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer *5*, 744-749.

Brembeck, F. H., Rosario, M., and Birchmeier, W. (2006). Balancing cell adhesion and Wnt signaling, the key role of beta-catenin. Curr Opin Genet Dev *16*, 51-59.

Brembeck, F. H., Schwarz-Romond, T., Bakkers, J., Wilhelm, S., Hammerschmidt, M., and Birchmeier, W. (2004). Essential role of BCL9-2 in the switch between beta-catenin's adhesive and transcriptional functions. Genes Dev *18*, 2225-2230.

Burk, U., Schubert, J., Wellner, U., Schmalhofer, O., Vincan, E., Spaderna, S., and Brabletz, T. (2008). A reciprocal repression between ZEB1 and members of the miR-200 family promotes EMT and invasion in cancer cells. EMBO Rep *9*, 582-589.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M., and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. Nature *395*, 604-608.

Clarke, M. F., Dick, J. E., Dirks, P. B., Eaves, C. J., Jamieson, C. H., Jones, D. L., Visvader, J., Weissman, I. L., and Wahl, G. M. (2006). Cancer stem cells--perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res *66*, 9339-9344.

Clevers, H. (2006). Wnt/beta-catenin signaling in development and disease. Cell *127*, 469-480.

Conacci-Sorrell, M., Simcha, I., Ben-Yedidia, T., Blechman, J., Savagner, P., and Ben-Ze'ev, A. (2003). Autoregulation of E-cadherin expression by cadherin-cadherin interactions: the roles of beta-catenin signaling, Slug, and MAPK. J Cell Biol *163*, 847-857.

Conacci-Sorrell, M., Zhurinsky, J., and Ben-Ze'ev, A. (2002). The cadherin-catenin adhesion system in signaling and cancer. J Clin Invest *109*, 987-991.

Crosnier, C., Stamataki, D., and Lewis, J. (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet *7*, 349-359.

Dalerba, P., Dylla, S. J., Park, I. K., Liu, R., Wang, X., Cho, R. W., Hoey, T., Gurney, A., Huang, E. H., Simeone, D. M.*, et al.* (2007). Phenotypic characterization of human colorectal cancer stem cells. Proc Natl Acad Sci U S A *104*, 10158-10163.

Dallas, N. A., Xia, L., Fan, F., Gray, M. J., Gaur, P., van Buren, G., 2nd, Samuel, S., Kim, M. P., Lim, S. J., and Ellis, L. M. (2009). Chemoresistant colorectal cancer cells, the cancer stem cell phenotype, and increased sensitivity to insulin-like growth factor-I receptor inhibition. Cancer Res *69*, 1951-1957.

de la Chapelle, A. (2004). Genetic predisposition to colorectal cancer. Nat Rev Cancer *4*, 769-780.

Dieter, S. M., Ball, C. R., Hoffmann, C. M., Nowrouzi, A., Brand, K., Schmidt, M., Koch, M., Weitz, J., von Kalle, C., and Glimm, H. (2010). Distinct types of human colon cancer initiating cells contribute to primary tumor and metastasis formation. AACR 101st Annual Meeting 2010 in Washington, DC, USA *Abstract #9*.

Du, L., Wang, H., He, L., Zhang, J., Ni, B., Wang, X., Jin, H., Cahuzac, N., Mehrpour, M., Lu, Y., and Chen, Q. (2008). CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res *14*, 6751-6760.

Dvorak, P., Dvorakova, D., and Hampl, A. (2006). Fibroblast growth factor signaling in embryonic and cancer stem cells. FEBS Lett *580*, 2869-2874.

Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., and Hampl, A. (2005). Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells *23*, 1200-1211.

Dylla, S. J., Beviglia, L., Park, I. K., Chartier, C., Raval, J., Ngan, L., Pickell, K., Aguilar, J., Lazetic, S., Smith-Berdan, S.*, et al.* (2008). Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. PLoS One *3*, e2428.

Easwaran, V., Lee, S. H., Inge, L., Guo, L., Goldbeck, C., Garrett, E., Wiesmann, M., Garcia, P. D., Fuller, J. H., Chan, V.*, et al.* (2003). beta-Catenin regulates vascular endothelial growth factor expression in colon cancer. Cancer Res *63*, 3145-3153.

Eiselleova, L., Matulka, K., Kriz, V., Kunova, M., Schmidtova, Z., Neradil, J., Tichy, B., Dvorakova, D., Pospisilova, S., Hampl, A., and Dvorak, P. (2009). A complex role for FGF-2 in self-renewal, survival, and adhesion of human embryonic stem cells. Stem Cells *27*, 1847-1857.

Esquela-Kerscher, A., and Slack, F. J. (2006). Oncomirs - microRNAs with a role in cancer. Nat Rev Cancer *6*, 259-269.

Faber, C., Kirchner, T., and Hlubek, F. (2009). The impact of microRNAs on colorectal cancer. Virchows Arch *454*, 359-367.

Fang, D., Hawke, D., Zheng, Y., Xia, Y., Meisenhelder, J., Nika, H., Mills, G. B., Kobayashi, R., Hunter, T., and Lu, Z. (2007). Phosphorylation of beta-catenin by AKT promotes beta-catenin transcriptional activity. J Biol Chem *282*, 11221-11229.

Fearon, E. R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759-767.

Fevr, T., Robine, S., Louvard, D., and Huelsken, J. (2007). Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol Cell Biol *27*, 7551-7559.

Fillmore, C. M., and Kuperwasser, C. (2008). Human breast cancer cell lines contain stem-like cells that self-renew, give rise to phenotypically diverse progeny and survive chemotherapy. Breast Cancer Res *10*, R25.

Fodde, R., and Brabletz, T. (2007). Wnt/beta-catenin signaling in cancer stemness and malignant behavior. Curr Opin Cell Biol *19*, 150-158.

Garcia, M., Jemal, A., Ward, E. M., Center, M. M., Hao, Y., Siegel, R. L., and Thun, M. J. (2007). Global Cancer Facts and Figures 2007. 1-52.

George, M. L., Tutton, M. G., Abulafi, A. M., Eccles, S. A., and Swift, R. I. (2002). Plasma basic fibroblast growth factor levels in colorectal cancer: a clinically useful assay? Clin Exp Metastasis *19*, 735-738.

Gilles, C., Polette, M., Mestdagt, M., Nawrocki-Raby, B., Ruggeri, P., Birembaut, P., and Foidart, J. M. (2003). Transactivation of vimentin by beta-catenin in human breast cancer cells. Cancer Res *63*, 2658-2664.

Gottardi, C. J., and Gumbiner, B. M. (2004). Distinct molecular forms of beta-catenin are targeted to adhesive or transcriptional complexes. J Cell Biol *167*, 339-349.

Gradl, D., Kuhl, M., and Wedlich, D. (1999). The Wnt/Wg signal transducer beta-catenin controls fibronectin expression. Mol Cell Biol *19*, 5576-5587.

Gregorieff, A., and Clevers, H. (2005). Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev *19*, 877-890.

Gregory, P. A., Bert, A. G., Paterson, E. L., Barry, S. C., Tsykin, A., Farshid, G., Vadas, M. A., Khew-Goodall, Y., and Goodall, G. J. (2008). The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. Nat Cell Biol *10*, 593-601.

Grohmann, A., Tanneberger, K., Alzner, A., Schneikert, J., and Behrens, J. (2007). AMER1 regulates the distribution of the tumor suppressor APC between microtubules and the plasma membrane. J Cell Sci *120*, 3738-3747.

Gupta, P. B., Onder, T. T., Jiang, G., Tao, K., Kuperwasser, C., Weinberg, R. A., and Lander, E. S. (2009). Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell *138*, 645-659.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harris, T. J., and Peifer, M. (2005). Decisions, decisions: beta-catenin chooses between adhesion and transcription. Trends Cell Biol *15*, 234-237.

Hase, K., Shatney, C., Johnson, D., Trollope, M., and Vierra, M. (1993). Prognostic value of tumor "budding" in patients with colorectal cancer. Dis Colon Rectum *36*, 627-635.

Hastie, N. D., Dempster, M., Dunlop, M. G., Thompson, A. M., Green, D. K., and Allshire, R. C. (1990). Telomere reduction in human colorectal carcinoma and with ageing. Nature *346*, 866-868.

Hatzis, P., van der Flier, L. G., van Driel, M. A., Guryev, V., Nielsen, F., Denissov, S., Nijman, I. J., Koster, J., Santo, E. E., Welboren, W*., et al.* (2008). Genome-wide pattern of TCF7L2/TCF4 chromatin occupancy in colorectal cancer cells. Mol Cell Biol *28*, 2732-2744.

Hayflick, L. (1965). The Limited in Vitro Lifetime of Human Diploid Cell Strains. Exp Cell Res *37*, 614-636.

He, B., Reguart, N., You, L., Mazieres, J., Xu, Z., Lee, A. Y., Mikami, I., McCormick, F., and Jablons, D. M. (2005). Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. Oncogene *24*, 3054-3058.

He, T. C., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., da Costa, L. T., Morin, P. J., Vogelstein, B., and Kinzler, K. W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509-1512.

Hecht, A., Vleminckx, K., Stemmler, M. P., van Roy, F., and Kemler, R. (2000). The p300/CBP acetyltransferases function as transcriptional coactivators of beta-catenin in vertebrates. Embo J *19*, 1839-1850.

Hiendlmeyer, E., Regus, S., Wassermann, S., Hlubek, F., Haynl, A., Dimmler, A., Koch, C., Knoll, C., van Beest, M., Reuning, U*., et al.* (2004). Beta-catenin up-regulates the expression of the urokinase plasminogen activator in human colorectal tumors. Cancer Res *64*, 1209-1214.

Hirohashi, S. (1998). Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol *153*, 333-339.

Hlubek, F., Jung, A., Kotzor, N., Kirchner, T., and Brabletz, T. (2001). Expression of the invasion factor laminin gamma2 in colorectal carcinomas is regulated by beta-catenin. Cancer Res *61*, 8089-8093.

Horst, D., Kriegl, L., Engel, J., Jung, A., and Kirchner, T. (2009a). CD133 and nuclear beta-catenin: the marker combination to detect high risk cases of low stage colorectal cancer. Eur J Cancer *45*, 2034-2040.

Horst, D., Kriegl, L., Engel, J., Kirchner, T., and Jung, A. (2008). CD133 expression is an independent prognostic marker for low survival in colorectal cancer. Br J Cancer *99*, 1285-1289.

Horst, D., Kriegl, L., Engel, J., Kirchner, T., and Jung, A. (2009b). Prognostic significance of the cancer stem cell markers CD133, CD44, and CD166 in colorectal cancer. Cancer Invest *27*, 844-850.

Horst, D., Scheel, S. K., Liebmann, S., Neumann, J., Maatz, S., Kirchner, T., and Jung, A. (2009c). The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. J Pathol *219*, 427-434.

Houchen, C. W., George, R. J., Sturmoski, M. A., and Cohn, S. M. (1999). FGF-2 enhances intestinal stem cell survival and its expression is induced after radiation injury. Am J Physiol *276*, G249-258.

Huang, E. H., Hynes, M. J., Zhang, T., Ginestier, C., Dontu, G., Appelman, H., Fields, J. Z., Wicha, M. S., and Boman, B. M. (2009). Aldehyde dehydrogenase 1 is a

marker for normal and malignant human colonic stem cells (SC) and tracks SC overpopulation during colon tumorigenesis. Cancer Res *69*, 3382-3389.

Huang, J., Papadopoulos, N., McKinley, A. J., Farrington, S. M., Curtis, L. J., Wyllie, A. H., Zheng, S., Willson, J. K., Markowitz, S. D., Morin, P.*, et al.* (1996). APC mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci U S A *93*, 9049-9054.

Jass, J. R. (2007). Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology *50*, 113-130.

Jones, S., Chen, W. D., Parmigiani, G., Diehl, F., Beerenwinkel, N., Antal, T., Traulsen, A., Nowak, M. A., Siegel, C., Velculescu, V. E.*, et al.* (2008). Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A *105*, 4283-4288.

Jung, A., Schrauder, M., Oswald, U., Knoll, C., Sellberg, P., Palmqvist, R., Niedobitek, G., Brabletz, T., and Kirchner, T. (2001). The invasion front of human colorectal adenocarcinomas shows co-localization of nuclear beta-catenin, cyclin D1, and p16INK4A and is a region of low proliferation. Am J Pathol *159*, 1613-1617.

Kaplan, K. B., Burds, A. A., Swedlow, J. R., Bekir, S. S., Sorger, P. K., and Nathke, I. S. (2001). A role for the Adenomatous Polyposis Coli protein in chromosome segregation. Nat Cell Biol *3*, 429-432.

Katoh, Y., and Katoh, M. (2007). Comparative genomics on PROM1 gene encoding stem cell marker CD133. Int J Mol Med *19*, 967-970.

Kemper, K., Sprick, M. R., de Bree, M., Scopelliti, A., Vermeulen, L., Hoek, M., Zeilstra, J., Pals, S. T., Mehmet, H., Stassi, G., and Medema, J. P. (2010). The AC133 epitope, but not the CD133 protein, is lost upon cancer stem cell differentiation. Cancer Res *70*, 719-729.

Kinzler, K. W., and Vogelstein, B. (1996). Lessons from hereditary colorectal cancer. Cell *87*, 159-170.

Knudson, A. G. (1993). Antioncogenes and human cancer. Proc Natl Acad Sci U S A *90*, 10914-10921.

Kolquist, K. A., Ellisen, L. W., Counter, C. M., Meyerson, M., Tan, L. K., Weinberg, R. A., Haber, D. A., and Gerald, W. L. (1998). Expression of TERT in early premalignant lesions and a subset of cells in normal tissues. Nat Genet *19*, 182-186.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet *19*, 379-383.

Kyo, S., Takakura, M., Fujiwara, T., and Inoue, M. (2008). Understanding and exploiting hTERT promoter regulation for diagnosis and treatment of human cancers. Cancer Sci *99*, 1528-1538.

LaBarge, M. A. (2010). The difficulty of targeting cancer stem cell niches. Clin Cancer Res *16*, 3121-3129.

Lanza, G., Gafa, R., Matteuzzi, M., and Santini, A. (1999). Medullary-type poorly differentiated adenocarcinoma of the large bowel: a distinct clinicopathologic entity characterized by microsatellite instability and improved survival. J Clin Oncol *17*, 2429-2438.

Lapham, A., Adams, J. E., Paterson, A., Lee, M., Brimmell, M., and Packham, G. (2009). The Bcl-w promoter is activated by beta-catenin/TCF4 in human colorectal carcinoma cells. Gene *432*, 112-117.

Le, N. H., Franken, P., and Fodde, R. (2008). Tumour-stroma interactions in colorectal cancer: converging on beta-catenin activation and cancer stemness. Br J Cancer *98*, 1886-1893.

Liu, W., Dong, X., Mai, M., Seelan, R. S., Taniguchi, K., Krishnadath, K. K., Halling, K. C., Cunningham, J. M., Boardman, L. A., Qian, C.*, et al.* (2000). Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling. Nat Genet *26*, 146-147.

Ma, L., Young, J., Prabhala, H., Pan, E., Mestdagh, P., Muth, D., Teruya-Feldstein, J., Reinhardt, F., Onder, T. T., Valastyan, S.*, et al.* (2010). miR-9, a MYC/MYCN-activated microRNA, regulates E-cadherin and cancer metastasis. Nat Cell Biol *12*, 247-256.

Major, M. B., Camp, N. D., Berndt, J. D., Yi, X., Goldenberg, S. J., Hubbert, C., Biechele, T. L., Gingras, A. C., Zheng, N., Maccoss, M. J.*, et al.* (2007). Wilms tumor suppressor WTX negatively regulates WNT/beta-catenin signaling. Science *316*, 1043-1046.

Mani, S. A., Guo, W., Liao, M. J., Eaton, E. N., Ayyanan, A., Zhou, A. Y., Brooks, M., Reinhard, F., Zhang, C. C., Shipitsin, M.*, et al.* (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133*, 704-715.

Marotta, A., Parhar, K., Owen, D., Dedhar, S., and Salh, B. (2003). Characterisation of integrin-linked kinase signalling in sporadic human colon cancer. Br J Cancer *88*, 1755-1762.

Martin-Berenjeno, I., and Vanhaesebroeck, B. (2009). PI3K regulatory subunits lose control in cancer. Cancer Cell *16*, 449-450.

Melton, D. A., and Cowan, C. (2004). ´Stemness´: definition, criteria and standards., Vol 1, (USA: Elesevier Academic Press).

Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996). XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. Cell *86*, 391-399.

Moon, R. T., Kohn, A. D., De Ferrari, G. V., and Kaykas, A. (2004). WNT and beta-catenin signalling: diseases and therapies. Nat Rev Genet *5*, 691-701.

Muto, T., Bussey, H. J., and Morson, B. C. (1975). The evolution of cancer of the colon and rectum. Cancer *36*, 2251-2270.

O'Brien, C. A., Pollett, A., Gallinger, S., and Dick, J. E. (2007). A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. Nature *445*, 106-110.

Ogino, S., and Goel, A. (2008). Molecular classification and correlates in colorectal cancer. J Mol Diagn *10*, 13-27.

Ong, C. W., Kim, L. G., Kong, H. H., Low, L. Y., Iacopetta, B., Soong, R., and Salto-Tellez, M. (2010). CD133 expression predicts for non-response to chemotherapy in colorectal cancer. Mod Pathol *Epub ahead of print*.

Ootani, A., Li, X., Sangiorgi, E., Ho, Q. T., Ueno, H., Toda, S., Sugihara, H., Fujimoto, K., Weissman, I. L., Capecchi, M. R., and Kuo, C. J. (2009). Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. Nat Med *15*, 701-706.

Pang, R., Law, W. L., Chu, A. C., Poon, J. T., Lam, C. S., Chow, A. K., Ng, L., Cheung, L. W., Lan, X. R., Lan, H. Y.*, et al.* (2010). A subpopulation of CD26+ cancer stem cells with metastatic capacity in human colorectal cancer. Cell Stem Cell *6*, 603-615.

Park, S. M., Gaur, A. B., Lengyel, E., and Peter, M. E. (2008). The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev *22*, 894-907.

Pendas-Franco, N., Garcia, J. M., Pena, C., Valle, N., Palmer, H. G., Heinaniemi, M., Carlberg, C., Jimenez, B., Bonilla, F., Munoz, A., and Gonzalez-Sancho, J. M. (2008). DICKKOPF-4 is induced by TCF/beta-catenin and upregulated in human colon cancer, promotes tumour cell invasion and angiogenesis and is repressed by 1alpha,25-dihydroxyvitamin D3. Oncogene *27*, 4467-4477.

Perotti, D., Gamba, B., Sardella, M., Spreafico, F., Terenziani, M., Collini, P., Pession, A., Nantron, M., Fossati-Bellani, F., and Radice, P. (2008). Functional inactivation of the WTX gene is not a frequent event in Wilms' tumors. Oncogene *27*, 4625-4632.

Pino, M. S., and Chung, D. C. (2010). The chromosomal instability pathway in colon cancer. Gastroenterology *138*, 2059-2072.

Pinto, D., Gregorieff, A., Begthel, H., and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev *17*, 1709-1713.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev *14*, 1837-1851.

Potten, C. S., Gandara, R., Mahida, Y. R., Loeffler, M., and Wright, N. A. (2009). The stem cells of small intestinal crypts: where are they? Cell Prolif *42*, 731-750.

Potten, C. S., and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development *110*, 1001-1020.

Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843-850.

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Ricci-Vitiani, L., Lombardi, D. G., Pilozzi, E., Biffoni, M., Todaro, M., Peschle, C., and De Maria, R. (2007). Identification and expansion of human colon-cancer-initiating cells. Nature *445*, 111-115.

Rivera, M. N., Kim, W. J., Wells, J., Driscoll, D. R., Brannigan, B. W., Han, M., Kim, J. C., Feinberg, A. P., Gerald, W. L., Vargas, S. O.*, et al.* (2007). An X chromosome gene, WTX, is commonly inactivated in Wilms tumor. Science *315*, 642-645.

Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998). The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. Nature *395*, 608-612.

Rosen, J. M., and Jordan, C. T. (2009). The increasing complexity of the cancer stem cell paradigm. Science *324*, 1670-1673.

Sansom, O. J., Meniel, V. S., Muncan, V., Phesse, T. J., Wilkins, J. A., Reed, K. R., Vass, J. K., Athineos, D., Clevers, H., and Clarke, A. R. (2007). Myc deletion rescues Apc deficiency in the small intestine. Nature *446*, 676-679.

Sato, H., Suzuki, H., Toyota, M., Nojima, M., Maruyama, R., Sasaki, S., Takagi, H., Sogabe, Y., Sasaki, Y., Idogawa, M.*, et al.* (2007). Frequent epigenetic inactivation of DICKKOPF family genes in human gastrointestinal tumors. Carcinogenesis *28*, 2459-2466.

Scheel, S. K., Das, B., Kriegl, L., Mukhtari, R. B., Tsui, M., Yeger, H., Brabletz, T., Kirchner, T., and Jung, A. (2010a). The beta-Catenin target gene FGF-2 is a

mediator of stemness, EMT and chemoresistance in human colorectal tumor cells. Submitted to Cancer Cell.

Scheel, S. K., Hiendlmeyer, E., Brabletz, T., Haynl, A., Herbst, H., Kirchner, T., and Jung, A. (2010b). The human TERT (telomerase RT component) is a beta-CATENIN target gene in colorectal cancer. Submitted to Molecular Cancer.

Scheel, S. K., Porzner, M., Pfeiffer, S., Ormanns, S., Kirchner, T., and Jung, A. (2010c). Mutations in the WTX - gene are found in some high-grade microsatellite instable (MSI-H) colorectal cancers. BMC Cancer *10*, 413.

Shimono, Y., Zabala, M., Cho, R. W., Lobo, N., Dalerba, P., Qian, D., Diehn, M., Liu, H., Panula, S. P., Chiao, E.*, et al.* (2009). Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell *138*, 592-603.

Shitoh, K., Furukawa, T., Kojima, M., Konishi, F., Miyaki, M., Tsukamoto, T., and Nagai, H. (2001). Frequent activation of the beta-catenin-Tcf signaling pathway in nonfamilial colorectal carcinomas with microsatellite instability. Genes Chromosomes Cancer *30*, 32-37.

Shmelkov, S. V., Butler, J. M., Hooper, A. T., Hormigo, A., Kushner, J., Milde, T., St Clair, R., Baljevic, M., White, I., Jin, D. K.*, et al.* (2008). CD133 expression is not restricted to stem cells, and both CD133+ and CD133- metastatic colon cancer cells initiate tumors. J Clin Invest *118*, 2111-2120.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A *96*, 5522-5527.

Smith, G., Carey, F. A., Beattie, J., Wilkie, M. J., Lightfoot, T. J., Coxhead, J., Garner, R. C., Steele, R. J., and Wolf, C. R. (2002). Mutations in APC, Kirsten-ras, and p53--alternative genetic pathways to colorectal cancer. Proc Natl Acad Sci U S A *99*, 9433-9438.

Snippert, H. J., van Es, J. H., van den Born, M., Begthel, H., Stange, D. E., Barker, N., and Clevers, H. (2009). Prominin-1/CD133 marks stem cells and early progenitors in mouse small intestine. Gastroenterology *136*, 2187-2194 e2181.

Song, S., Wientjes, M. G., Gan, Y., and Au, J. L. (2000). Fibroblast growth factors: an epigenetic mechanism of broad spectrum resistance to anticancer drugs. Proc Natl Acad Sci U S A *97*, 8658-8663.

Spaderna, S., Schmalhofer, O., Wahlbuhl, M., Dimmler, A., Bauer, K., Sultan, A., Hlubek, F., Jung, A., Strand, D., Eger, A.*, et al.* (2008). The transcriptional repressor ZEB1 promotes metastasis and loss of cell polarity in cancer. Cancer Res *68*, 537-544.

Spirio, L. N., Samowitz, W., Robertson, J., Robertson, M., Burt, R. W., Leppert, M., and White, R. (1998). Alleles of APC modulate the frequency and classes of mutations that lead to colon polyps. Nat Genet *20*, 385-388.

Stein, U., Arlt, F., Walther, W., Smith, J., Waldman, T., Harris, E. D., Mertins, S. D., Heizmann, C. W., Allard, D., Birchmeier, W.*, et al.* (2006). The metastasis-associated gene S100A4 is a novel target of beta-catenin/T-cell factor signaling in colon cancer. Gastroenterology *131*, 1486-1500.

Sukhthankar, M., Yamaguchi, K., Lee, S. H., McEntee, M. F., Eling, T. E., Hara, Y., and Baek, S. J. (2008). A green tea component suppresses posttranslational expression of basic fibroblast growth factor in colorectal cancer. Gastroenterology *134*, 1972-1980.

Sussman, R. T., Ricci, M. S., Hart, L. S., Sun, S. Y., and El-Deiry, W. S. (2007). Chemotherapy-resistant side-population of colon cancer cells has a higher

sensitivity to TRAIL than the non-SP, a higher expression of c-Myc and TRAIL-receptor DR4. Cancer Biol Ther *6*, 1490-1495.

Suzuki, H., Watkins, D. N., Jair, K. W., Schuebel, K. E., Markowitz, S. D., Chen, W. D., Pretlow, T. P., Yang, B., Akiyama, Y., Van Engeland, M.*, et al.* (2004). Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat Genet *36*, 417-422.

Takahashi-Yanaga, F., and Kahn, M. (2010). Targeting Wnt signaling: can we safely eradicate cancer stem cells? Clin Cancer Res *16*, 3153-3162.

Takakura, M., Kyo, S., Kanaya, T., Hirano, H., Takeda, J., Yutsudo, M., and Inoue, M. (1999). Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res *59*, 551-557.

Tan, C., Costello, P., Sanghera, J., Dominguez, D., Baulida, J., de Herreros, A. G., and Dedhar, S. (2001). Inhibition of integrin linked kinase (ILK) suppresses beta-catenin-Lef/Tcf-dependent transcription and expression of the E-cadherin repressor, snail, in APC-/- human colon carcinoma cells. Oncogene *20*, 133-140.

Tatsumoto, N., Hiyama, E., Murakami, Y., Imamura, Y., Shay, J. W., Matsuura, Y., and Yokoyama, T. (2000). High telomerase activity is an independent prognostic indicator of poor outcome in colorectal cancer. Clin Cancer Res *6*, 2696-2701.

Tetsu, O., and McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422-426.

Thiery, J. P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer *2*, 442-454.

Todaro, M., Alea, M. P., Di Stefano, A. B., Cammareri, P., Vermeulen, L., Iovino, F., Tripodo, C., Russo, A., Gulotta, G., Medema, J. P., and Stassi, G. (2007). Colon cancer stem cells dictate tumor growth and resist cell death by production of interleukin-4. Cell Stem Cell *1*, 389-402.

Turner, N., and Grose, R. (2010). Fibroblast growth factor signalling: from development to cancer. Nat Rev Cancer *10*, 116-129.

Ueno, H., Murphy, J., Jass, J. R., Mochizuki, H., and Talbot, I. C. (2002). Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. Histopathology *40*, 127-132.

Valenta, T., Lukas, J., and Korinek, V. (2003). HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells. Nucleic Acids Res *31*, 2369-2380.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A. P.*, et al.* (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241-250.

van den Brink, G. R., Bleuming, S. A., Hardwick, J. C., Schepman, B. L., Offerhaus, G. J., Keller, J. J., Nielsen, C., Gaffield, W., van Deventer, S. J., Roberts, D. J., and Peppelenbosch, M. P. (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. Nat Genet *36*, 277-282.

van der Flier, L. G., and Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol *71*, 241-260.

Vermeulen, L., De Sousa, E. M. F., van der Heijden, M., Cameron, K., de Jong, J. H., Borovski, T., Tuynman, J. B., Todaro, M., Merz, C., Rodermond, H.*, et al.* (2010). Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol *12*, 468-476.

Vermeulen, L., Todaro, M., de Sousa Mello, F., Sprick, M. R., Kemper, K., Perez Alea, M., Richel, D. J., Stassi, G., and Medema, J. P. (2008). Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci U S A *105*, 13427-13432.

Wassermann, S., Scheel, S. K., Hiendlmeyer, E., Palmqvist, R., Horst, D., Hlubek, F., Haynl, A., Kriegl, L., Reu, S., Merkel, S.*, et al.* (2009). p16INK4a is a beta-catenin target gene and indicates low survival in human colorectal tumors. Gastroenterology *136*, 196-205 e192.

Weinberg, R. A. (2007). The Biology of Cancer, (New York: Garland Sciences).

Weisenberger, D. J., Siegmund, K. D., Campan, M., Young, J., Long, T. I., Faasse, M. A., Kang, G. H., Widschwendter, M., Weener, D., Buchanan, D.*, et al.* (2006). CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet *38*, 787-793.

Wellner, U., Schubert, J., Burk, U. C., Schmalhofer, O., Zhu, F., Sonntag, A., Waldvogel, B., Vannier, C., Darling, D., zur Hausen, A.*, et al.* (2009). The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. Nat Cell Biol *11*, 1487-1495.

Wielenga, V. J., Smits, R., Korinek, V., Smit, L., Kielman, M., Fodde, R., Clevers, H., and Pals, S. T. (1999). Expression of CD44 in Apc and Tcf mutant mice implies regulation by the WNT pathway. Am J Pathol *154*, 515-523.

Yamada, T., Takaoka, A. S., Naishiro, Y., Hayashi, R., Maruyama, K., Maesawa, C., Ochiai, A., and Hirohashi, S. (2000). Transactivation of the multidrug resistance 1 gene by T-cell factor 4/beta-catenin complex in early colorectal carcinogenesis. Cancer Res *60*, 4761-4766.

Yeung, T. M., Gandhi, S. C., Wilding, J. L., Muschel, R., and Bodmer, W. F. (2010). Cancer stem cells from colorectal cancer-derived cell lines. Proc Natl Acad Sci U S A *Epub ahead of print*.

Zhang, T., Otevrel, T., Gao, Z., Gao, Z., Ehrlich, S. M., Fields, J. Z., and Boman, B. M. (2001a). Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer. Cancer Res *61*, 8664-8667.

Zhang, X., Gaspard, J. P., and Chung, D. C. (2001b). Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia. Cancer Res *61*, 6050-6054.

Zhu, L., Gibson, P., Currle, D. S., Tong, Y., Richardson, R. J., Bayazitov, I. T., Poppleton, H., Zakharenko, S., Ellison, D. W., and Gilbertson, R. J. (2009). Prominin 1 marks intestinal stem cells that are susceptible to neoplastic transformation. Nature *457*, 603-607.

6 ABBILDUNGSVERZEICHNIS

7 ANHANG

7.1 Abkürzungen

5-FU	5-Fluoro-Uracil
AKF	Aberrante Kryptenfoci
ALDH1	Aldehyd Dehydrogenase 1
APC	Adenomatous Polyposis Coli
al.	alia
c-AMP	cyclic Adenosin Mono-phosphate
CBP	C-AMP RESPONSE ELEMENT BINDING PROTEIN
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomale Instabilität
CKIα	Casein Kinase I α
CTBP	C-terminal Binding Protein
DCC	Deleted in Colon Cancer
DKK	Dickkopf
DNA	Desoxyribonukleinsäure (*desoxyribonucleic acid*)
DSH	Dishevelled
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epitheliale-Mesenchymale Transition
FACS	Fluorescent Activated Cell Sorting
FAP	Familiäre Adenomatöse Polyposis coli
FGF	Fibroblast Growth Factor
FRZ	Frizzled
GRO	Groucho

GSK3β	Glycogen Synthase Kinase 3 β
HAT	Histon Acetyl Transferase
HDAC	Histon Deacetylase
HGF	Hepatocyte Growth Factor
HH	Hedgehog
HMG	High Mobility Group
HNPCC	Hereditary Non Polyposis Colorectal Carcinoma
IL4	Interleukin 4
ILK	Integrin linked kinase
INK4A	Inhibitor of Cyclin dependent Kinase 4
ISEMF	Intestinale SubEpitheliale Myofibroblasten
koKSZ	Kolorektale Tumor-/Krebsstammzelle (*colorectal cancer stem cell)*
KRK	Kolorektales Karzinom (*colorectal carcinoma*)
KSZ	Tumor-/Krebsstammzelle (*cancer stem cell*)
LEF	Lymphoid Enhancer Factor
Lgr5	Leucine rich repeat containing G-Protein coupled Receptor 5
LOH	Loss of heterozigosity
LRP	Low density lipoprotein Receptor related Protein
mKSZ	migrierende Krebsstammzelle (*migrating cancer stem cell*)
MDR1	Multi Drug Resistance 1
MET	Mesenchymale-Epitheliale Transition
miRNA	micro RNA
MLH1	Mut-L homologue 1
MMP7	Matrix Metalloproteinase 7
MMR	Mismatch Repair System
mRNA	Messenger RNA
MSI-H	High-grade MicroSatellite Instability
N-Terminus	Amino-Terminus

PCR	Polymerase Kettenreaktion (*polymerase chain reaction)*
RNA	Ribonukleinsäure (*ribonucleic acid*)
RNAi	RNA Interferenz
s. S.	siehe Seite
SC	Stammzelle (*stem cell*)
SFRP	Secreted Frizzled Related Protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMAD	Sma- and Drosophila homolog of Mothers Against Decapentaplegic 2
SNF	Sucrose Non-fermenting
sog.	sogenannte
SP	Side Population
TBE	TCF Binde-Element
TCF	T-Cell Factor
TLE	Transduction-Like Enhancer of split
TERT	Telomerase Reverse Transkriptase
TrCP	Beta-Transducin repeat-Containing Protein
u. a.	unter anderem
UICC	Union Internationale Contre le Cancer
UPA	Urokinase Plasminogen Activator
UTR	Untranslatierte Region (*untranslated region*)
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
WNT	Wingless-type MMTV integration site family
WTX	Wilms Tumor gene on the X chromosome
z. B.	zum Beispiel
ZEB1	Zinc finger E box Binding Homeobox

7.2 Manuskript-Beiträge

3.1 In einigen hochgradig mikrosatelliteninstabilen (MSI-H) kolorektalen Tumoren finden sich Mutationen im WTX – Gen

In diesem Projekt war ich an der Entwicklung und der Koordination der Studie beteiligt. Darüber hinaus entwickelte und optimierte ich die analytischen Methoden, generierte einen Großteil der Daten und wertete diese aus. Außerdem fertigte ich die Abbildungen an, schrieb den Entwurf des Manuskripts und optimierte und submittierte das Manuskript zusammen mit Andreas Jung.

3.2 Dickkopf-4 ist in kolorektalen Krebszellen oft herunterreguliert und inhibiert ihr Wachstum

In diesem Projekt analysierte ich mittels von mir etablierter Real Time PCR die DKK4 Expression im Tumor verglichen zum Normalgewebe und wertete die hier gewonnenen Daten aus (Abbildung 1E). Ich war zudem an Diskussionen das Projekt betreffend und am Korrekturlesen des Manuskripts beteiligt.

3.3 Der Krebsstammzellmarker CD133 hat eine hohe prognostische Aussagekraft aber unbekannte funktionelle Relevanz für die Metastasierung von humanem Dickdarmkrebs

Mein alleiniger Beitrag zu dieser Veröffentlichung lag in der Entwicklung und Durchführung sämtlicher zell- und molekularbiologischen Analysen, deren Auswertung und der Anfertigung der entsprechenden Abbildungen. Zusammen mit David Horst und Andreas Jung plante und koordinierte ich das Projekt, schrieb Teile des Manuskriptes und war am Submissions- und Reviewprozess beteiligt. Zusammen mit David Horst bin ich *equally contributing* Erstautor dieser Publikation.

3.4 Die humane TERT (Telomerase RT-Komponente) ist in kolorektalen Tumoren ein Zielgen von β-CATENIN

In diesem Projekt war ich an der Planung der experimentellen Strategie sowie der Koordination der Experimente beteiligt. Darüber hinaus führte ich einen Großteil der Experimente aus (insbesondere die der Abbildung 3), analysierte die Daten und fertigte die Abbildungen an. Zusammen mit Andreas Jung erarbeitete und submittierte ich das Manuskript.

3.5 p16INK4A ist ein β-Catenin Zielgen und korreliert mit schlechtem Überleben in humanen kolorektalen Tumoren

Für diese Veröffentlichung in *Gastroenterology* lag mein Beitrag in der Durchführung eines Teils der molekularbiologischen Analysen (Abbildung 1 und 2). Außerdem wertete ich die Daten aus, fertigte Abbildungen an und war an der Erstellung des Manuskripts beteiligt. Zusammen mit Stella Wassermann bin ich *equally contributing* Erstautor dieser Veröffentlichung.

3.6 Das β-CATENIN Zielgen FGF-2 ist ein Mediator von Stemness, EMT und Chemoresistenz in humanen kolorektalen Tumorzellen

Die Arbeit an diesem Projekt und Manuskript stellt den Hauptanteil meiner Promotion dar. Zusammen mit Andreas Jung entwickelte ich die Idee zu diesem Projekt, plante die experimentelle Strategie und führte alle Arbeiten mit Ausnahme der FACS-Analysen und den Mausexperimenten aus. Auf einem Workshop in Snowmass Village, Colorado, USA initiierte ich die Kooperation mit meinem *equally contributing* Erstautor Bikul Das vom Hospital for Sick Children, aus Toronto, Kanada (jetzt Stanford University, Kalifornien, USA) und koordinierte diese. Ich fertigte alle Abbildungen an, schrieb den Entwurf des Manuskripts und überarbeitete und submittierte diesen zusammen mit Andreas Jung.

7.3 Erklärung gemäß der "Promotionsordnung der LMU München für die Fakultät Biologie"

Betreuung: Hiermit erkläre ich, dass die vorgelegte Arbeit an der LMU von Herrn PD Dr. Andreas Jung (med. Fak.) betreut und von Herrn Professor Dr. Heinrich Leonhardt vor der Fakultät für Biologie vertreten wurde.

Anfertigung: Hiermit versichere ich ehrenwörtlich, dass die Dissertation selbstständig und ohne unerlaubte Hilfsmittel angefertigt wurde. Über Beiträge, die im Rahmen der kumulativen Dissertation in Form von Manuskripten in der Dissertation enthalten sind, wurde im Kapitel 7.2 Rechenschaft abgelegt und die eigenen Leistungen wurden aufgelistet.

Prüfung: Hiermit erkläre ich, dass die Dissertation weder als Ganzes noch in Teilen an einem anderen Ort einer Prüfungskommission vorgelegt wurde. Weiterhin habe ich weder an einem anderen Ort eine Promotion angestrebt oder angemeldet oder versucht eine Doktorprüfung abzulegen.

München, den 12.07.2010

……

(Silvio Scheel)

7.4 Danksagung

Zuallererst möchte ich mich bei meinem Betreuer und Doktorvater Dr. Andreas Jung bedanken. Es war mir eine sehr große Ehre und Freude mit Dir zusammenzuarbeiten. Die Jahre mit Dir haben mich in vielerlei Hinsicht unheimlich geprägt! Du hast es dabei geschafft mich mit Deiner immer positiven Einstellung in **allen** Lebenslagen und selbst den verzwicktesten Situationen im Forschungsalltag immer wieder zu motivieren am Ball zu bleiben. Vielenvielen Dank für Deine besondere Unterstützung und Deine Förderung!

Allen derzeitigen und ehemaligen Mitgliedern der AG Jung danke ich von ganzem Herzen für die (meist) tolle, kollegiale aber auch freundschaftliche Labor-, Büro- und Feierabend-Atmosphäre. Ganz besonderer Dank geht an Jana, Achim, Steffen, Sabine und Sabine, Angie, Alex, Martina, Steffi und Laura. Ich hoffe Ihr werdet Euren Silliboy ordentlich vermissen!

Professor Kirchner und allen Kollegen des Pathologischen Instituts danke ich dafür meine Dr.-Arbeit unter herausragenden Bedingungen und mit optimaler Unterstützung angefertigt haben zu können. Besonders erwähnen möchte ich hier auch die sehr angenehme Zusammenarbeit mit Sibylle, Evelyn, Lydia, Helge, René, Monika und vielen weiteren.

Bei Herrn Professor Dr. Leonhardt bedanke ich mich dafür meine Promotion vor der Fakultät für Biologie vertreten zu haben und für seine Bereitschaft als Gutachter zu fungieren. Genauso danke ich auch Herrn Professor Dr. Eick und allen weiteren Mitgliedern der Prüfungskommission.

Ein weiterer Dank geht an alle Kooperationspartner, insbesondere die AG Brabletz (vor allem Thomas, Simone und Otto), Bikul Das, Andreas Herbst und Harald Lahm.

Ferner danke ich allen meinen Freunden, die mich zwar oft „nervten“ wann ich denn endlich fertig sei, mir aber durch Ihre Freundschaft und durch Abwechslung vom Laboralltag immer wieder die Kraft gegeben haben, meine Ziele zu erreichen. Besonderer Dank geht an Wolfgang, ohne dessen Gunst und Weitsicht ich wohl niemals in der Patho gelandet wäre und an Maria fürs Einfachdasein in der langen Anfangsphase in der gar nichts voranging. Meinem iPod und meiner Susi danke ich für ihre immerwährende Treue und Zuverlässigkeit, außer ihre Reifen waren mal wieder aufgeschlitzt.

Für viele schöne, entspannende, lustige aber auch frustbewältigende Situationen geht ein feuchtfröhlicher Dank an Augustiner, Shiraz und Ramazzotti, keinesfalls aber an Löwenbräu.

Zu guter Letzt geht der größte Dank an meine Familie, dafür trotz allem einfach zu wissen wo man herkommt und wo man hingehört. Ich bin froh, dass ich Euch alle habe! Danke Mama, danke Papa!

7.5 Curriculum Vitae

Persönliche Daten

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Studium

Seit 05 / 2005	Promotion in der Arbeitsgruppe von PD Dr. Andreas Jung
	Pathologisches Institut, LMU München und FAU Erlangen
04 / 2005	Diplom in Biologie (Abschlussnote 1,5)
07 / 2004 - 03 / 2005	Diplomarbeit in der Arbeitsgruppe von Prof. Dr. Robert Slany
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Thema:	„Charakterisierung des transformierenden Potentials von MLL2“
10 / 1999 - 04 / 2005	Studium der Biologie, FAU Erlangen - Nürnberg

Zivildienst

07 / 1998 - 07 / 1999	Apotheke des Klinikums Nürnberg, Deutschland

Schulbildung

07 / 1998	Allgemeine Hochschulreife (Abschlussnote 1,7)
09 / 1989 - 07 / 1998	Emil-von-Behring-Gymnasium, Spardorf, Deutschland
09 / 1985 - 07 / 1989	Grundschule Eckental-Brand, Deutschland

7.6 Kongress - und Workshop - Teilnahmen

April 2006 **„β-CATENIN regulates the expression of TENASCIN-C in human colorectal tumors."** Posterpräsentation auf der 90. Jahrestagung der Deutschen Gesellschaft für Pathologie in Berlin, Deutschland

Oktober 2006 **„β-CATENIN regulates the expression of TENASCIN-C in human colorectal tumors."** Posterpräsentation auf dem 35. Kongress der Gesellschaft für Gastroenterologie in Bayern e.V., in Nürnberg, Deutschland

Juni 2007 **"The cell cycle inhibitor $p16^{INK4A}$ is a β-CATENIN target gene and its expression correlates with low survival in human colorectal carcinomas."** Vortrag auf der 91. Jahrestagung der Deutschen Gesellschaft für Pathologie in Magdeburg, Deutschland

Juli 2007 **„The pleiotropic factor FGF-2 is a β-CATENIN target gene in human colorectal adenocarcinomas."** Posterpräsentation auf dem AACR Edward A. Smuckler Memorial Pathobiology of Cancer workshop in Snowmass Village, Colorado, USA

April 2008 **„The β-CATENIN target gene FGF-2 is expressed in a small fraction of tumor cells with characteristics of cancer stem cells in human colorectal cancer."** Vortrag auf dem 99. Annual Meeting 2008 der American Association for Cancer Research (AACR) in San Diego, Kalifornien, USA

Mai 2008 **„The β-CATENIN target gene FGF-2 is expressed in a small fraction of tumor cells with characteristics of cancer stem cells in human colorectal cancer."** Vortrag auf der 92. Jahrestagung der Deutschen Gesellschaft für Pathologie in Berlin, Deutschland

Juni 2009 **„CD133 in colorectal carcinomas: a clinical relevant cancer stem cell marker with unknown functional relevance."** Vortrag und Moderation der Sitzungen „State of the art - Lectures: Invasion and Metastasierung" und "Grundlagen von Invasion und Metastasierung" auf der 93. Jahrestagung der Deutschen Gesellschaft für Pathologie in Freiburg, Deutschland

April 2010 **“Expression of FGF-2 is essential for the maintenance of cancer stem cell characteristics in human colorectal tumor cell lines.”** Posterpräsentation (*late breaking abstract*) auf dem 101. Annual Meeting 2010 der American Association for Cancer Research (AACR) in Washington, DC, USA

Mai 2010 **“Expression of FGF-2 is essential for the maintenance of cancer stem cell characteristics in human colorectal tumor cell lines.”** Posterpräsentation auf der 94. Jahrestagung der Deutschen Gesellschaft für Pathologie in Berlin, Deutschland

“hTERT (human telomerase RT-component) expression is regulated by β-CATENIN in human colorectal cancer.” Posterpräsentation (vertreten durch Jana Reiche, AG Jung) auf der 94. Jahrestagung der Deutschen Gesellschaft für Pathologie in Berlin, Deutschland

“WTX mutations in high-grade microsatellite instable (MSI-H) colorectal cancers.” Vortrag (vertreten durch Achim Schäffauer, AG Jung) auf der 94. Jahrestagung der Deutschen Gesellschaft für Pathologie in Berlin, Deutschland

7.7 Auszeichnungen

April 2008 **AACR International Scholar-in-Training Award**

der American Association for Cancer Research (AACR), San Diego, CA, USA

Mai 2010 **Posterpreisträger, 1. Preis**

der 94. Jahrestagung der Deutschen Gesellschaft für Pathologie, Berlin, Deutschland

7.8 Eigene Publikationen

Wassermann, S., **Scheel, S. K**., Hiendlmeyer, E., Palmqvist, R., Horst, D., Hlubek, F., Haynl, A., Kriegl, L., Reu, S., Merkel, S*., et al.* (2009). p16INK4a is a beta-catenin target gene and indicates low survival in human colorectal tumors. *Gastroenterology 136, 196-205 e192.* (*equally contributing first author*)

Baehs, S., Herbst, A., Thieme, S. E., Perschl, C., Behrens, A., **Scheel, S**., Jung, A., Brabletz, T., Goke, B., Blum, H., and Kolligs, F. T. (2009). Dickkopf-4 is frequently down-regulated and inhibits growth of colorectal cancer cells. *Cancer Lett 276, 152-159.*

Horst, D., **Scheel, S. K**., Liebmann, S., Neumann, J., Maatz, S., Kirchner, T., and Jung, A. (2009). The cancer stem cell marker CD133 has high prognostic impact but unknown functional relevance for the metastasis of human colon cancer. *J Pathol 219, 427-434*. (*equally contributing first author*)

Scheel, S. K., Pfeiffer, S., Ormanns, S., Kirchner, T., and Jung, A. (2010). Mutations in the WTX - gene are found in some high-grade microsatellite instable (MSI-H) colorectal cancers. *BMC Cancer, 10, 413*.

Scheel, S. K., Hiendlmeyer, E., Brabletz, T., Haynl, A., Herbst, H., Kirchner, T., and Jung, A. (2010). The human TERT (telomerase RT component) is a beta-CATENIN target gene in colorectal cancer. *Under Review at Molecular Cancer*.

Scheel, S. K., Das, B., Kriegl, L., Tsui, M., Mukhtari, R. B., Brabletz, T., Yeger, H., Kirchner, T., and Jung, A. (2010). The beta-CATENIN target gene FGF-2 is essential for the maintenance of cancer stem cell characteristics in human colorectal tumor cells. *Under Review at Cancer Cell*.