Short Article

Methylation of Cancer-Stem-Cell-Associated Wnt Target Genes Predicts Poor Prognosis in Colorectal Cancer Patients

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SUMMARY

Gene signatures derived from cancer stem cells (CSCs) predict tumor recurrence for many forms of cancer. Here, we derived a gene signature for colorectal CSCs defined by high Wnt signaling activity, which in agreement with previous observations predicts poor prognosis. Surprisingly, however, we found that elevated expression of Wnt targets was actually associated with good prognosis, while patient tumors with low expression of Wnt target genes segregated with immature stem cell signatures. We discovered that several Wnt target genes, including *ASCL2* and *LGR5*, become silenced by CpG island methylation during progression of tumorigenesis, and that their re-expression was associated with reduced tumor growth. Taken together, our data show that promoter methylation of Wnt target genes is a strong predictor for recurrence of colorectal cancer, and suggest that CSC gene signatures, rather than reflecting CSC numbers, may reflect differentiation status of the malignant tissue.

INTRODUCTION

Colorectal cancer (CRC) is a major contributor to cancer-related death. Although patients with local disease (stage I and II) have a favorable prognosis, a small fraction of these patients will inevitably develop a recurrence after intentionally curative surgery (Edge and Compton, 2010). Identifying these poor-prognosis patients will allow optimized selection of individuals that would benefit from adjuvant chemotherapy. Exciting new insights in tumor biology might facilitate this selection; of particular interest in this respect is the recent discovery of cancer stem cells (CSCs) in CRC, a subset of cells that are defined by their capacity to

transplant the human malignancy to immuno-compromised mice (O'Brien et al., 2007; Ricci-Vitiani et al., 2007). CSCs share many features with normal intestinal stem cells (ISCs) (Medema and Vermeulen, 2011), and can be identified based on cell surface markers, such as CD133 (Todaro et al., 2007; Vermeulen et al., 2008), or functionally, using high Wnt-signaling activity levels (Vermeulen et al., 2010). Importantly, CSCs are suggested to fuel tumor growth and as such are hypothesized to cause tumor recurrence and metastasis. Accordingly, recent reports address the relation between stem cells, CSCs, and patient prognosis in different malignancies, including CRC (Liu et al., 2007). For example, in a recent study by Merlos-Suárez et al. (2011) a mouse ISC signature has been derived by identifying genes that are highly expressed in EphB2^{high} ISCs compared with that of the more differentiated epithelial cells. This profile encompasses known ISC markers, such as the Wnt target gene *LGR5*, and is strongly associated with both CRC disease stage and the occurrence of tumor relapse and metastasis formation. This has led to the suggestion that an increased number of CSCs is predictive for prognosis.

Here we show that CSC-derived gene signatures can indeed predict tumor recurrence. However, the positive association is not due to expression of specific CSC and CSC-associated Wnt target genes, which rather inversely correlate with prognosis. The subset of patients identified in this manner display decreased expression of a wide range of ISC/Wnt target genes. This is not due to decreased Wnt pathway activity, but is a result of selective promoter methylation. Moreover, Wnt target methylation levels, by themselves, can be used to effectively identify patients at risk of recurrence, and re-expression of these methylated genes lowers tumorigenicity in vitro and in vivo.

RESULTS

ISC and CSC Profiles Predict Poor Prognosis in CRC

Previously we have shown that colon-CSCs can be identified in primary human CRC using Wnt signaling intensity levels and can

be isolated by employing a Wnt reporter construct (TOP-GFP, Figure 1A) (Vermeulen et al., 2010). Gene expression profiling of the TOP-GFP[high] human colon-CSCs indicated high expression of the stem cell marker *LGR5*, as well as several other Wnt targets (*APCDD1*, *LEF1*), while typical intestinal differentiation markers (e.g., *MUC2* and *FABP1*) displayed low expression (Figure 1A). Based on two primary isolated spheroid cultures, we generated a colon-CSC signature comprising 187 genes most differentially expressed between the CSCs and the more differentiated cells (Table S1A, available online). This profile was subsequently validated in several independent freshly isolated CRCs (Figure S1A, available online).

Crucially, by employing gene set enrichment analysis (GSEA) (Subramanian et al., 2005), we found that this colon-CSC gene expression signature was intimately associated with disease recurrence in a set of 90 stage II CRC patients that underwent intentionally curative surgery at our institute (AMC-AJCCII-90, see Table S1B for patient characteristics), a finding we confirmed in an unrelated, publically available data set (Figure 1B and Figure S1B). Similar results were obtained using two independent ISC profiles that have previously been shown to relate to disease recurrence, lending strength to the validity of both the CSC signature and our patient set (Figure 1B and Figure S1B) (Merlos-Suárez et al., 2011). Using a simple rank-sum approach, we stratified the AMC-AJCCII-90 patients into two groups, which further established the prognostic power of the CSC profile because it revealed that especially early relapses in stage II CRC patients were characterized by a strong resemblance to the CSC signature (Figure 1C, see Experimental Procedures for details). Importantly, however, the nature and biological implication of this correlation remain unclear.

CSC-Associated Wnt Target Genes Inversely Correlate with Prognosis

In an attempt to analyze the biological mechanism behind the prognostic power of CSC profiles in more depth and to identify genes that are most predictive regarding tumor relapse and metastasis formation, we employed a cluster analysis. Unsupervised K-means clustering of the AMC-AJCCII-90 set using the TOP-GFP[high]/CSC-derived gene expression profile resulted in two distinct patient groups with a significant difference in relapse-free survival, as the Kaplan-Meier curve illustrates (Figure 1D). Gene tree analysis revealed that segregation of these two clusters is accompanied by generation of two major subgroups of genes. The majority of genes were upregulated in the patient cluster that was associated with poor prognosis (blue). However, a clearly distinct subset of genes, at the bottom region of the gene tree, inversely correlated with disease relapse (Figure 1D). To our surprise this gene cluster contained many well-known Wnt target genes of which the expression is intimately linked to ISCs and CSCs. (For a list of genes and their differential expression between the clusters, see Tables S1C and S1D). Similar results were obtained by employing different stem cell signatures, including the LGR5− and EphB2 ISC signatures that have been described previously (Figures S1C and S1D) (Merlos-Suárez et al., 2011). In all cases high expression of the Wnt-driven ISC marker genes present in the different signatures, like *ASCL2*, *LGR5*, *AXIN2*, and *APCDD1*, were associated with the favorable prognosis cluster (Figure 1D and

Figure S1C and S1D). We confirmed the expression level differences between the two groups by qPCR (Figure S1E) and found that expression was not related to oncogenic mutations present within the different patients (Figure S2A). Multivariate Cox regression analysis indicated the profile to be an independent prognostic factor that was much more predictive than the presence of different mutations (Figure S2A). Strikingly, we also observed that the expression of Wnt target genes was not a simple reflection of patient-to-patient variation in Wnt signaling activity as measured by nuclear localized β-catenin levels (Figure 1F).

Intrigued by this counterintuitive finding, which indicates that high Wnt target gene expression is linked to favorable rather than poor prognosis, we repeated this analysis with a more defined set of Wnt target genes, previously identified by overexpression of dominant-negative TCF4 (dnTCF4) in CRC cell lines (van de Wetering et al., 2002). The clear majority of genes in this dnTCF4 signature are also markedly lower expressed in the poor-prognosis patient cluster (Figure 1E). Even single Wnt target genes, including the validated ISC markers *EPHB2* (Merlos-Suárez et al., 2011), *LGR5* (Barker et al., 2007), and *ASCL2* (van der Flier et al., 2009), but also more general Wnt targets such as *AXIN2* and *APCDD1*, can identify poor-prognosis patients based on their low expression levels both in our patient set and in publically available data sets (Figure S2B and results not shown).

The finding that high expression of genes intimately associated with the CSC phenotype is associated with good prognosis in CRC immediately challenges the conventional interpretation as to why (cancer-) stem-cell-associated profiles define poor prognosis in cancer. Mostly it is believed that association with a CSC profile reflects the number of CSC-like cells in the malignancy. However, when we use FACS staining to define the fraction of cells positive for CD133 in several freshly isolated colon cancer specimens, which so far is the best studied and validated means to identify colon-CSCs, we could not correlate the number of CSCs to the overall expression of CSC-associated Wnt target genes within these tumors (Figure S2C). In addition, the fraction of CRC cells positive for nuclear β-catenin, which has been used before as a trait to identify colon-CSCs, also does not correlate significantly with Wnt target gene expression in our patient data set (Figure S2D). These findings both indicate that CSC numbers in CRC are not causal determinants in the patient stratification obtained with the CSC-associated expression signature. More importantly, the lack of correlation between nuclear β-catenin levels and Wnt target gene expression indicates that additional regulatory mechanisms are in place to regulate Wnt target gene expression.

CSC-Associated Wnt Targets Are Downregulated during Progression

In order to better understand why CSC-associated Wnt targets are inversely correlated with prognosis, we determined the expression of five Wnt target genes at multiple stages during the adenoma-carcinoma sequence. As expected, comparison of normal tissue with adenoma tissue revealed a marked increase in expression of most Wnt target genes, in line with the notion that activation of the Wnt cascade is the initiating event in CRC development (Figure 2A) (Morin et al., 1997). It is well accepted that further genetic and epigenetic alterations in
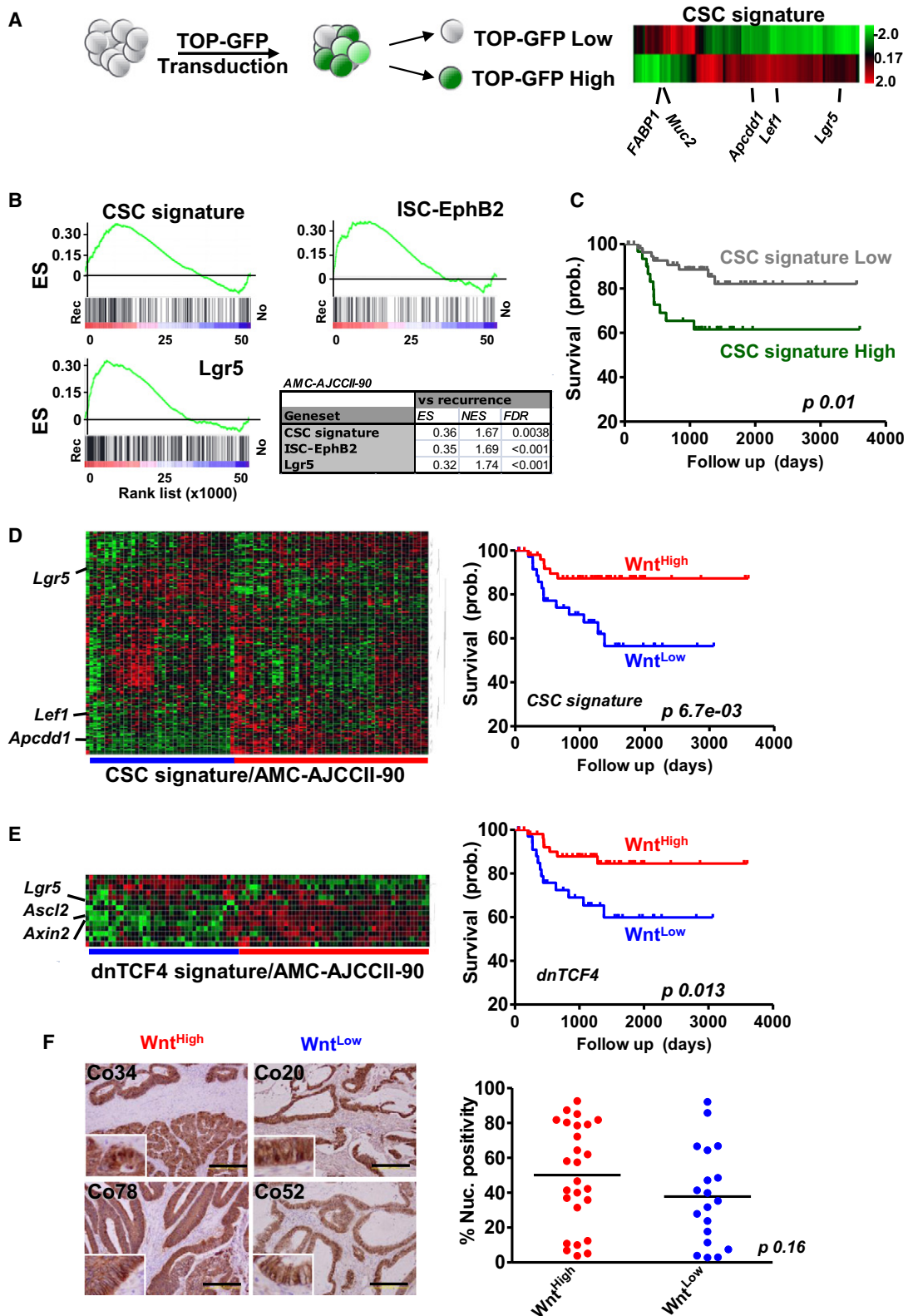
Figure 1. CSC Profile Predicts Poor Prognosis; Wnt Targets and ISC Markers Predict Favorable Prognosis
(A) Schematic representation of CSC spheroid transduction with TOP-GFP lentiviral vector. Microarray analysis was performed on 10% lowest and highest TOP-GFP sorted fractions to generate a colon-CSC signature. The heat map depicts the most differentially regulated genes, including differentiation markers (*FABP1*, *MUC2*) and Wnt canonical targets (*LGR5*, *LEF1*, and *APCDD1*). See Table S1A for the complete gene set.

the premalignant adenoma tissue mark the transition to an invasively growing CRC (Fearon and Vogelstein, 1990). However, evaluation of Wnt target gene expression in CRC samples strikingly indicated a downregulation in the majority of patients compared with the adenoma stage (Figure 2A). This was also observed in an independent data set containing normal, adenoma, and carcinoma samples (Figures S3A and S3B). The relevance of this suppressed Wnt target gene expression was immediately evident: patients that developed a tumor relapse displayed the lowest expression levels (Figure 2A, red triangles). This confirms our findings that a low Wnt target gene expression profile is related to poor prognosis. In addition, we obtained evidence that CRC tissue characterized by a low Wnt target expression signature demonstrates other characteristics of advanced disease as well. In this respect we analyzed the association of the patients in the Wnt target-low cluster with immature stem cell signatures (based on SOX2, OCT4, and Nanog targets), which have been used previously to determine disease grade and prognosis in cancer patients (Figure S3C) (Ben-Porath et al., 2008). GSEA showed a clear correlation between the Wnt target-low cluster and these signatures (Figure S3D). This correlation was not detected when a direct comparison was made between the expression of pluripotency genes in the Wnt target high clusters with that of low clusters (Figure S3D), but the association with immature stem cell signatures does suggest that segregation based on the CSC signature reflects a more immature trait of the malignant tissue in the poor-prognosis cluster. In agreement, the association of the Wnt target-low cluster with this molecular immature fingerprint is also reflected by a significant enrichment for tumors presenting with a poorly differentiated histology (Figure 2B).

CSC-Associated Wnt Target Genes Are Subject to Methylation-Dependent Regulation

Although the above indicates that the tumors that cluster in the Wnt-low group have a more immature phenotype, this does not provide insight into the apparent discrepancy between the observed low Wnt target gene expression and the lack of reduction in nuclear β-catenin localization (Figure S2). To explain this conundrum we sought to determine whether epigenetic regulatory mechanisms might act to regulate Wnt target gene expression and thereby promote progression. Methylation is involved in many biological processes, including stem cell maintenance and cancer progression. In addition, CRC development is accompanied by global changes in methylation status of a plethora of genes (Morin et al., 1997; Rai et al., 2010). A subtype of CRC, the CpG Island Methylation Phenotype (CIMP), has even been

identified that is characterized by extensive methylation (Toyota et al., 1999). Several natural Wnt inhibitors, such as AXIN2 and SFRP1, have previously been shown to be methylated in CRC (Koinuma et al., 2006; Suzuki et al., 2004). We therefore analyzed the DNA methylation status of Wnt target genes first in a series of CRC cell lines. Methylation-specific PCR analysis revealed that several Wnt target genes including LGR5, APCDD1, DKK1, and ASCL2 are, to a different extent, methylated in a panel of CRC cell lines, suggesting epigenetic silencing of these genes (Figure 2C). In agreement, treatment of CRC cells with the demethylating agent 5-Azacytidine (5-Aza) resulted in marked upregulation of these genes specifically in those cell lines where methylation was evident (Figure 2D). For instance, APCDD1 expression was enhanced by 5-Aza in the lines where APCDD1 promoter methylation was clearly detectable. These data enforce the notion that Wnt targets are regulated, at least in part, by a mechanism that involves promoter methylation in CRC cell lines.

Functional Relevance of Wnt Target Gene Methylation

Next we determined the functional relevance of this methylation-dependent silencing of Wnt target genes. Treatment of CRC cell lines with a demethylating agent resulted in markedly decreased clonogenicity of these lines (Figure 3A). Importantly, this was a general observation; primary isolated colon-CSC cultures treated with 5-Aza also demonstrated significantly lower clonogenicity as determined by limiting dilution analysis, suggesting that the fraction of CSCs in these cultures decreased (Figure 3B). Also, in an in vivo model system in which primary CSC-induced xenograft tumors were growing subcutaneously, we observed that 5-Aza treatment resulted in markedly suppressed tumor growth (Figure 3C). Importantly, analysis of these 5-Aza-treated xenografts confirmed the efficacy of 5-Aza on re-expression of the Wnt target genes in vivo (Figure 3D), validating the methylation-dependent regulation of Wnt target genes in this in vivo model as well. To strengthen our hypothesis that re-expression of Wnt target genes has important functional consequences, we analyzed the effect of 5-Aza on Wnt activity levels. In this light it is important to realize that several of these repressed target genes normally serve as feedback inhibitors of the same pathway and as such could repress the activity of the Wnt pathway. In agreement, in several CRC cell lines, 5-Aza-mediated expression of genes previously suppressed by methylation resulted in decreased Wnt activity (Figure 3E).

Although these data are suggestive for a role of Wnt target gene methylation in CRC, the effects of 5-Aza are rather generic,

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(B) GSEA reveals a strong relationship between CSC signature and tumor relapse in the AMC-AJCCII-90 patient set. Also, previously described LGR5− and ISC-EphB2 signatures associate with recurrence in our set. ES, enrichment score; NES, normalized enrichment score; FDR, false discovery rate.

(C) Kaplan-Meier graph (relapse-free survival) based on overall adherence to the TOP-GFP^high/CSC profile as identified by gene ranking analysis (see Experimental Procedures for details).

(D and E) K-means clustering analysis of CRC samples from the AMC-AJCCII-90 patient set according to the colon-CSC signature (D) and the dnTCF4 signature (E) representing a defined set of Wnt target genes. Several canonical Wnt target genes are denoted for each signature. Kaplan-Meier analysis on relapse-free survival is drawn for each corresponding signature. Note that the poor-prognosis cluster (blue) is associated with low expression of indicated Wnt targets (see also Figures S1C and S1D for similar conclusions based on the LGR5− and ISC-EphB2 signatures). p values are calculated with the log-rank test.

(F) Representative β-catenin stainings are shown for two patients in both the Wnt-target-High (Wnt^High; red) and Wnt-target-Low (Wnt^Low; blue) clusters. Automated scoring of nuclear β-catenin fractions in patients from the AMC-AJCCII-90 set is shown. Scale bars represent 200 μm.

In (D–F), blue represents Wnt-target-low cluster (Wnt^Low); red, Wnt-target-high cluster (Wnt^High).
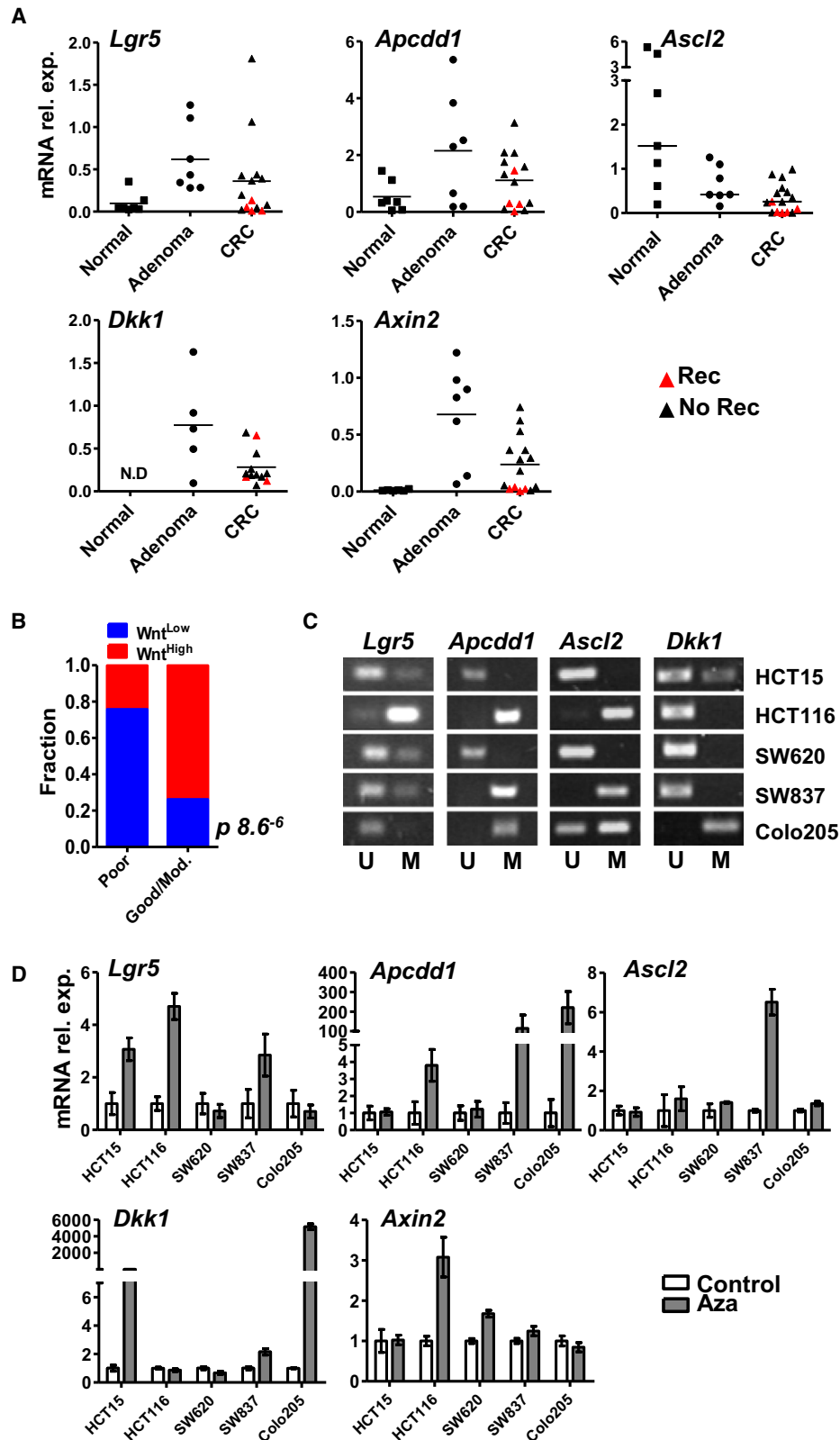
Figure 2. Wnt Target Genes Are Regulated by Methylation in CRC

(A) Relative expression of established Wnt target genes (*LGR5*, *APCDD1*, *ASCL2*, *DKK1*, and *AXIN2*) in normal, adenoma, and CRC tissue. Patients in the CRC group that developed a recurrence are highlighted in red. Horizontal line indicates mean value.

affecting all methylated CpG islands, and can therefore not be considered specific for Wnt target genes. We therefore questioned whether growth inhibition could be achieved directly by specific re-expression of methylated Wnt target genes. Indeed, we observed that re-expression of either *AXIN2* or *APCDD1*, both methylated in CRC, was sufficient to decrease Wnt signaling levels (Figure 3F). This is not only observed in CRC cell lines, but confirmed in a primary CSC culture as well (Figure 3F, right bars, Co100). This Wnt activity modulation directly suggests a potential functional explanation of why suppression of a large set of Wnt target genes occurs during the adenoma to carcinoma sequence and why Wnt target gene inactivation could be associated with poor prognosis.

Wnt Target Gene Methylation Predicts Prognosis in CRC

So far our data indicate that low expression of CSC-associated Wnt target genes is related to poor prognosis and that methylation-dependent downregulation of these genes has a functionally relevant impact on the clonogenicity of CRC cells. However, it is unclear whether in-patient material suppression of a stem-cell-associated Wnt expression program is also dependent on methylation. To this end we determined the relative methylation levels of the Wnt target genes in our AMC-AJCCII-90 patient set by either methylation-specific PCR or bisulphite sequencing (Figure 4). Intriguingly, also in the tumors from our patient cohort, low Wnt target gene expression was associated with increased methylation of the promoter regions of these genes (Figure 4A). That is, in the tumors that cluster in the Wnt-low group (Figure 4A, right black bars), the fraction of DNA methylation in the *APCDD1*, *ASCL2*, *AXIN2*, *DKK1*, and *LGR5* promoter regions is much higher as compared with that of the tumors that cluster in the Wnt-high group. Moreover, tumors in the Wnt-high group that did show methylation were, in several cases, derived from patients that eventually developed recurrences or metastases, as indicated by the asterisks in Figure 4A, suggesting that methylation of Wnt target genes is an even better marker for recurrences. In agreement, we found that methylation levels of a small subset of these CSC-associated Wnt target genes resulted in a highly predictive association with disease recurrence and metastasis using unsupervised cluster analysis based on the relative methylation levels (Figures 4B and 4C). Indeed, all recurrences cluster within the Wnt target gene methylation high group. These findings lend further support to the idea that methylation-dependent tuning of the Wnt expression program is related to disease progression and increased risk for recurrent disease.

DISCUSSION

Our findings have three major implications for the use and interpretation of (cancer-) stem-cell-associated profiles in risk stratification of CRC.

First, we confirm previous observations that ISC signatures can predict recurrence of CRC (Merlos-Suárez et al., 2011) and extend these findings with a novel colon-CSC-derived signature that has similar predictive properties, as might be expected based on the partial overlap between the various stem cell signatures. In particular both ISC and CSC signatures were characterized by a clear enrichment in Wnt target genes, consistent with the major role of the Wnt cascade in both ISC and colon-CSC biology.

Second, to our surprise, our data unequivocally show that expression of many well-defined canonical Wnt target genes, including prominent ISC markers, was inversely correlated with prognosis. This unexpected finding, which was verified in multiple patient sets, significantly changes the conclusions of earlier studies of stem-cell-derived predictive signatures (Merlos-Suárez et al., 2011). In previous reports the association of a (cancer) stem cell signature with poor prognosis is often attributed to a relative high number of CSCs present in the malignant tissue (Merlos-Suárez et al., 2011; Pardal et al., 2003; Charafe-Jauffret et al., 2009), which was thought to enhance the chance of CSCs shedding from the primary tumor. Indeed, adherence to an ISC profile of individual CRCs, but also similarity of breast cancers to a breast-CSC signature, was translated into an increased risk of metastasis and tumor recurrence (Liu et al., 2007; Merlos-Suárez et al., 2011). It is important to realize though that CSC numbers in primary tumors are on average suggested to constitute a minority of the tumor cells. It therefore appears rather unlikely that gene profiling of a complete tumor specimen would yield detailed information on a small minority of the cells. This would only be feasible when stem cell genes in the signatures are unique to the CSC population and additionally highly expressed. Instead, it is apparent from our data that these signatures identify poor-prognosis patients *despite* the presence of key (cancer) stem cell markers and canonical Wnt target genes, which we find to inversely correlate with tumor relapse and disease stage in CRC. Indeed, deletion of Wnt targets from the CSC signature improves the association with malignancies harboring a poor prognosis as predicted (Figure S1F). We believe our data indicate that CSC signatures identify tumors with a relatively immature signature as suggested by the association with a poorly differentiated histology. In agreement, progression of disease seems to be accompanied by adapting a more primitive, immature expression program defined by SOX2, OCT4, and Nanog signatures, as opposed to the more intestinal-tissue-specific stem cell signature with genes such as *ASCL2* and *LGR5*. The fact that this association is strongest with the Wnt-target low cluster (Figure S3C), which has the poorest prognosis, substantiates this hypothesis. Whether this is directly related to methylation of ISC/CSC-associated Wnt target genes or occurs in parallel is unclear at this point, but suppression of the Wnt target expression profile during the adenoma-carcinoma sequence clearly links this Wnt regulation to disease progression

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(B) The Wnt-target-Low (Wnt^Low) cluster of patients is enriched in patients displaying a poorly differentiated morphology. p value was calculated by Chi-square test.

(C) Methylation-specific PCR for Wnt target genes *LGR5*, *APCDD1*, *ASCL2*, and *DKK1* in a panel of CRC lines. U, unmethylated; M, methylated.

(D) Relative expression of indicated Wnt target genes following 48 hr demethylating treatment with 5-Aza in CRC cell lines. Confidence intervals represent standard deviations.
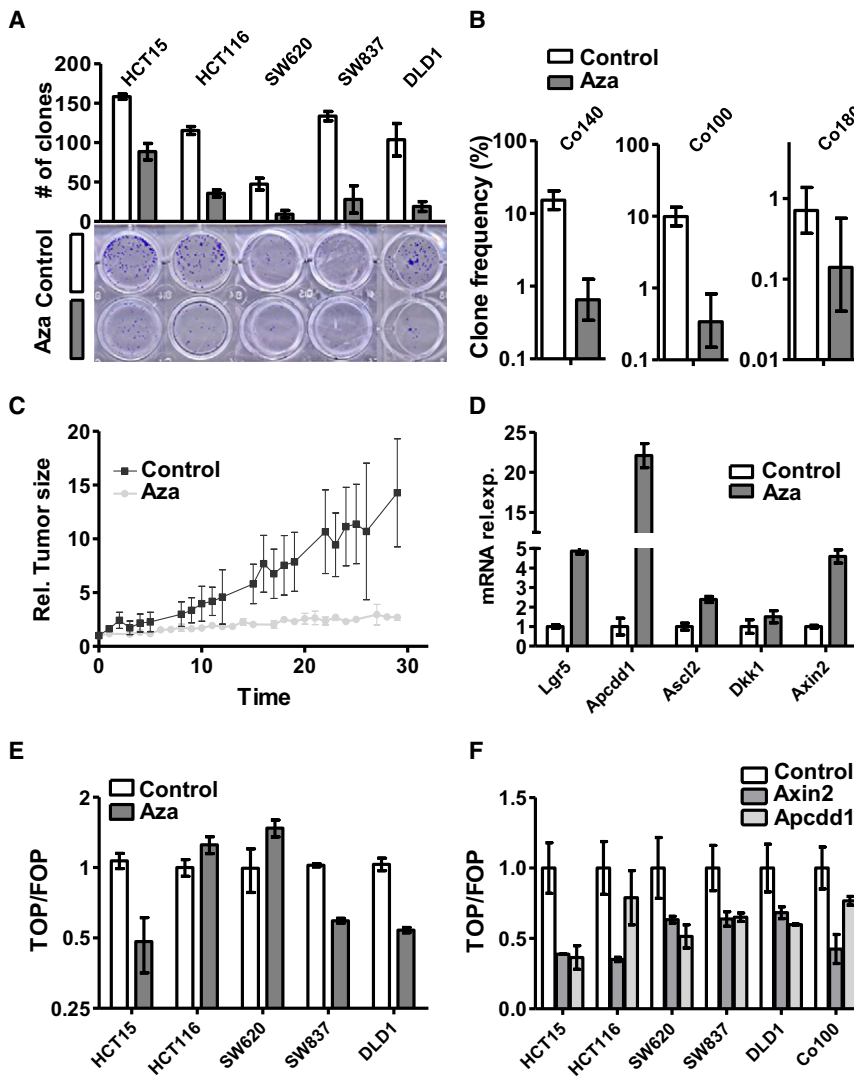
Figure 3. Functional Relevance of Wnt Target Gene Methylation

(A and B) Clonogenic analysis of indicated CRC cell lines (A) and primary human colon-CSC cultures (B) in the absence or presence of demethylating treatment with 5-Aza for 48 hrs.

(C) Subcutaneous xenografts of human primary CSC cultures treated with 5-Aza or PBS intraperitoneally.

(D) Expression of Wnt target genes in 5-Aza-treated primary human CSC culture-derived xenografts from the experiment depicted in (C).

(E) TOP/FOP analysis to determine Wnt signaling levels following 5-Aza treatment.

(F) Effect of transient overexpression of *APCDD1* or *AXIN2* on TOP/FOP activity in CRC cell lines and in primary human CSC cultures (Co100, right bars). Error bars represent standard deviation.

In this light it is important to realize that many Wnt target genes (e.g., *AXIN2* and *APCDD1*) function as negative feedback regulators of the pathway (Shimomura et al., 2010), and therefore suppression of these genes by methylation might in fact increase Wnt activity levels and contribute to disease progression and relapse via activation of yet unknown positive targets. This is corroborated by our observation that demethylating treatment with 5-Aza, or more specifically re-expression of *AXIN2* or *APCDD1*, decreases Wnt signaling activity in vitro (Figures 3F and 3G). In general, this implies that re-expression of suppressed Wnt targets by demethylating agents might provide an exciting therapeutic strategy that deserves further exploration.

in a manner that is unexpected (Figure 2A and Figure S3A). Our results therefore support a different interpretation of prognosis prediction by (cancer) stem cell profiles; an overall resemblance to a CSC signature does not simply reflect CSC numbers, but rather reflects a clonal, immature trait of the tissue as a whole and points to more advanced disease. This is also in line with the lack of correlation between established colon-CSC markers, such as CD133 or nuclear localized β-catenin, and the expression of CSC-associated genes in primary human CRC (Figures S2C and S2D). Interestingly, a similar conclusion can be drawn when analyzing breast cancer specimens using a breast-CSC signature, which also appears to identify basal, i.e., more immature, breast cancers (Bertucci et al., 2007).

Third, our results indicate that suppression of the Wnt expression program is related to metastatic spread, which supports recent findings that Wnt blockage by dnTCF4 expression is promoting metastasis formation in CRC (Varnat et al., 2010). However, these observations are not completely in line with the current data, because we do not detect decreased Wnt pathway activity as evidenced by nuclear β-catenin localization.

To conclude, by using CSC-derived gene signatures, we unraveled a fundamental change during the progression of CRC that is characterized by methylation of a set of key Wnt targets. This methylation allows a relatively easy way of identifying patients at risk of recurrence, which would likely benefit most from adjuvant therapy. Moreover, our data also point to novel means that could take advantage of the modulated Wnt target gene expression and/or more immature phenotype to design more effective therapies.

EXPERIMENTAL PROCEDURES

Cell Culture and Generation of the Colon-CSC Signature

The generation and culture of colon-CSCs has been previously described (Vermeulen et al., 2010). CRC cell lines were purchased at the ATCC. All lines were maintained in DMEM (supplemented with 10% FCS/1% glutamine) except for Colo205, which was cultured in RPMI-1640 (10% FCS/1% glutamine). CSC signature was derived from the 10% highest and lowest TOP-GFP fraction of cells in two independent colon-CSC cultures (Vermeulen et al., 2010) using Human Genome U133 Plus 2.0 microarrays (see Supplemental Information). The ISC-EphB2, LGR5, and dnTCF4 signatures were described elsewhere (Merlos-Suárez et al., 2011; van de Wetering et al., 2002).
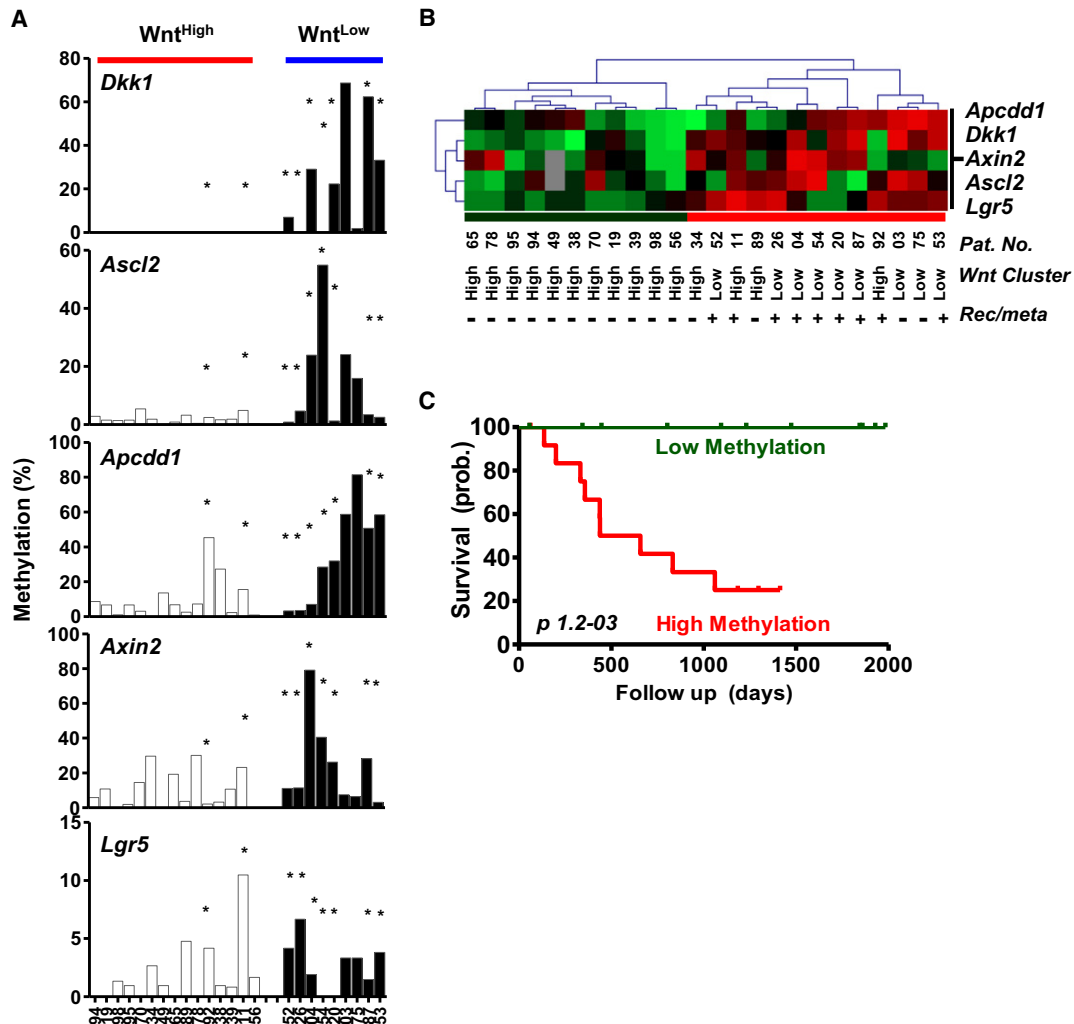
Figure 4. Methylation of Wnt Target Genes Identifies Poor-Prognosis Patients

(A) Percentage of CpG island methylation in the promoter region of the indicated Wnt target genes for a subset of patients from the AMC-AJCCII-90 set.

(B) Unsupervised cluster analysis using the methylation levels (ranking) of Wnt target genes reveals two clusters. Patient number, High or Low Wnt-target cluster, and recurrence are indicated. Colors depict rank order of methylation level within the patient set for each gene: green, lowly methylated; red, highly methylated; grey, data not available.

(C) Kaplan-Meier curve depicting the two different patient groups as identified in (B). p value is calculated with the log-rank test.

Patient Cohorts

Two different CRC patient series were used for this study. The first one consisted of 90 AJCC stage II CRC patients that underwent intentionally curative surgery in the Academic Medical Center in Amsterdam, The Netherlands in the years 1997–2006 (AMC-AJCCII-90). Extensive medical records are kept of these patients and long-term clinical follow-up is available for the large majority. Both paraffin-embedded and fresh frozen tissue is available from all these patients for analysis, which was used to derive gene expression profiles. The second patient set is composed of two merged cohorts that form a metacohort of 345 CRC patients and has been described elsewhere (Merlos-Suárez et al., 2011). In addition, a separate panel of normal and adenoma fresh-frozen tissues was obtained in the AMC and these observations were validated with a publically available data set (Galamb et al., 2008).

Clustering and Survival Analysis

Unsupervised K-means cluster analysis and Kaplan-Meier survival curves were generated in the different expression data sets with the different gene signatures using the software package R2 (http://r2.amc.nl), a web-based microarray analysis application developed by J.K. (data not shown). For single-

gene survival prediction, the median expression value of each gene was used as a cutoff to generate two groups of 45 patients having either a low or high relative expression. p value was calculated using the log-rank test. For cluster analysis (Euclidian Distance, average linkage in the MultiExperiment Viewer package v4.5, www.TM4.org) of methylation levels of Wnt target gene sets, we used the rank value for each individual patient for each gene.

Prediction Power of Signature and Multivariate Analysis

For the predictive power of the CSC signature, we selected the 134 upregulated genes. Every individual gene was ranked according to their expression in each patient. The rank score for all the genes per patient was summed to define the rank for each patient. A high expression of a gene is translated into a low rank score. Patients that have an overall high expression of the genes in the signature have an overall low rank score and therefore are highly associated with the CSC profile. A Kaplan-Meier survival curve was generated to plot the relapse-free survival of patients having a high (n = 30) versus low (n = 60) correlation with the CSC profile. For multivariate analysis, the Cox proportional hazard model was used. All p values are two-sided. Statistical analysis was performed in SPSS.

In Vivo 5-Aza Treatment

Murine experiments were performed in accordance with the ethical committee of the AMC. For transplantation of CSCs, 5,000 cells suspended in 100 μl of PBS/BSA admixed with Matrigel at a 1:1 ratio were injected subcutaneously into nude mice (Hsd:Athymic Nude/Nude) (Harlan). When tumors were palpable, mice were injected i.p. with 5-Aza (5 mg/kg) or PBS vehicle. Injections were performed every 2 days.

β-catenin Staining and Spectral Imaging Quantification

Paraffin-embedded primary human specimens were stained with anti-β-catenin (Transduction Labs) and then incubated with anti-mouse-HRP (Powervison) (1:1). Multispectral data sets from slides stained for β-catenin were acquired using a Nuance camera system (Caliper Life Science, Hopkinton, MA) from 420–720 nm at intervals of 20 nm. To analyze the frequency of hematoxylin-β-catenin colocalization, spectral data sets were analyzed with the tissue segmentation and machine-learning Inform 1.2 software (Caliper Life Science), similar to methods described elsewhere (Al-Kofahi et al., 2011). The software was trained to recognize tumor and stroma areas, and next, all spectral data sets were segmented into these two tissue categories. All data sets were analyzed using the nuclear algorithm, scoring the percentage of β-catenin positive nuclei out of all nuclei in the tumor tissue category.

RNA, PCR, and Methylation Analysis

For all methods on RNA isolation, PCR, and mutation analysis, and for all primer sequences, please see the Supplemental Information.

ACCESSION NUMBERS

The GenBank accession number for the CSC signature is GSE33112: gene expression in CSC cultures identified by Wnt signaling levels. The GenBank accession number for the patient dataset is GSE33113: AMC colon cancer AJCCII.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.stem.2011.10.008.

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