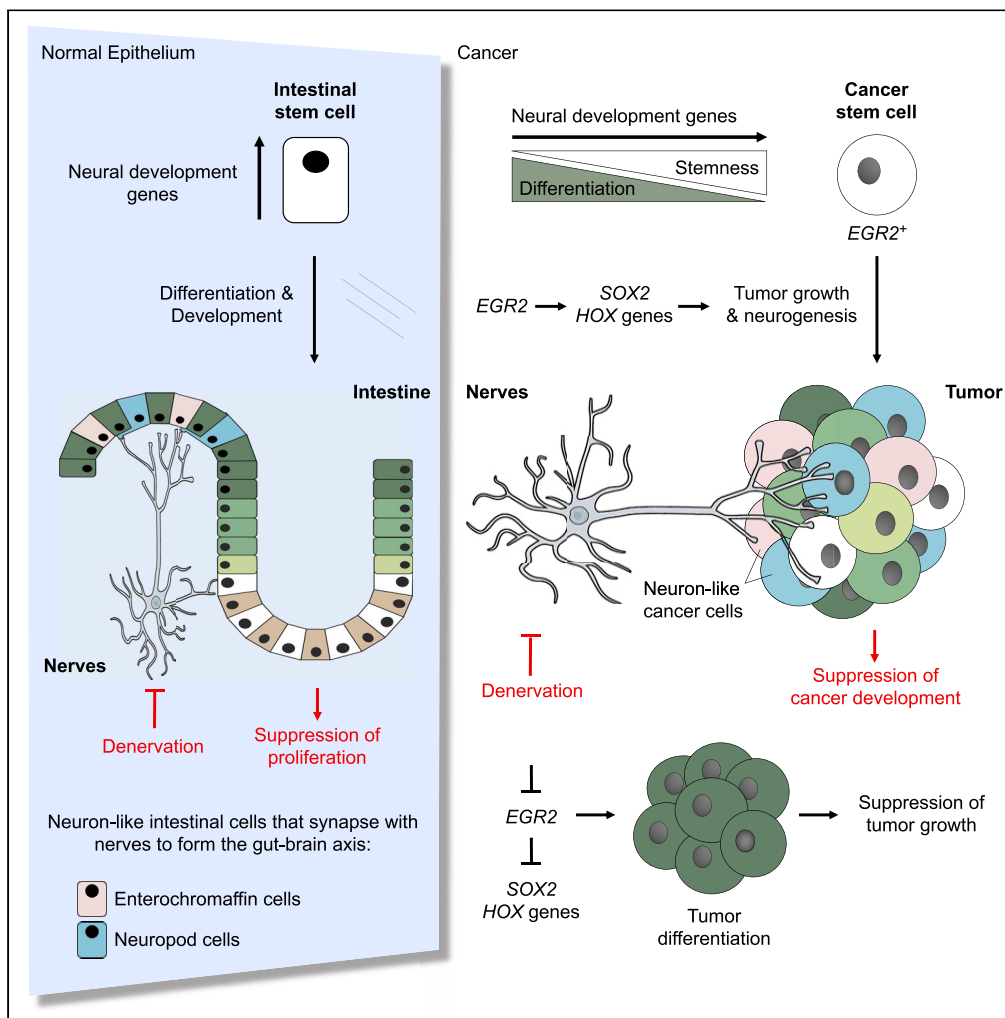


Article

Identification of a neural development gene expression signature in colon cancer stem cells reveals a role for *EGR2* in tumorigenesis



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Highlights

Colon cancer stem cells (CSCs) are enriched for nervous system development genes

Colon cancer cells express nerve cell markers

EGR2 is required for CSC survival and tumor growth and regulates *SOX2* and *HOX* genes

Targeting *EGR2* may block cancer neurogenesis and stop disease progression

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Article

Identification of a neural development gene expression signature in colon cancer stem cells reveals a role for *EGR2* in tumorigenesis

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SUMMARY

Recent evidence demonstrates that colon cancer stem cells (CSCs) can generate neurons that synapse with tumor innervating fibers required for tumorigenesis and disease progression. Greater understanding of the mechanisms that regulate CSC driven tumor neurogenesis may therefore lead to more effective treatments. RNA-sequencing analyses of ALDH^{Positive} CSCs from colon cancer patient-derived organoids (PDOs) and xenografts (PDXs) showed CSCs to be enriched for neural development genes. Functional analyses of genes differentially expressed in CSCs from PDO and PDX models demonstrated the neural crest stem cell (NCSC) regulator *EGR2* to be required for tumor growth and to control expression of homebox superfamily embryonic master transcriptional regulator *HOX* genes and the neural stem cell and master cell fate regulator *SOX2*. These data support CSCs as the source of tumor neurogenesis and suggest that targeting *EGR2* may provide a therapeutic differentiation strategy to eliminate CSCs and block nervous system driven disease progression.

INTRODUCTION

Colorectal cancer (CRC), the third most common cancer and fourth most common cause of cancer deaths worldwide (Siegel et al., 2014), is a heterogeneous tumor initiated and driven by CSCs, which are also the source of relapse following treatment (O'Brien et al., 2007; Regan et al., 2017, 2021; Reya et al., 2001; Ricci-Vitiani et al., 2007; Vermeulen et al., 2008). Elucidating the mechanisms that regulate CSCs may therefore lead to novel treatments and improved patient outcomes.

Neural-cancer cell cross-talk is required for tumor initiation and progression (Cheng et al., 2021; Magnon et al., 2013; Pan et al., 2021) and cancer neurogenesis has recently been proposed as a new hallmark of cancer (Senga and Grose, 2022). The nervous system branches throughout the body to innervate tissues and regulate organogenesis, homeostasis, cellular plasticity, response to injury, and normal tissue function. The nervous system also controls tumor growth and metastasis (Amit et al., 2020; Hayakawa et al., 2017; Jobling et al., 2015; Magnon et al., 2013; Monje et al., 2020; Rademakers et al., 2017; Saloman et al., 2018; Venkataramani et al., 2019), and tumor innervation is an indicator of poor clinical outcome in CRC and other tumor entities (Albo et al., 2011; Chatterjee et al., 2012; Horn et al., 1991; Huang et al., 2014; Liebl et al., 2013; Magnon et al., 2013; Shao et al., 2016; Tan et al., 2021). Recently, Lu et al. (2017) demonstrated that CSCs are the source of neurogenesis in CRC (Lu et al., 2017). Targeting the ability of CSCs to generate neurons or neuron-like cells that synapse with nerves could therefore be of therapeutic benefit to patients (Faulkner et al., 2019; Jiang et al., 2020; Saloman et al., 2016; Schonkeren et al., 2021). However, the molecular mechanisms that regulate CSC neurogenesis remain largely unknown.

CSCs are undifferentiated cancer cells that share many of the attributes of stem cells, such as multipotency, self-renewal, and the ability to produce daughter cells that differentiate (Clarke and Fuller, 2006; Reya et al., 2001; Wicha et al., 2006). Stem cells are controlled by core gene networks that include the embryonic master transcriptional regulator *HOX* genes (Gouti and Gavalas, 2007; Nolte et al., 2014) and neural stem cell

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Continued



and master cell fate regulator SOX2 (Ellis et al., 2004; Feng et al., 2013; Julian et al., 2017; Pevny and Nicolis, 2010; Sarkar and Hochedlinger, 2013; Suh et al., 2007; Thiel, 2013), whose misregulation can result in aberrant stem cell function, developmental defects, and cancer (Chew and Gallo, 2009; Grimm et al., 2020; Shah and Sukumar, 2010). These genes are crucial for embryonic development and their expression is maintained in adult tissue stem cells where they regulate self-renewal and differentiation (Arnold et al., 2011; Gouti and Gavalas, 2007; Kamachi and Kondoh, 2013; Seifert et al., 2015). *HOX* genes and SOX2 are aberrantly expressed in several cancer entities, including CRC, and are involved in the transformation of tissue stem cells into CSCs (Basu-Roy et al., 2012; Bhatlekar et al., 2014, 2018a, 2018b; Gangemi et al., 2009; Lundberg et al., 2016; Mansouri et al., 2016; Novak et al., 2019; Schaefer and Lengerke, 2020; Shah and Sukumar, 2010; Takeda et al., 2018). Modulation of *HOX* genes and SOX2 could therefore provide novel therapeutic strategies to block tumorigenesis and overcome therapy resistance in CRC and other CSC driven cancers.

During embryonic development of the neural crest, which gives rise to the peripheral nervous system (PNS) and several nonneuronal cell types (Bronner and LeDouarin, 2012), *HOX* and *SOX* genes are regulated by retinoic acid (Duester, 2008; Nolte et al., 2014; Tremblay et al., 2012), a product of the normal tissue stem cell and CSC marker aldehyde dehydrogenase (*ALDH1A1*) (Douville et al., 2008; Huang et al., 2009; Vassalli, 2019), and by the neural crest stem cell (NCSC) zinc finger transcription factor and wound response gene *EGR2* (*KROX20*) (Chavrier et al., 1990; Desmazières et al., 2009; Ghislain et al., 2003; Grose et al., 2002; Jang et al., 2010; Kim et al., 1998; Manzanares et al., 2002; Nonchev et al., 1996a, 1996b; Sham et al., 1993; Topilko et al., 1994; Vesque et al., 1996).

Here, we carried out whole transcriptome analyses of functionally tested ALDH^{Positive} CSCs from a panel of colon PDOs and PDX models and show that colon CSCs and Lgr5^{Positive} intestinal stem cells (ISCs) are highly enriched for nervous system development and neural crest genes. Furthermore, we demonstrate that the NCSC gene *EGR2* is a marker of poor prognosis in CRC and modulates expression of *HOX* genes and SOX2 in CSCs to regulate tumorigenicity and differentiation.

RESULTS

Colon cancer PDOs are heterogeneous and enriched for ALDH^{Positive} self-renewing CSCs

Colon cancer PDO models were established from freshly isolated primary tumors and metastases from colon cancer patients (Table S1) by embedding in growth-factor reduced Matrigel and cultivating in serum free media as described previously (Regan et al., 2021; Sato et al., 2011). Immunostaining of PDOs for the structural proteins EZRIN and EPCAM demonstrated that PDOs retain the apical-basal polarity and structural adhesion of the normal intestine (Figure 1A). Immunostaining of PDOs and equivalent PDX models for stem cell regulator Wnt signaling protein β -catenin demonstrated differences in nuclear localization of β -catenin and confirmed previous data demonstrating heterogeneous Wnt signaling activity within the tumors (Regan et al., 2017) (Figure 1B). Increased aldehyde dehydrogenase (ALDH) activity, as measured using the Aldefluor™ assay, is a marker of CSCs in colon cancer and many other cancer types (Huang et al., 2009; Ma and Allan, 2011). We previously carried out limiting dilution serial xenotransplantation of ALDH^{Negative} and ALDH^{Positive} cells and demonstrated that colon CSCs are ALDH^{Positive} and enriched for Wnt signaling activity (Regan et al., 2017). However, ALDH^{Negative} cells also gave rise to tumors when transplanted at higher cell numbers. To determine if ALDH^{Negative} and ALDH^{Positive} cells maintained their self-renewal and tumorigenic capacity, we performed additional rounds of limiting dilution serial xenotransplantation of ALDH^{Negative} and ALDH^{Positive} cells (Figure 1E). These data confirmed that PDOs are enriched for ALDH^{Positive} cells compared to equivalent PDX models, supporting their efficacy as models for the study of CSC biology (Figures 1C and 1D) and that ALDH^{Positive} CSCs self-renew to maintain their tumorigenic capacity over extended rounds of xenotransplantation, but that ALDH^{Negative} cells do not (Figure 1E).

Colon CSCs are enriched for embryonic and nervous system development gene expression signatures

To identify modulators of colon CSCs, ALDH^{Negative} cells and ALDH^{Positive} CSCs were isolated from PDO and PDX models and subjected to whole transcriptome analysis by RNA-sequencing. Gene set enrichment analysis (GSEA) of ALDH^{Negative} and ALDH^{Positive} cells from PDO and PDX models demonstrated that ALDH^{Positive} CSCs are enriched for stem cell associated gene sets, including TNF α via NF- κ B signaling, epithelial mesenchymal transition (EMT), organ and embryonic development, and Wnt signaling (Figure 2A

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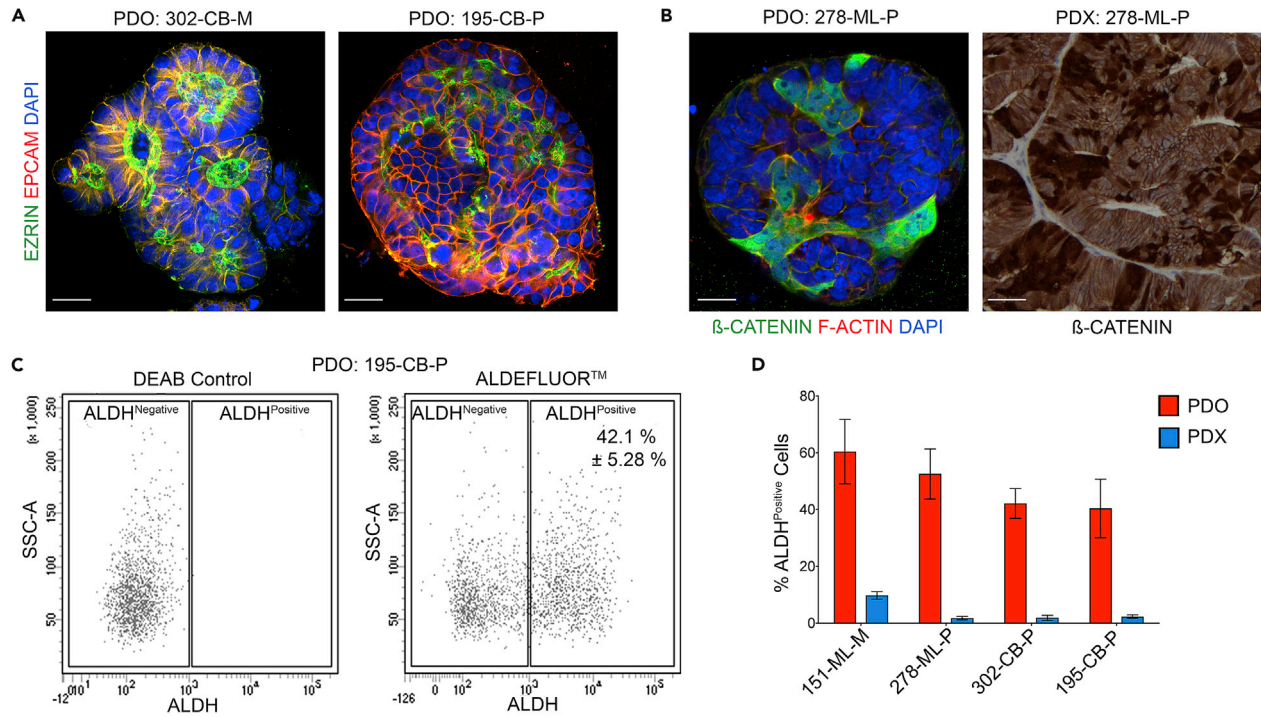
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E

Serial PDX Transplant 1

| Cell dilution | 151-ML-M | | 278-ML-P | | 302-CB-M | | 195-CB-P | |
|--------------------------------|--------------------------|--------------------------|-----------------------------|--------------------------|----------------------------|--------------------------|-----------------------------|--------------------------|
| | ALDH ^{Negative} | ALDH ^{Positive} | ALDH ^{Negative} | ALDH ^{Positive} | ALDH ^{Negative} | ALDH ^{Positive} | ALDH ^{Negative} | ALDH ^{Positive} |
| 100 cells | 0/0 | 2/6 | 0/0 | 1/6 | 0/0 | 5/6 | 0/0 | 5/6 |
| 1000 cells | 0/6 | 1/6 | 1/6 | 0/6 | 1/6 | 6/6 | 0/6 | 6/6 |
| 10000 cells | 0/6 | 0/0 | 0/6 | 0/0 | 3/6 | 0/0 | 1/6 | 0/0 |
| Frequency of Cancer Stem Cells | 0 | 1 in 1,986 | 1 in 65,499 | 1 in 6,550 | 1 in 12,113 | 1 in 55.8 | 1 in 60,863 | 1 in 55.8 |
| (95% confidence limits) | NA | (1 in 591 - 1 in 6,667) | (1 in 8,664 - 1 in 495,152) | (1 in 866 - 1 in 49,515) | (1 in 4,350 - 1 in 33,733) | (1 in 20.6 - 1 in 151) | (1 in 8,614 - 1 in 43,0042) | (1 in 20.6 - 1 in 151) |

Serial PDX Transplant 2

| Cell dilution | 278-ML-P | | 302-CB-M | | 195-CB-P | |
|--------------------------------|-----------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| | ALDH ^{Negative} | ALDH ^{Positive} | ALDH ^{Negative} | ALDH ^{Positive} | ALDH ^{Negative} | ALDH ^{Positive} |
| 100 cells | 0/0 | 3/6 | 0/0 | 6/6 | 0/0 | 5/6 |
| 1000 cells | 1/6 | 3/6 | 0/6 | 6/6 | 0/6 | 5/6 |
| 10000 cells | 0/6 | 0/0 | 0/6 | 0/0 | 0/6 | 0/0 |
| Frequency of Cancer Stem Cells | 1 in 65,499 | 1 in 772 | 0 | 1 in 1 | 0 | 1 in 247 |
| (95% confidence limits) | (1 in 8,664 - 1 in 495,152) | (1 in 307 - 1 in 1,940) | NA | (1 in 1 - 1 in 107) | NA | (1 in 90 - 1 in 686) |

Figure 1. Colon cancer PDOs are heterogeneous and enriched for self-renewing ALDH^{Positive} CSCs

(A) Immunofluorescence staining of colon cancer PDOs for EZRIN (green) and EPCAM (red). Nuclei are stained blue with DAPI (Bars = 20 μ m).
 (B) Immunofluorescence staining of a PDO for β -CATENIN (green) and F-ACTIN (red) (left hand side) and immunostaining of a PDX for β -CATENIN (right hand side) (Bars = 20 μ m).
 (C) Representative Aldefluor Assay FACS plots of cells derived from PDO model 195-CB-P (data from 10 independent experiments). DEAB (diethylaminobenzaldehyde) is a specific inhibitor of ALDH and is used to control background fluorescence.
 (D) Frequency (\pm SD) of ALDH^{Positive} cells in PDOs and corresponding PDX models (data from 10 independent experiments).
 (E) Tables show results of two rounds of limiting dilution serial xenotransplantation of ALDH^{Positive} and ALDH^{Negative} cells from previously established PDO derived xenograft models. The number of successfully established tumors as a fraction of the number of animals transplanted is given. p values for pairwise tests of differences in CSC frequencies between ALDH^{Positive} versus ALDH^{Negative} cells in 151-ML-M, 278-ML-P, 302-CB-M, and 195-CB-P in serial transplant round one tumors are 1.12×10^{-4} , 1.37×10^{-1} , 8.39×10^{-14} , and 2.92×10^{-17} , respectively, and in 278-ML-P, 302-CB-M, and 195-CB-P serial transplant round two tumors are 3.82×10^{-7} , 3.67×10^{-22} and 3.78×10^{-15} , respectively. (See also Table S1).

and Table S2). Interestingly, at the same time as possessing a molecular profile associated with epithelial development and wound response, the CSCs were highly enriched for neural development genes, including nervous system development and neurotrophin signaling (Figures 2A and Table S2).

In addition, gene ontology analysis revealed that CSCs from PDO and PDX models were significantly enriched for nervous system development terms, including neural crest cell differentiation (p value 3.81E-20), modification of postsynaptic structure (p value 4.74E-02), peripheral nervous system development (p value 2.7E-02), axonogenesis (p value 2.79E-03), and neurogenesis (p value 7.65E-05) (Figure 2B). In agreement with this, gene ontology analysis of Lgr5^{Positive} crypt stem cell transcriptomes from earlier studies by, Yan et al. (2017), Muñoz et al. (2012) and Merlos-Suárez et al. (2011) demonstrated that normal ISCs are also enriched for nervous system development genes (Figure S1).

Significantly, PDO and PDX cells were found to express the exclusive nerve cell markers *ENO2* (neuron specific enolase [NSE]) (Bai et al., 2019; Chung, 2015) and *TUBB3* (Jiang and Oblinger, 1992; Latremoliere et al., 2018), the molecular marker of neural activity *c-FOS* (Chung, 2015) and the exclusive neuroendocrine cell marker *CLU* (Andersen et al., 2007), which we have also previously demonstrated to be enriched in quiescent CSCs (Regan et al., 2021) (Figure 2C). Importantly, the PDOs showed ubiquitous staining for the epithelial cell marker EPCAM (Figure 1A), demonstrating that they do not contain a separate non-epithelial neural cell lineage that could be the origin of the nervous system gene expression. These data demonstrate that CSCs are enriched for nervous system development genes and support recent studies showing that CSCs are the source of cancer neurogenesis (Lu et al., 2017; Magnon, 2015; Monje et al., 2020; Pan et al., 2021).

Differential gene expression analysis identified 218 genes upregulated in ALDH^{Positive} cells from PDOs and 250 genes upregulated in ALDH^{Positive} cells from PDX models, compared to ALDH^{Negative} cell fractions. Of these, 30 genes were found to be differentially expressed in both ALDH^{Positive} PDO and PDX cells, including *ALDH1A1* (Figures 2D and 2E). Interestingly, many of these differentially expressed and common PDO-PDX genes are expressed during embryogenesis and have a role in neural crest cell (NCC) and CNS development. Many of the same stem cell processes that contribute to normal development and response to injury are required for tumorigenesis, disease progression, and resistance to therapy. Furthermore, the expression of neural crest genes in CSCs demonstrates their cell state plasticity (Hanahan, 2022). We therefore selected ten genes, *ALDH1A1* (Douville et al., 2008; Huang et al., 2009; Vassalli, 2019), *EGR2* (Desmazières et al., 2009; Dillon et al., 2007; Ghislain et al., 2003; O'Donovan et al., 1999; To et al., 2013; Topilko et al., 1994; Yao et al., 2016; Young et al., 2016), *EGR3* (Baron et al., 2015; Inoue et al., 2004; O'Donovan et al., 1999; Quach et al., 2013; Shin et al., 2014; Tourtellotte and Milbrandt, 1998; Xi and Kersh, 2004; Yao et al., 2016), *HDGFRP3* (Cai et al., 2015; El-Tahir et al., 2009; Fok et al., 2012; Ortega-Paino et al., 2008; Xiao et al., 2013; Yun et al., 2013), *OLFM2* (Anholt, 2014; Lee et al., 2008; Shi et al., 2014; Shi and Chen, 2015), *OLFML3* (Anholt, 2014; Mijlkovic-Licina et al., 2012; Torres et al., 2013), *PCP4* (Hamada et al., 2014; Harashima et al., 2011; Yoshimura et al., 2016), *PEG10* (Akamatsu et al., 2015; Kainz et al., 2007; Li et al., 2006, 2016; Okabe et al., 2003; Ono et al., 2006; Peng et al., 2017; Tsou et al., 2003; Xie et al., 2018), *PRKACB* (Furuta et al., 2011; Livshits et al., 2015), and *THBS1* (Adams and Tucker, 2000; Daubon et al., 2019; Huang et al., 2017; Iruela-Arispe et al., 2018; Kazerounian et al., 2008; Ostankova et al., 2011), for functional analysis based on their expression and roles in development and cancer (Figures 2E, S2 and S3).

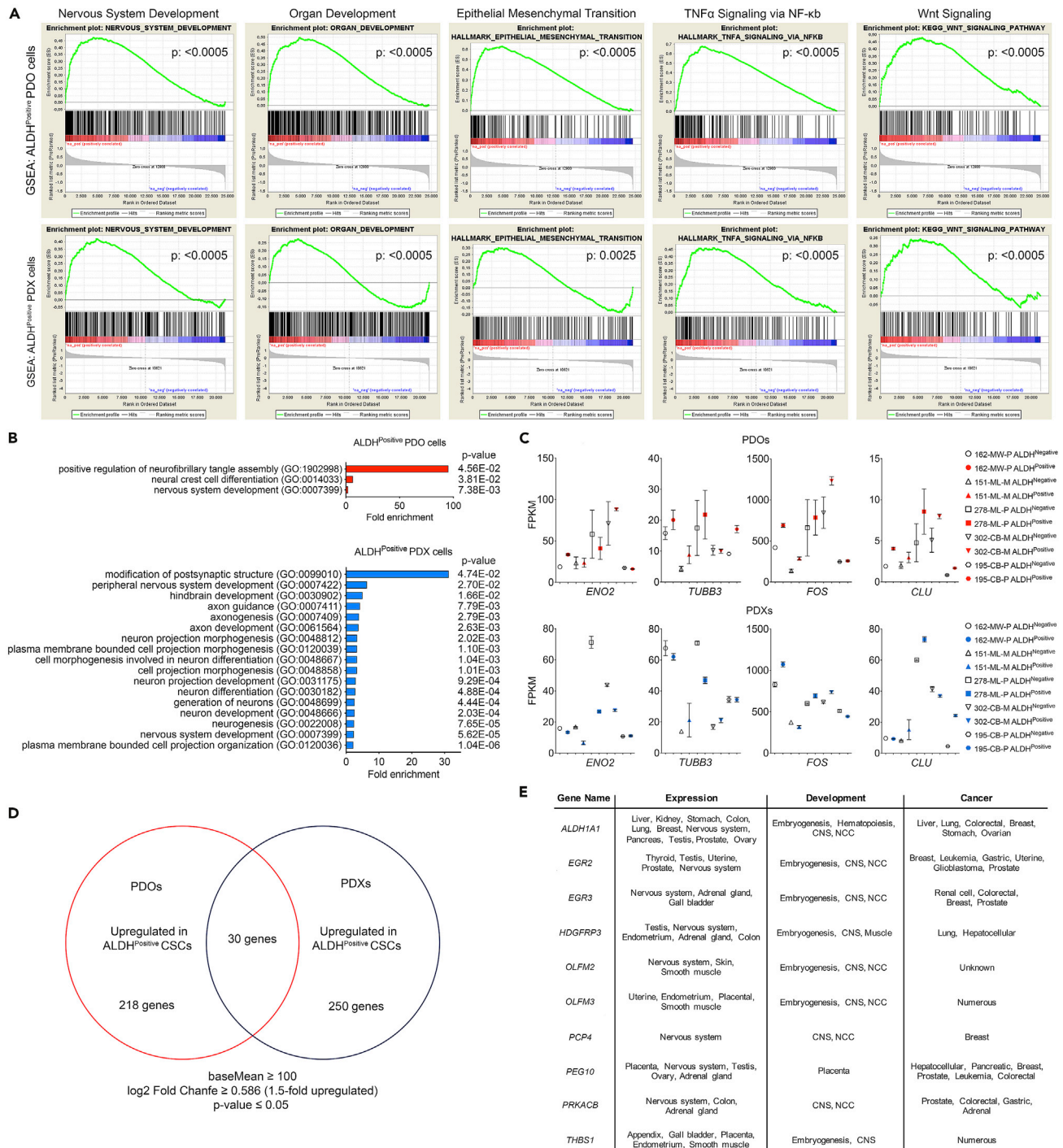


Figure 2. ALDH^{Positive} CSCs are enriched for nervous system development gene sets and neural crest stem cell genes

(A) Gene set enrichment analysis for nervous system development (nominal p values <0.0005), organ development (nominal p values <0.0005), epithelial to mesenchymal transition (nominal p values <0.0005 and 0.0025), TNF α signaling via NF κ B (nominal p values <0.0005), and Wnt signaling (nominal p values <0.0005) in ALDH^{Positive} cells (compared to ALDH^{Negative} cells) from PDO models (top panels) and PDX models (bottom panels) (See also Table S2).

(B) Gene ontology analysis of ALDH^{Positive} cells from PDOs (red bars) and PDX models (blue bars) reveals CSCs are enriched for neural crest and nervous system development terms (See also Figure S1).

(C) RNA sequencing generated FPKM values for nerve cell markers *ENO2*, *TUBB3*, *FOS* and the neuroendocrine cell marker *CLU* in ALDH^{Positive} CSCs and ALDH^{Negative} cells from PDO models (top panels) and PDX models (bottom panels).

Figure 2. Continued

(D) Venn diagram shows the number of RNA-sequencing generated transcripts upregulated in PDO ALDH^{Positive} CSCs (218 genes) and PDX ALDH^{Positive} CSCs (250 genes) and upregulated in both PDO ALDH^{Positive} CSCs and PDX ALDH^{Positive} CSCs (30 genes) (n = 4 separate cell preparations, basemean greater than or equal to 100, log2 fold change = 1.5-fold upregulated, p value <0.05).

(E) Table shows 10 genes upregulated in both PDO ALDH^{Positive} CSCs and PDX ALDH^{Positive} CSCs selected for functional analysis by RNAi (relevant literature is cited in brackets below gene names). (See also [Figures S1](#), [S2](#), and [S3](#)).

EGR2 is required for CSC survival in nonadherent cell culture

The ability of CSCs to survive and form spheroids in nonadherent cell culture is the gold standard assay for the assessment of normal stem cells and CSCs *in vitro* ([Ricci-Vitiani et al., 2007](#); [Weiswald et al., 2015](#)). To functionally test the effect of the candidate gene set on CSCs, cells were transfected with siRNAs against *ALDH1A1*, *EGR2*, *EGR3*, *HDGFRP3*, *OLFM2*, *OLFML3*, *PCP4*, *PEG10*, *PRKACB*, and *THBS1* ([Figures 3B](#), [S4](#) and [Table S3](#)), serially plated at limiting dilution into low-attachment plates and assessed for spheroid formation. siRNA *EGR2* caused a significant decrease in spheroid formation and proliferation in all models ([Figures 3A](#), [3C](#), [3D](#) and [3E](#)). Immunostaining of PDO, PDX and clinical samples demonstrated *EGR2* to be ubiquitously expressed, with increased cytoplasmic and nuclear expression in cancer compared to normal mucosa ([Figure S5](#)). Significantly, single cell RNA-sequencing analyses (scRNA-seq) demonstrated that *EGR2* is cell type enhanced in intestinal neuropod (enteroendocrine) cells and stem cells of the colon ([Figure 3F](#)). Neuropod cells form neuroepithelial circuits by directly synapsing with vagal neurons ([Bohórquez et al., 2015](#); [Kaelberer et al., 2018, 2020](#); [Liddle, 2019](#)) and possess injury-inducible stem cell activity ([Yan et al., 2017](#)). These data further support a role for *EGR2* in regulating stem cells, CSCs, and neural differentiation in the gut.

shRNA EGR2 cells are less tumorigenic, more differentiated and have decreased expression of HOX genes and SOX2

shRNA *EGR2* similarly affected spheroid formation and proliferation in non-adherent culture ([Figure 3G](#)). Limiting dilution xenotransplantation of control virus-transduced and shRNA *EGR2*-transduced 195-CB-P cells was carried out to determine if *EGR2* regulates tumorigenesis *in vivo*. Control virus transduced cells generated xenografts at each cell dilution tested, but shRNA *EGR2*-transduced cells were significantly impaired in their ability to generate tumors when transplanted at low cell number ([Figure 3H](#)). In addition, shRNA *EGR2* tumors grew more slowly than control transduced cells ([Figure 3I](#)). These data demonstrate that loss of *EGR2* in CSCs significantly decreased their tumorigenic capacity. Quantitative RT-PCR analysis of three shRNA *EGR2* tumors confirmed that the shRNA *EGR2* knockdown was present ([Figure 4A](#)). Significantly, expression of proliferation (*MKI67*, *MYC*), stem cell genes (*ALCAM*, *ALDH1A1*, *BMI1*, *EPHA4*, *EPHB2*, *LRIG1*, *OLFM4*, and *PROM1*) and Wnt signaling genes (*AXIN2*, *CTNNB1*, *LGR5*, and *RUNX2*) were decreased, whereas the expression of differentiation markers, including the tumor suppressor and Wnt signaling target *ATOH1*, were strongly increased. The decreased expression of *ALDH1A1* in shRNA *EGR2* tumors suggests that *EGR2* may also regulate *ALDH* activity, although it is also likely that the effect on *ALDH1A1* expression is because of diminished CSC frequency and increased differentiation of the shRNA *EGR2* tumors.

During embryogenesis *EGR2* has a conserved role in regulating embryonic master transcriptional regulator *HOX* genes and the stem cell regulator *SOX2* ([Chavrier et al., 1990](#); [Desmazières et al., 2009](#); [Ghislain et al., 2003](#); [Jang et al., 2010](#); [Kim et al., 1998](#); [Manzanares et al., 2002](#); [Nonchev et al., 1996a, 1996b](#); [Sham et al., 1993](#); [Topilko et al., 1994](#); [Vesque et al., 1996](#)). In addition, several *HOX* genes and *SOX2* have recently been shown to regulate colon CSCs ([Bhatlekar et al., 2014, 2018b](#); [Novak et al., 2019](#); [Schaefer and Lengerke, 2020](#); [Takeda et al., 2018](#)). We therefore investigated whether these genes were similarly regulated by *EGR2* in colon PDX tumors. Notably, we found that *SOX2* and several *HOX* genes, namely *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXB2*, *HOXB3*, and *HOXD10*, were downregulated by shRNA *EGR2* ([Figure 4A](#)). siRNA *EGR2* demonstrated a similar effect on gene expression in PDOs, although due to the undifferentiated culture conditions in which PDOs are maintained ([Sato et al., 2011](#)), there was no effect on the expression of differentiation genes ([Figure S6](#)).

EGR2, ATOH1, HOXA2, HOXA4, HOXA5, HOXA7, HOXB2, and HOXB3 are predictors of patient outcome in colorectal cancer

To characterize *EGR2*, *ATOH1*, *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXB2*, *HOXB3*, *HOXD10*, and *SOX2* expression in clinical samples, we analyzed expression across different colorectal tumor stages ([Figure 4B](#)).

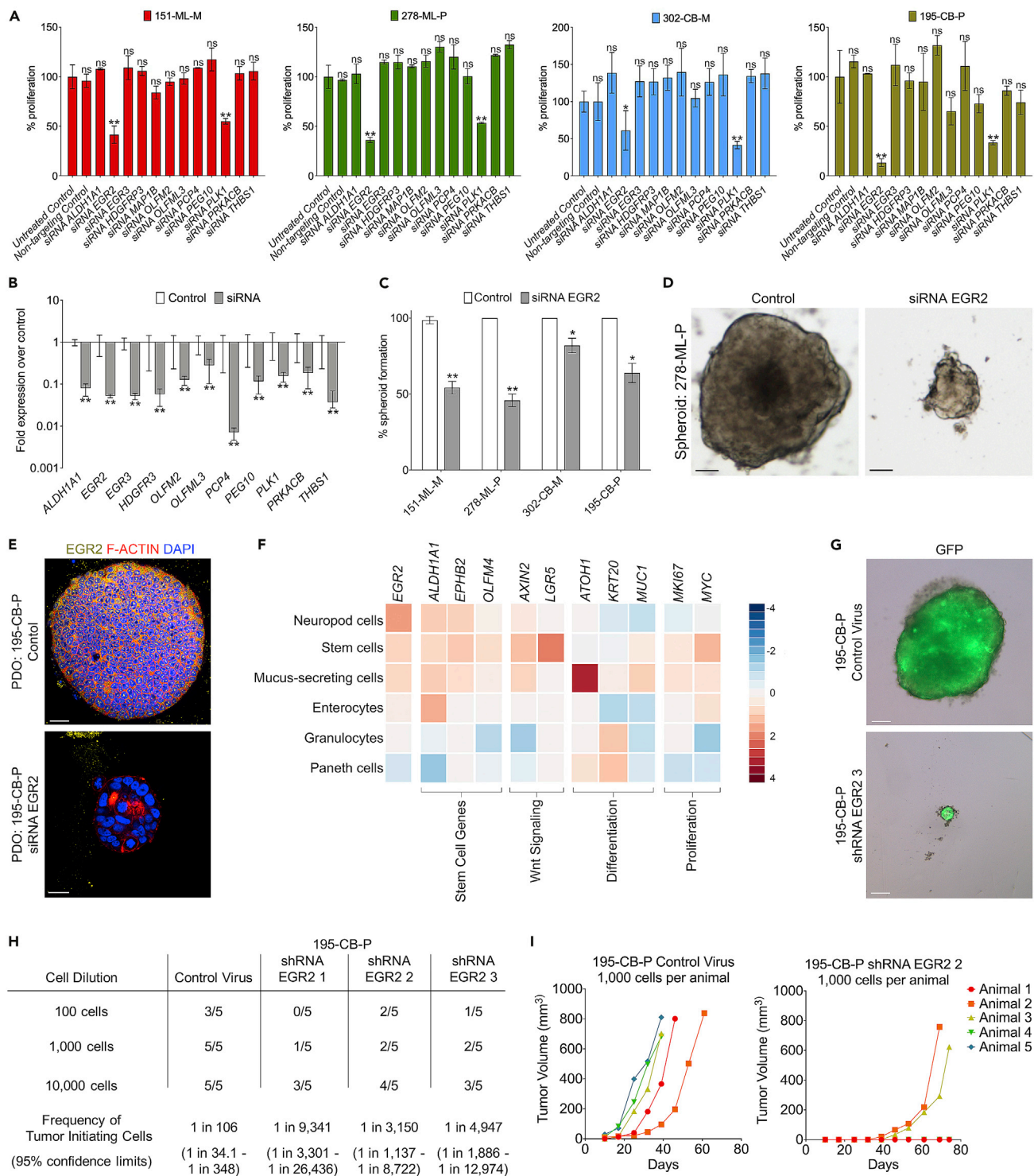


Figure 3. EGR2 is required for CSC survival and tumorigenicity

(A) Proliferation of siRNA transfected patient-derived colon cancer cells in nonadherent cell culture compared to untreated control cells (mean \pm SD; data from three independent experiments). *p value < 0.05; **p value < 0.01 (t-test).

(B) Fold expression of *ALDH1A1*, *EGR2*, *EGR3*, *HDGFR3*, *OLFM2*, *OLFM3*, *PCP4*, *PEG10*, *PLK1*, *PRKACB*, and *THBS1* RT-PCR gene expression data (\pm 95% confidence intervals) in siRNA transfected 278-ML-P cells (n = 3 independent cell preparations) compared to untreated control cells. Nontargeting siRNA and siRNA PLK1 were used as negative and positive controls, respectively (see also Figure S4, Tables S3 and S5).

Figure 3. Continued

- (C) Frequency of siRNA EGR2 spheroid formation in nonadherent cell culture compared to control transfected cells (mean \pm SD; data from three independent experiments). ns = not significant; *p value < 0.05; **p value < 0.01 (t-test).
- (D) Representative images of a 278-ML-P control spheroid (LHS) and a siRNA EGR2 spheroid (RHS) in nonadherent cell culture (scale bars = 100 μ m).
- (E) Immunofluorescence staining of control (top panel) and siRNA EGR2 (lower panel) PDOs for EGR2 (yellow) and F-ACTIN (red) in Matrigel culture. Nuclei are stained blue with DAPI (scale bars, 20 μ m).
- (F) Heatmap showing scRNA-seq generated log(nTPM) z-scores for EGR2, stem cell genes (ALDH1A1, EPHB2, OLFM2), Wnt signaling genes (AXIN2, LGR5), differentiation (ATOH1, KRT20, MUC1), and proliferation genes (MKI67, MYC) in neuropod cells, stem cells, mucus-secreting cells, enterocytes, granulocytes, and Paneth cells from healthy human colon tissue. Results based on Single Cell Type information from the Human Protein Atlas (Karlsson et al., 2021) (<https://www.proteinatlas.org/ENSG00000122877-EGR2/single+cell+type/colon>).
- (G) Representative images of control virus (top panel) and shRNA EGR2 (lower panel) transduced 195-CB-P cells in non-adherent cell culture (scale bars, 100 μ m).
- (H) Table shows results of limiting dilution transplantation of control virus transduced and shRNA EGR2 transduced 195-CB-P cells. The number of established tumors as a fraction of the number of animals transplanted is given. P values for pairwise tests of differences in CSC frequencies between control virus versus shRNA EGR2 1, shRNA EGR2 two, and shRNA EGR2 3 195-CB-P cells are 6.9×10^{-9} , 4.9×10^{-6} , and 6.92×10^{-8} , respectively.
- (I) Growth curves for xenografts derived from control virus transduced cells and shRNA EGR2 transduced cells. (See also Table S4).

These data demonstrated that EGR2 (p value 0.027), HOXA2 (p value 0.026), HOXA4 (p value 0.000075), HOXA5 (p value 0.001), HOXA7 (p value 0.009), HOXB3 (p value 0.0016), and HOXD10 (p value 0.043) expression are more enhanced in late stage T4 clinical tumors. Of these, HOXA4, HOXA5, HOXA7, and HOXB3 are significant at FDR <5%. Analysis of Kaplan-Meier survival curves showed that patients with higher EGR2, HOXA2, HOXA4, HOXA5, HOXA7, HOXB2, and HOXB3 expression have a poorer clinical outcome (p values 0.00017, 0.0028, 0.0006, 0.0043, 0.0022, 0.00025, and 0.019, respectively) (Figure 4C). Of these, higher EGR2, HOXA2, HOXA4, HOXA5, and HOXA7 are significant at FDR <5%. Furthermore, these data demonstrated that high levels of ATOH1 are predictive of good prognosis (p value 0.0013). These data support ATOH1, EGR2 and its target genes HOXA2, HOXA4, HOXA5, HOXA7, and HOXB3 as potential new biomarkers for CRC prognosis.

DISCUSSION

We previously demonstrated that colon cancer PDOs are enriched for CSCs and preserve the functional and molecular heterogeneity found *in vivo* (Regan et al., 2017, 2021). However, the defined conditions of the PDO culture media results in reduced cell type diversity (Sato et al., 2011). Conversely, the *in vivo* environment promotes differentiation and reduces CSCs to a minority population. Therefore, to identify genes that regulate CSC survival and differentiation we carried out whole transcriptome analyses of functionally defined ALDH^{Negative} cells and ALDH^{Positive} CSCs from colon cancer PDO and PDX models and performed functional analyses of genes differentially expressed and common to ALDH^{Positive} CSCs from both models. Interestingly, these analyses revealed transcripts associated with nervous system development and NCSCs to be highly enriched in both PDO and PDX CSCs (Figure 2).

A growing body of evidence has demonstrated a gut-neural axis (de Araujo et al., 2012; Bohórquez and Liddle, 2015; Clemmensen et al., 2017; Han et al., 2018; Mayer, 2011; Sharon et al., 2016) in which various intestinal cells, including stem cells, interact with the autonomic nervous system (ANS), either directly (Bohórquez et al., 2015; Callaghan, 1991; Davis et al., 2018; Kennedy et al., 1983; Lachat and Goncalves, 1978; Musso et al., 1975; Tutton and Helme, 1974) or via the enteric nervous system (ENS) (Hernandes et al., 2000; Puzan et al., 2018; Zucoloto et al., 1997), a network of neurons and glia within the bowel wall that regulates most aspects of intestinal function (Sasselli et al., 2012), to control stem cell proliferation and differentiation (Davis and Dailey, 2018; Greig and Cowles, 2017; Lundgren et al., 2011; Schaak et al., 2000; Takahashi et al., 2014; Valet et al., 1993). For example, differentiated cell types, such as intestinal enterochromaffin (EC) cells have been found to be electrically excitable and to modulate serotonin-sensitive primary afferent nerve fibers via synaptic connections, enabling them to detect and transduce environmental, metabolic, and homeostatic information from the gut directly to the nervous system (Bellono et al., 2017). Recent studies have also demonstrated that enteroendocrine cells form neuroepithelial circuits by directly synapsing with vagal neurons and called for a renaming of these cells from enteroendocrine to neuropod cells (Bohórquez et al., 2015; Kaelberer et al., 2018, 2020; Liddle, 2019). Neuropod cells and EC cells, like all differentiated intestinal cells (enteroendocrine, enterocyte, goblet, and Paneth) and CSCs, derive from multipotent Lgr5^{Positive} crypt base stem cells (Barker et al., 2007, 2009). Significantly, colorectal CSCs themselves have been shown to be capable of generating neurons when transplanted intraperitoneally in nude mice (Lu et al., 2017). Intestinal stem cells and CSCs should therefore possess the capacity to

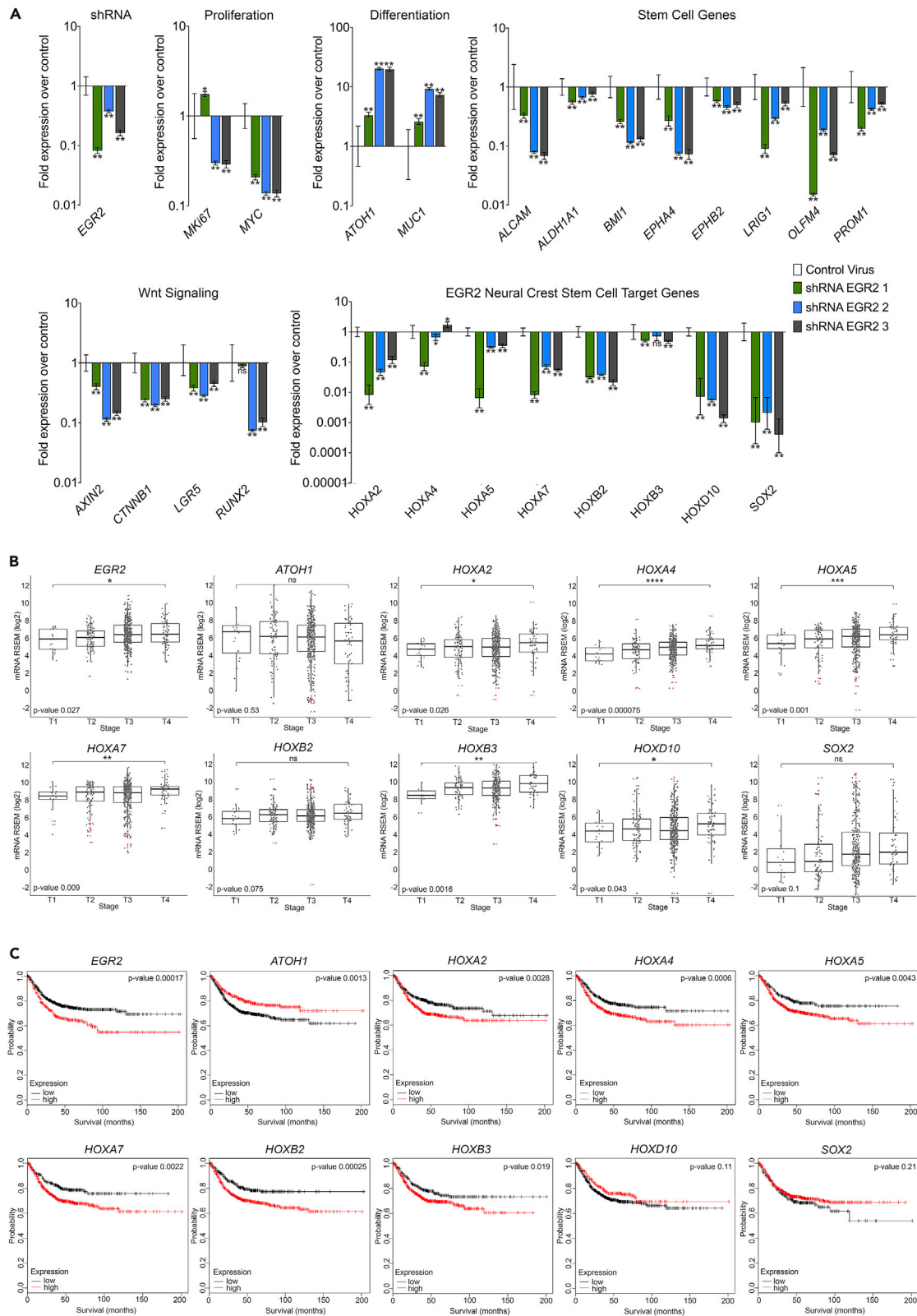


Figure 4. *EGR2* regulates CSC differentiation and expression of NCSC *HOX* genes and *SOX2* and, along with *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXB2*, and *HOXB3*, is an indicator of poor prognosis in clinical samples

(A) Fold expression of *EGR2*, proliferation, differentiation, stem cell genes, Wnt signaling, and *EGR2* NCSC target genes RT-PCR gene expression data (\pm 95% confidence intervals) in three separate 195-CB-P shRNA *EGR2* tumors over the comparator population (three control virus transduced 195-CB-P xenografts). Significant differences are ns: not significant, * $p < 0.05$, and ** $p < 0.01$ and were determined by inspection of error bars as described by Cumming et al. (2007) (Cumming et al., 2007) (See also Table S5).

(B) Expression of *EGR2*, *ATOH1*, *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXB2*, *HOXB3*, *HOXD10*, and *SOX2* in colorectal cancer patients across different tumor stages (T1 v T4, p values 0.027, 0.53, 0.026, 0.000075, 0.001, 0.009, 0.075, 0.0016, 0.043, and 0.1, respectively). Of these, *HOXA4*, *HOXA5*, *HOXA7*, and *HOXB3* are significant at FDR <5%. RNA-seq and clinical data of 533 patients ($n = 378$ colon adenocarcinoma, $n = 155$ rectal adenocarcinoma) was extracted from cBioPortal. The upper whisker extends from the hinge to the highest value that is within 1.5 * interquartile range (IQR) of the hinge. The lower whisker extends from the hinge to the lowest value within 1.5 * IQR of the hinge. Data beyond the end of the whiskers are outliers and plotted as red points (as specified by Tukey).

(C) Kaplan-Meier survival curves for *EGR2*, *ATOH1*, *HOXA2*, *HOXA4*, *HOXA5*, *HOXA7*, *HOXB2*, *HOXB3*, *HOXD10*, and *SOX2* in colorectal cancer patients comparing lower third percentile to upper third percentile (log rank p values = 0.00017, 0.0013, 0.0028, 0.0006, 0.0043, 0.0022, 0.00025, 0.019, 0.11, and 0.21, respectively). Of these, higher *EGR2*, *HOXA2*, *HOXA4*, *HOXA5*, and *HOXA7* are significant at FDR <5%. Results based upon data generated by the Kaplan-Meier Plotter (www.kmplot.com/analysis) (Nagy et al., 2018).

express nervous system genes, because they are the progenitors of cells with neural function. However, until now, nervous system gene enrichment has not been reported in ISCs or CSCs.

Therefore, we carried out gene ontology analysis of Lgr5^{Positive} crypt stem cell transcriptomes from earlier studies (Merlos-Suárez et al., 2011; Muñoz et al., 2012; Yan et al., 2017). In agreement with our CSC data (Figure 2), this analysis revealed normal ISCs to also be enriched for nervous system development gene ontology terms (Figure S1). Overall, these data support CSCs as the source of the neural connections that interact with the ANS and peripheral nervous system (PNS) to drive tumor growth and metastasis (Amit et al., 2020; Lu et al., 2017; Magnon et al., 2013; Rademakers et al., 2017; Venkataramani et al., 2019). Denervation of the ANS and PNS, which causes loss of autonomic neurotransmitters in the gut, results in loss of crypt stem cell proliferation and suppression of tumorigenesis (Callaghan, 1991; Hernandez et al., 2000; Kennedy et al., 1983; Lachat and Goncalves, 1978; Musso et al., 1975; Tutton and Helme, 1974; Zhao et al., 2014; Zucoloto et al., 1997). The inhibition of nervous system gene transcription in CSCs and their progeny, or drugs that block neural interactions, may therefore provide novel therapeutic strategies in colorectal cancer, with results similar to denervation (Demir et al., 2020; Jiang et al., 2020; Saloman et al., 2016; Schonkeren et al., 2021).

During embryonic development, the PNS, of which the ENS is a part, arises from NCSCs, multipotent and highly migratory stem cells that move throughout the embryo to colonize multiple organ primordia and differentiate into numerous cell types (Bronner and LeDouarin, 2012; Le Douarin and Teillet, 1973; Nagy and Goldstein, 2017; Yntema and Hammond, 1954). Recently, self-renewing NCSCs have been discovered in postnatal tissue (Adameyko et al., 2009; Dyachuk et al., 2014; Espinosa-Medina et al., 2014; Jinno et al., 2010; Morrison et al., 1999), including the adult gut (Kruger et al., 2002; Uesaka et al., 2015), and can generate enteric neurons that innervate the intestine in adult mice (Yuan et al., 2021). However, the degree to which these cells contribute to the adult tissue is not known.

EGR2 is a conserved regulator and marker of NCSCs that acts upstream of several *HOX* genes and *SOX2* to control cell fate in embryonic and nervous system stem cells (Chavrier et al., 1990; Desmazières et al., 2009; Ghislain et al., 2003; Grose et al., 2002; Jang et al., 2010; Kim et al., 1998; Manzanares et al., 2002; Nonchev et al., 1996a, 1996b; Sham et al., 1993; Topilko et al., 1994; Vesque et al., 1996). In addition, *EGR2* expression is rapidly activated after wounding in embryonic and adult mouse skin (Grose et al., 2002) and is a marker of hair follicle matrix progenitor cells that give rise to the hair shaft (Liao et al., 2017). More recently, scRNA-seq of the mammary gland found *EGR2* expression to mark a dynamic transcriptional stem cell state from/through which all basal cells in the mammary gland are derived and to be upregulated during developmental periods of morphogenesis and ductal expansion (Gutierrez et al., 2022). The same cellular processes that drive wound response and cellular plasticity persist in cancer and are governed by stress-induced transcription factors, such as *EGR2* (Ge et al., 2017). Significantly, and supporting our data, *EGR2* has recently been found to drive tumor innervation in oral squamous cell carcinoma and melanoma (Pascual et al., 2021). Here, we demonstrate that *EGR2* is enriched in colon CSCs, neuropod cells and intestinal stem cells and is required for tumorigenicity and to maintain CSCs in an undifferentiated neural development primed state by regulating *HOX* genes and *SOX2*.

SOX2 is one of the early genes activated in the developing neural crest and has a broad role as a transcriptional regulator in embryonic and adult stem cells (Aquino et al., 2006; Arnold et al., 2011; Avilion et al., 2003; Favaro et al., 2009; Masui et al., 2007; Rogers et al., 2009; Wakamatsu et al., 2004). In embryonic and adult neural stem cells it is required for the maintenance of neural stem cell properties, including proliferation, survival, self-renewal, and neurogenesis (Ellis et al., 2004; Feng et al., 2013; Pevny and Nicolis, 2010; Suh et al., 2007; Thiel, 2013). In the intestine, its expression results in cell fate conversion and redirects the intestinal epithelium to a more undifferentiated phenotype (Hagey et al., 2018; Kuzmichev et al., 2012; Raghoebir et al., 2012). In addition, SOX2 has been associated with a stem cell state in several cancer types (Bareiss et al., 2013; Herreros-Villanueva et al., 2013; Lee et al., 2014; Mu et al., 2017), where it confers lineage plasticity and therapy resistance (Grimm et al., 2020; Julian et al., 2017), and is aberrantly expressed in CRC (Fang et al., 2010; Park et al., 2008; Raghoebir et al., 2012). Overall, these data combined with our own, support a role for SOX2 in CRC tumor initiation, neurogenesis, and disease progression, possibly by promoting lineage plasticity and an NCSC state.

HOX genes have been reported to be enriched in and required for the maintenance of normal stem cells and CSCs in various adult tissues (Bhatlekar et al., 2018a; Eoh et al., 2017; Hassan et al., 2007; Ben Khadra et al., 2014; Leucht et al., 2008; Liedtke et al., 2010; Seifert et al., 2015; Shah et al., 2017; Shah and Sukumar, 2010; Tabuse et al., 2011). Recently, HOXA4, HOXA9, and HOXD10 were shown to be selectively expressed in ALDH^{Positive} intestinal crypt stem cells and colon CSCs, to promote self-renewal and regulate expression of stem cell markers (Bhatlekar et al., 2014, 2018b). Here, we demonstrate that the same HOX genes that are regulated by EGR2 in NCSCs are also regulated by EGR2 in colon CSCs and that several of these, HOXA2, HOXA4, HOXA5, HOXA7, HOXB2, HOXB3, along with EGR2, are indicators of poor prognosis in CRC. However, in contrast to other studies, our RNA-seq analyses did not show differential expression of these genes CSCs.

These data demonstrate that colon CSCs are enriched for neural crest and nervous system development genes, including the NCSC regulator EGR2, which controls SOX2 and HOX genes to maintain CSCs in an undifferentiated state. Targeting EGR2 to induce differentiation and potentially block cancer neurogenesis, e.g., by downregulating the neural stem cell regulator SOX2, may offer a novel therapeutic strategy to eliminate colon CSCs and prevent nervous system driven proliferation and metastasis.

Limitations of the study

Our study demonstrates that ISCs and CSCs are enriched for nervous system development genes and proposes that CSCs are the source of cancer neurogenesis. However, the analysis of neuron-like cell/nerve interactions and tumor innervation was limited by the absence of an integrated PNS in the PDO models used here. Model systems that co-culture epithelial organoids with nerves have been reported by others (Pastuła et al., 2016; Workman et al., 2017) and should be adapted for future studies to a) track the evolution of the proposed CSC-derived neural-like tumor cells/nerves, b) characterize tumor cell interactions with the PNS, and c) identify drugs that block cancer neurogenesis and/or neural-tumor cell cross talk. In addition, our study demonstrated that colon cancer cells express nerve cell markers (e.g., ENO2 and TUBB3); however, we did not evaluate the effects of EGR2 RNAi on these markers or other neural genes. The direct effect of EGR2 and its target SOX2 and HOX genes on cancer neurogenesis also remains to be fully determined.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

A.S., T.J., D.M., and D.H. are employees of Bayer AG. R.L., J.T., and M.L. are employees of Nuvisan ICB GmbH. C.R.A.R. is the founder of CELLphenomics GmbH.

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STAR★METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--|--|--|
| Antibodies | | |
| Mouse monoclonal anti- α -Tubulin | Sigma | T5168; RRID:AB_477579 |
| Rabbit monoclonal anti-non-phospho β -Catenin | Cell Signaling Technology | #8814; RRID:AB_11127203 |
| Rabbit polyclonal anti-EGR2 | Abcam | ab43020; RRID:AB_942051 |
| Mouse monoclonal EpCam | Cell Signaling Technology | #2929; RRID:AB_2098657 |
| Alexa Fluor® 647 Phalloidin | Thermo Fisher | #A22287 |
| Biological samples | | |
| Patient-derived organoids (PDOs) | Charité Universitätsmedizin Berlin, Germany | OncoTrack |
| Critical commercial assays | | |
| Aldefluor assay | STEMCELL Technologies | 01700 |
| RNeasy Mini Plus RNA extraction kit | Qiagen | ID:74136 |
| Sensiscript RT kit | Qiagen | ID:205213 |
| TruSeq RNA Library Preparation Kit | Illumina | 20020597 |
| CellTiter-Glo Cell Viability Assay | Fisher Scientific | G7571 |
| Deposited data | | |
| Array data | This paper; www.ebi.ac.uk/arrayexpress | ArrayExpress: E-MTAB-5209; ArrayExpress: E-MTAB-8927 |
| Experimental models: Organisms/strains | | |
| <i>Mus musculus</i> : NMRI nude | Taconic | MGI:5653040 |
| Oligonucleotides | | |
| EGR2 MISSION shRNA Lentiviral Transduction Particles: TRCN0000013839, TRCN000013840, TRCN000013841 | Sigma-Aldrich | SHCLNV-NM_000399 |
| <i>ATOH1</i> (Hs00245453_s1) | Thermo Fisher | 4331182 |
| <i>AXIN2</i> (Hs00610344_m1) | Thermo Fisher | 4331182 |
| <i>BMI1</i> (Hs00180411_m1) | Thermo Fisher | 4331182 |
| <i>CTNNB1</i> (Hs00355049_m1) | Thermo Fisher | 4331182 |
| <i>EGR2</i> (Hs00166165_m1) | Thermo Fisher | 4331182 |
| <i>EPHA4</i> (Hs00953178_m1) | Thermo Fisher | 4331182 |
| <i>EPHB2</i> (Hs00362096_m1) | Thermo Fisher | 4331182 |
| <i>GAPDH</i> (Hs02758991_g1) | Thermo Fisher | 4331182 |
| <i>HOXA2</i> (Hs00534579_m1) | Thermo Fisher | 4331182 |
| <i>HOXA5</i> (Hs00430330_m1) | Thermo Fisher | 4331182 |
| <i>HOXA7</i> (Hs00430330_m1) | Thermo Fisher | 4331182 |
| <i>HOXB2</i> (Hs01911167_s1) | Thermo Fisher | 4331182 |
| <i>HOXB3</i> (Hs05048382_s1) | Thermo Fisher | 4331182 |
| <i>HOXB10</i> (Hs00157974_m1) | Thermo Fisher | 4331182 |
| <i>LGR5</i> (Hs00969422_m1) | Thermo Fisher | 4331182 |
| <i>MKI67</i> (Hs04260396_g1) | Thermo Fisher | 4331182 |
| <i>MUC1</i> (Hs00159357_m1) | Thermo Fisher | 4331182 |

(Continued on next page)

Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|-----------------------------------|---------------|-------------|
| MYC (Hs00153408_m1) | Thermo Fisher | 4331182 |
| RUNX2 (Hs01047973_m1) | Thermo Fisher | 4331182 |
| SOX2 (Hs01053049_s1) | Thermo Fisher | 4331182 |
| HDGFRP3 (Hs00274988_m1) | Thermo Fisher | 4331182 |
| OLFM2 (Hs01017934_m1) | Thermo Fisher | 4331182 |
| OLFML3 (Hs01113293_g1) | Thermo Fisher | 4331182 |
| PCP4 (Hs01113638_m1) | Thermo Fisher | 4331182 |
| PEG10 (Hs00248288_s1) | Thermo Fisher | 4331182 |
| PLK1 (Hs00983227_m1) | Thermo Fisher | 4331182 |
| PPID (Hs00234593_m1) | Thermo Fisher | 4331182 |
| PRKACB (Hs01086757_m1) | Thermo Fisher | 4331182 |
| THBS1 (Hs00962908_m1) | Thermo Fisher | 4331182 |
| Non-Targeting Control siRNA | Dharmacon | D-001910-01 |
| Red Non-targeting Control siRNA | Dharmacon | D-001960-01 |
| PPID Control siRNA | Dharmacon | D-001930-01 |
| ALDH1A1 (216) siRNA - SMARTpool | Dharmacon | E-008722-00 |
| EGR2 (1959) siRNA - SMARTpool | Dharmacon | E-006527-01 |
| EGR3 (1960) siRNA - SMARTpool | Dharmacon | E-006528-00 |
| HDGFRP3 (50810) siRNA - SMARTpool | Dharmacon | E-017093-00 |
| OLFM2 (93145) siRNA - SMARTpool | Dharmacon | E-015212-00 |
| OLFML3 (56944) siRNA - SMARTpool | Dharmacon | E-020325-00 |
| PCP4 (5121) siRNA - SMARTpool | Dharmacon | E-020122-00 |
| PEG10 (23089) siRNA - SMARTpool | Dharmacon | E-032579-00 |
| PLK1 (5347) siRNA - SMARTpool | Dharmacon | E-003290-01 |
| PRKACB (5567) siRNA - SMARTpool | Dharmacon | E-004650-00 |
| THBS1 (7057) siRNA - SMARTpool | Dharmacon | E-019743-00 |

Software and algorithms

| | | |
|--------------------------------|--|---|
| ELDA software | Hu and Smyth (2009) | http://bioinf.wehi.edu.au/software/elda/index.html |
| ImageJ | Schneider et al. (2012) | https://imagej.nih.gov/ij/ |
| DESeq2 | Love et al. (2014) | https://bioconductor.org/packages/release/bioc/html/DESeq2.html |
| STAR aligner (version 2.4.2a). | Dobin et al. (2013) | https://github.com/alexdobin/STAR |
| Kaplan-Meier Plotter | Nagy et al. (2018) | www.kmplot.com/analysis |
| Gene Ontology Resource | Ashburner et al. (2000) | (www.geneontology.org) |
| GSEA software | Liberzon et al. (2015); Subramanian et al. (2005) | https://www.gsea-msigdb.org/gsea/index.jsp |
| Prism 9.0 | GraphPad | https://www.graphpad.com/scientific-software/prism/ |

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact Joseph L. Regan (joseph.regan@charite.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- Array data have been deposited at ArrayExpress (www.ebi.ac.uk/arrayexpress) and are publicly available as of the date of publication. Accession numbers are listed in the [key resources table](#).
- Array data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession numbers ArrayExpress: E-MTAB-5209 and ArrayExpress: E-MTAB-8927.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human tissue samples

Tumor material was obtained with informed consent from colon cancer patients (2 male and 3 female; detailed in [Table S1](#)) under approval from the local Institutional Review Board of Charité University Medicine (Charité Ethics Cie: Charitéplatz 1, 10117 Berlin, Germany) (EA 1/069/11) and the ethics committee of the Medical University of Graz and the ethics committee of the St John of God Hospital Graz (23-015 ex 10/11). Tumor staging was carried out by experienced and board-certified pathologists ([Table S1](#)). Age of subjects can be inquired through info@oncotrack.eu.

Patient-derived organoids

Cancer organoid cultures were established and propagated as previously described ([Sato et al., 2011](#); [Schütte et al., 2017](#)). Briefly, resected tumor samples were enzymatically digested with Collagenase IV (C9407, Sigma-Aldrich), DNaseI (A3778,0050, AppliChem) and Dispase (07913, Stem Cell Technologies) at 37°C for 60 min. Suspensions were washed, filtered, and depleted of red blood cells using Red Blood Cell Lysis Solution (00-4333-57, Invitrogen). Cells were mixed with phenol-red free growth factor-reduced Matrigel (356231, Corning) and seeded into 24-well plates. Solidified droplets were overlaid with culture medium consisting of Advanced DMEM/F12 (12634-010, Gibco) supplemented with 1% penicillin/streptomycin, 1% HEPES buffer (1064859, Fisher Scientific), 1% Glutamax, 1x N2 (#17502-048, Invitrogen), 1x B27 (17504-044, Invitrogen), 50 ng/mL EGF (E9644, Sigma), and 1mM N-acetylcysteine (A9165-5G, Sigma) and maintained at 37°C. Organoids were released from Matrigel and passaged by adding 5 mL Advanced DMEM/F12 followed by centrifugation and digestion of pellets with TrypLE Express (12604-013, Gibco) ([Regan, 2022](#)).

Xenotransplantation

Housing and handling of animals followed European and German Guidelines for Laboratory Animal Welfare. Animal experiments were conducted in accordance with animal welfare law, approved by local authorities, and in accordance with the ethical guidelines of Bayer AG. PDO derived PDX models were processed to single cells and sorted by FACS (BD FACS Aria II) for ALDH activity (Aldefluor assay) and DAPI to exclude dead cells. Cells were then re-transplanted at limiting dilutions by injected subcutaneously in PBS and Matrigel (1:1 ratio) at limiting cell dilutions into female 8–10-week-old nude mice. The purity of the sorted cell populations was confirmed by post-sort FACS analysis.

METHOD DETAILS

Histology and immunohistochemistry

Tumors were fixed in 4% paraformaldehyde overnight for routine histological analysis and immunohistochemistry. Immunohistochemistry was carried out via standard techniques with non-phospho (Active) β -Catenin (#8814, rabbit monoclonal, Cell Signaling Technology; diluted 1:200) and EGR2 (ab43020, Abcam, rabbit IgG, polyclonal, diluted 1:1000) antibodies. Negative controls were performed using the same protocols with substitution of the primary antibody with IgG-matched controls (ab172730, rabbit IgG, monoclonal [EPR25A], Abcam). Colorectal cancer tissue microarrays from the OncoTrack patient cohort ([Schütte et al., 2017](#)) were obtained from The Institute of Pathology, Medical University Graz, Austria and analyzed using Aperio TMA Lab and Image software (Leica Biosystems).

Immunofluorescence staining

Immunofluorescence staining and imaging of PDOs was carried out as described in ([Regan, 2022](#)). Briefly, cancer organoid cultures were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.1% Triton X-100 for 30 min and blocked in phosphate-buffered saline (PBS) with 10% bovine serum albumin (BSA). Samples were incubated with primary antibodies overnight at 4°C. Antibodies used were Non-phospho (Active) β -Catenin (#8814, rabbit monoclonal, Cell Signaling Technology; diluted

1:200), EZRIN (ab40839, rabbit monoclonal, Abcam, diluted 1:200), EPCAM (#2929, mouse monoclonal, Cell Signaling Technology, diluted 1:500) and EGR2 (ab43020, rabbit polyclonal, Abcam, diluted 1:500). Samples were stained with a conjugated secondary antibody overnight at 4°C. F-actin was stained with Alexa Fluor® 647 Phalloidin (#A22287, Thermo Fisher; diluted 1:20) for 30 min at room temperature. Nuclei were counterstained with DAPI. Negative controls were performed using the same protocol with substitution of the primary antibody with IgG-matched controls. Cancer organoids were then transferred to microscope slides for examination using a Zeiss LSM 700 Laser Scanning Microscope.

Aldefluor Assay

Organoids and xenografts were processed to single cells and labelled using the Aldefluor Assay according to the manufacturer's (STEMCELL Technologies) instructions. ALDH levels were assessed by FACS on a BD LSR II analyzer.

RNA sequencing

Cells were lysed in RLT buffer and processed for RNA using the RNeasy Mini Plus RNA extraction kit (Qiagen). Samples were processed using Illumina's TruSeq RNA protocol and sequenced on an Illumina HiSeq 2500 machine as 2x125nt paired-end reads. The raw data in Fastq format were checked for sample quality using our internal NGS QC pipeline. Reads were mapped to the human reference genome (assembly hg19) using the STAR aligner (version 2.4.2a) (Dobin et al., 2013). Total read counts per gene were computed using the program "featureCounts" (version 1.4.6-p2) in the "subread" package, with the gene annotation taken from Gencode (version 19). The "DESeq2" Bioconductor package (Love et al., 2014) was used for the differential-expression analysis.

siRNA transfection

Cells were seeded in 100 µL volumes of Accell Delivery Media (B-005000-500, Dharmacon) at 1.0×10^5 cells per well in ultra-low attachment 96-well plates and transfected with 2 µM concentrations of Accell SMARTPool siRNAs (Table S3) by incubating for up to 96 h in Accell siRNA Delivery Media. The endogenous reporter gene Cyclophilin B (*PP1D*) (D-001920-01, Dharmacon) and the essential gene *PLK1* (E-003290-01, Dharmacon) were used as positive controls. Accell red fluorescent non-targeting siRNA (D-001960-01, Dharmacon) and non-targeting siRNA (D-001910-01, Dharmacon) were used as negative controls.

Viral transduction

Cells were seeded in 100 µL volumes of antibiotic free culture media at 1.0×10^5 cells per well in ultra-low attachment 96-well plates. Control and shRNA lentiviruses were purchased from Sigma-Aldrich (Table S4). Viral particles were added at a multiplicity of infection of 1. Cells were transduced for up to 96 h or until GFP positive cells were observed before being embedded in Matrigel for the establishment of lentiviral transduced cancer organoid cultures. Puromycin (2 µg/mL) was used to keep the cells under selection.

Limiting dilution spheroid assays

For siRNA spheroid assays, transfected live (DAPI^{Negative}) cells were sorted at 10 cells per well into 96-well ultra-low attachment plates. 20 days later wells containing spheroids were counted and used to calculate CSC frequency using ELDA software. Spheroid sizes were quantified using ImageJ software (Schneider et al., 2012). Proliferation was measured using the CellTiter-Glo® Luminescent Cell Viability Assay.

Gene expression analysis

For quantitative real-time RT-PCR analysis RNA was isolated using the RNeasy Mini Plus RNA extraction kit (Qiagen). cDNA synthesis was carried out using a Sensiscript RT kit (Qiagen). RNA was transcribed into cDNA using an oligo dTn primer (Promega) per reaction. Gene expression analysis was performed using TaqMan® Gene Expression Assays (Applied Biosystems) (Table S5) on an ABI Prism 7900HT sequence detection system (Applied Biosystems). GAPDH was used as an endogenous control and results were calculated using the $\Delta\text{-}\Delta\text{Ct}$ method. Data were expressed as the mean fold gene expression difference in three independently isolated cell preparations over a comparator sample with 95% confidence intervals. Pairwise comparison of gene expression was performed using R (R Core Team, 2020) together with package ggplot2 (Wickham, 2016) on log2 transformed RNA-seq data from 533 patients with clinical data (n = 378 colon adenocarcinomas, n = 155 rectal carcinomas staged T1-T4) extracted from the cBioPortal for

Cancer Genomics ([cancer.sanger.ac.uk](https://cancer.sanger.ac.uk/cancer-genomics)) ([Hoadley et al., 2018](#); [Liu et al., 2018](#)). Single cell type information based on scRNA-seq data from The Human Protein Atlas [proteinatlas.org](https://www.proteinatlas.org) ([Karlsson et al., 2021](#)). Survival curves were generated using the Kaplan-Meier Plotter (www.kmplot.com/analysis) ([Nagy et al., 2018](#)). Gene ontology enrichment analysis was carried out using the Gene Ontology Resource (www.geneontology.org) ([Ashburner et al., 2000](#); [The Gene Ontology Consortium, 2019](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 6.0 was used for data analysis and imaging. All data are presented as the means \pm SD, followed by determining significant differences using the two-tailed t test. Significance of RT-PCR data was determined by inspection of error bars as described by [Cumming et al. \(2007\)](#) ([Cumming et al., 2007](#)). Limiting-dilution frequency and probability estimates were analyzed by the single-hit Poisson model and pairwise tests for differences in stem cell frequencies using the ELDA software (<http://bioinf.wehi.edu.au/software/elda/index.html>, [Hu and Smyth, 2009](#)) ([Hu and Smyth, 2009](#)). Gene set enrichment analysis was carried out using pre-ranked feature of the Broad Institute GSEA software version 2 using msigdb v5.1 gene sets ([Liberzon et al., 2015](#); [Subramanian et al., 2005](#)). The ranking list was derived from the fold changes (1.5fold upregulated) calculated from the differential gene expression calculation and nominal p-values. p-values <0.05 were considered as statistically significant. For the final list of significant genes, False Discovery Rate was computed using the Benjamini-Hochberg method ([Benjamini and Hochberg, 1995](#)).