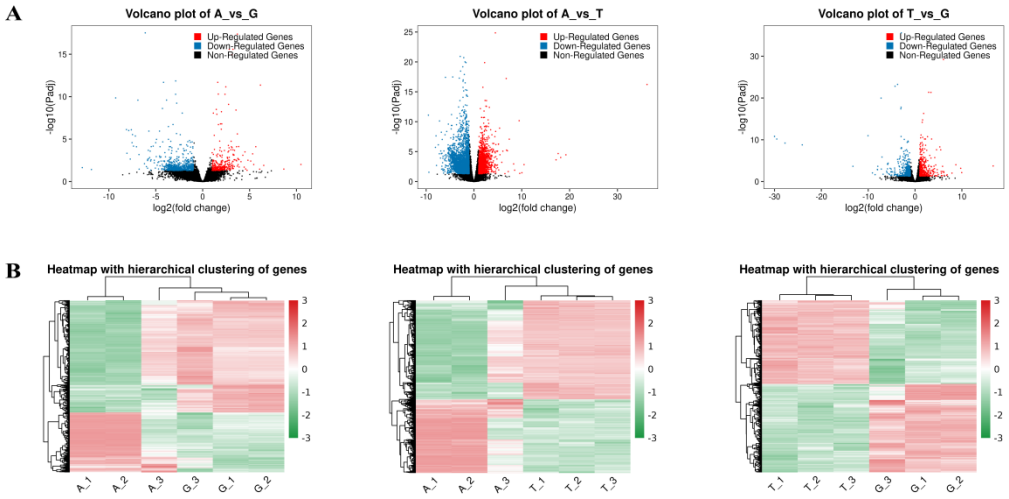
Supplementary Figures

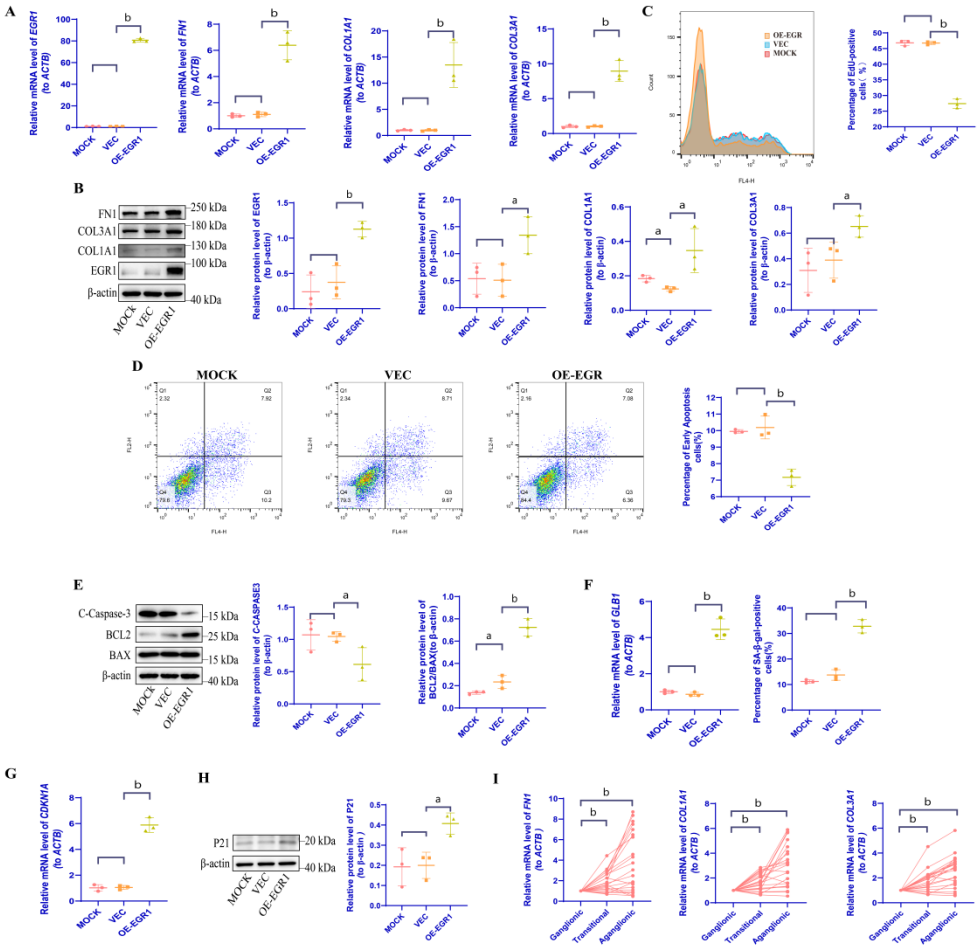


Supplementary Figure 1 Paired differential expression across matched aganglionic, transitional and ganglionic colonic segments.

(A) Volcano plots of DEGs for the three pairwise comparisons (A_vs_G, A_vs_T and T_vs_G), called under a paired design in DESeq2 (~ patient + segment, with patient as a blocking factor). Red, up-regulated; blue, down-regulated; black, non-significant. DEG counts under the paired model: 927 (A_vs_G), 3,635 (A_vs_T) and 828 (T_vs_G).

(B) Hierarchical clustering heatmaps of the same DEG sets across samples G_1–G_3, T_1–T_3 and A_1–A_3, annotated by patient and by segment. Colour, Z-score-normalized expression.

Statistics. DESeq2 Wald test with Benjamini–Hochberg correction; DEG cutoff $|\log_2 FC| \geq 1$ and adjusted $P < 0.05$; n = 3 patients (nine samples).



Supplementary Figure 2. Reproduction of EGR1-driven ECM, proliferation, apoptosis and senescence-like phenotypes in human HFL-1 fibroblasts; orthogonal patient-tissue qRT-PCR.

MOCK, untransduced cells; VEC, empty-vector lentivirus; OE-EGR1, EGR1-expressing lentivirus. Unlike the adenoviral system used in the primary mouse fibroblasts, this lentiviral vector carries no GFP reporter, so a MOCK arm could be included in the flow-cytometry panels.

(A) qRT-PCR in MOCK/VEC/OE-EGR1 HFL-1 cells (normalized to *ACTB*): *EGR1* 1.01 ± 0.16 / 0.91 ± 0.13 / 80.70 ± 1.66; *FN1* 1.01 ± 0.13 / 1.11 ± 0.17 / 6.39 ± 1.12; *COL1A1* 1.01 ± 0.14 / 0.98 ± 0.15 / 13.49 ± 4.29; *COL3A1* 1.01 ± 0.18 / 1.02 ± 0.10 / 8.96 ± 1.49.

(B) Western blot and quantification (relative to β-actin): EGR1 0.24 ± 0.24 / 0.37 ± 0.23 / 1.13 ± 0.11; FN1 0.54 ± 0.29 / 0.51 ± 0.30 / 1.34 ± 0.34; COL1A1 0.18 ± 0.02 / 0.12 ± 0.01 / 0.35 ± 0.13; COL3A1 0.31 ± 0.17 / 0.39 ± 0.14 / 0.65 ± 0.08.

(C) EdU flow cytometry: representative overlaid histograms (FL4-H, EdU-APC; key in the order MOCK, VEC, OE-EGR1, with a vertical line marking the EdU-positive gate) and the EdU^+ fraction 46.81 ± 0.88% / 46.76 ± 0.52% / 27.40 ± 1.50%.

(D) Annexin V-APC/PI flow cytometry: representative dot plots (Q1 necrotic, Q2 late-apoptotic, Q3 early-apoptotic, Q4 viable; *y*-axis FL2-H, PI; *x*-axis FL4-H, Annexin V-APC) and the Annexin V^+/PI^- early-apoptotic fraction 9.95 ± 0.11% / 9.74 ± 0.69% / 7.17 ± 0.50%.

(E) Western blot of cleaved caspase-3, BCL-2 and BAX with β-actin loading control, with molecular-weight markers in kDa. Cleaved caspase-3 / β-actin: 1.07 ± 0.24 / 1.05 ± 0.08 / 0.61 ± 0.26. BCL-2/BAX ratio: 0.14 ± 0.01 / 0.23 ± 0.06 / 0.72 ± 0.08.

(F) qRT-PCR of *GLB1* mRNA (normalized to *ACTB*): 1.00 ± 0.10 / 0.86 ± 0.11 / 4.46 ± 0.57, and SA-β-gal^+ HFL-1 cells: 11.20 ± 0.70% / 13.75 ± 2.06% / 32.82 ± 2.59%.

(G) qRT-PCR of *CDKN1A* mRNA (normalized to *ACTB*): 1.02 ± 0.25 / 1.05 ± 0.16 / 5.89 ± 0.57.

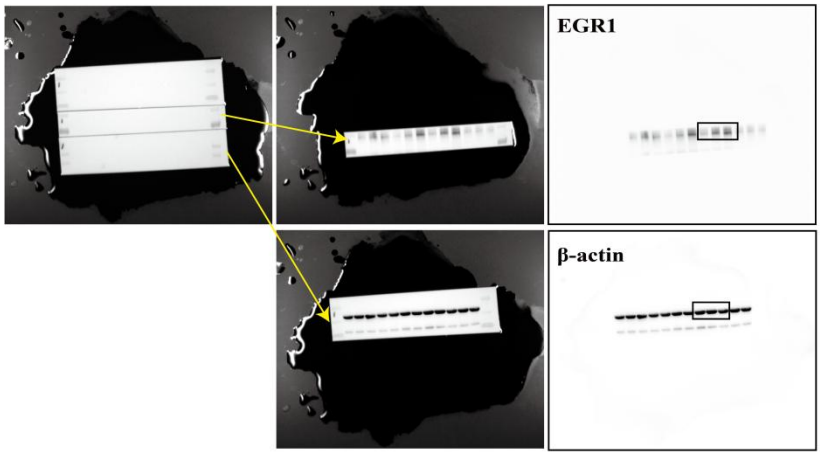
(H) Western blot of P21 with β-actin loading control, markers in kDa. P21 / β-actin: 0.19 ± 0.09 / 0.20 ± 0.07 / 0.41 ± 0.03.

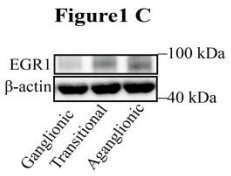
(I) qRT-PCR of *FN1*, *COL1A1* and *COL3A1* in matched G/T/A HSCR colonic segments (normalized to *ACTB*; each patient's ganglionic value set to 1.000 by definition, so that group carries no dispersion): *FN1* G 1.000 / T 2.009 ± 0.981 / A 3.697 ± 2.891; *COL1A1* 1.000 / 1.663 ± 0.689 / 2.919 ± 1.705; *COL3A1* 1.000 / 1.813 ± 0.854 / 2.557 ± 1.247. n = 16 paired patients; each connected line is one patient.

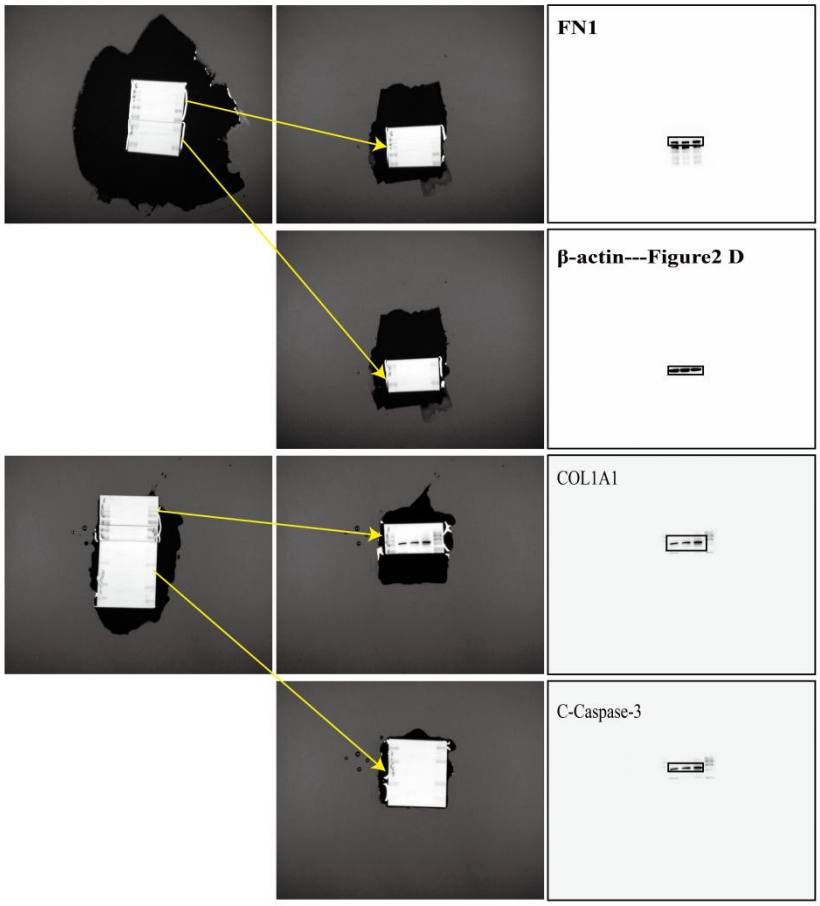
Cell-line panels: n = 3 biological replicates, one biological replicate = one independently transduced culture; Patient-tissue panels: n = 16 paired patients. Statistics. (A–H) One-way ANOVA with Tukey's post hoc test (MOCK/VEC/OE-EGR1); (I) repeated-measures one-way ANOVA with Geisser–Greenhouse correction and Tukey's post hoc test (paired G/T/A).

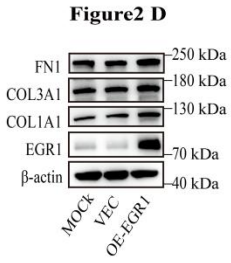
Data are mean ± SD; [a]$P < 0.05$, [b]$P < 0.01$ *vs* MOCK and [c]$P < 0.05$, [d]$P < 0.01$ *vs* VEC in (A–H); [a]$P < 0.05$, [b]$P < 0.01$ *vs* the ganglionic segment in (I). Comparisons that are not annotated did not reach $P < 0.05$.

Original Western Blot Images









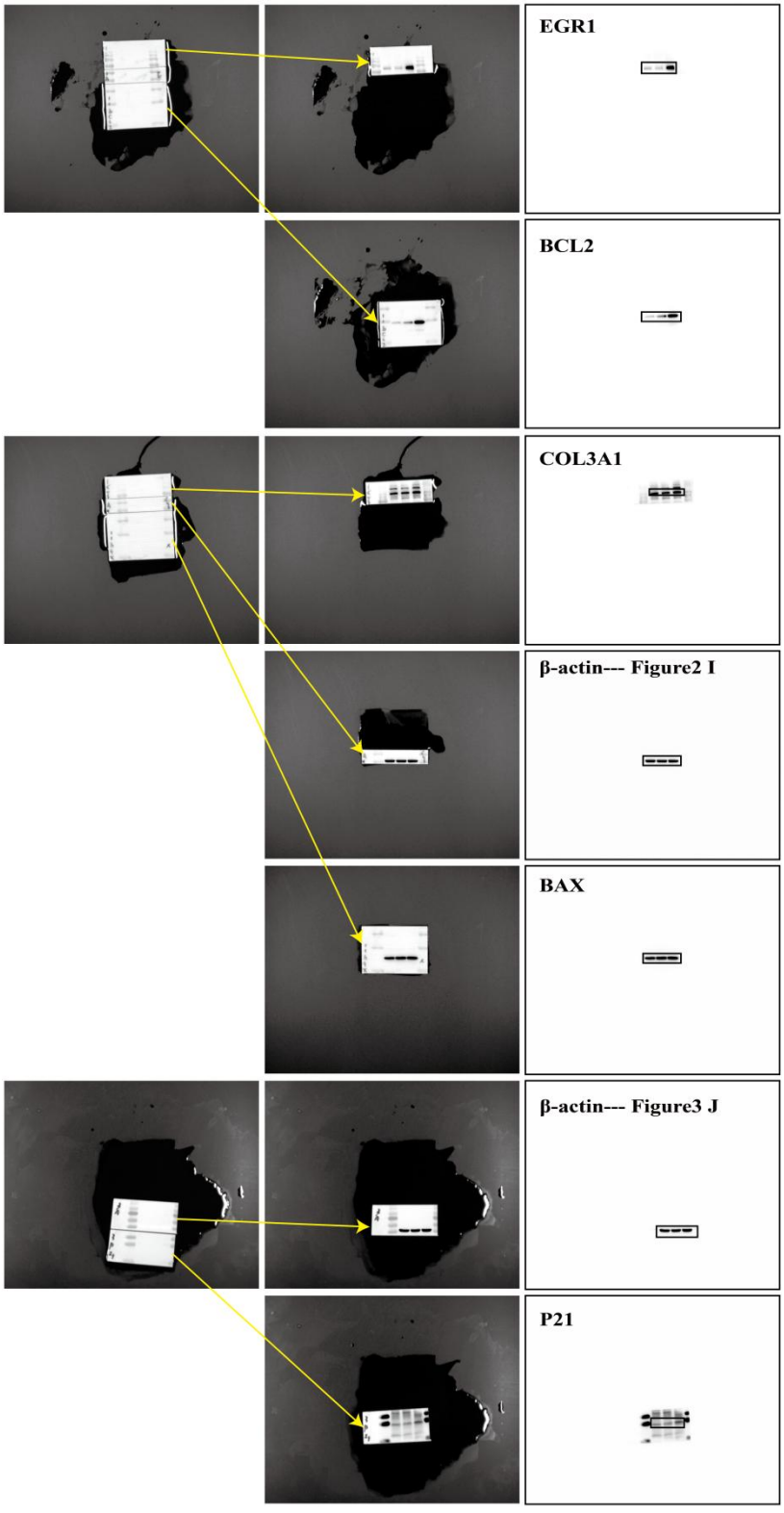


Figure2 I

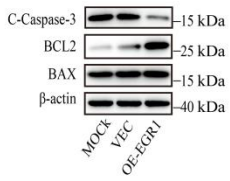
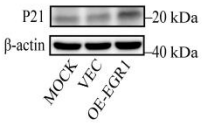


Figure3 J



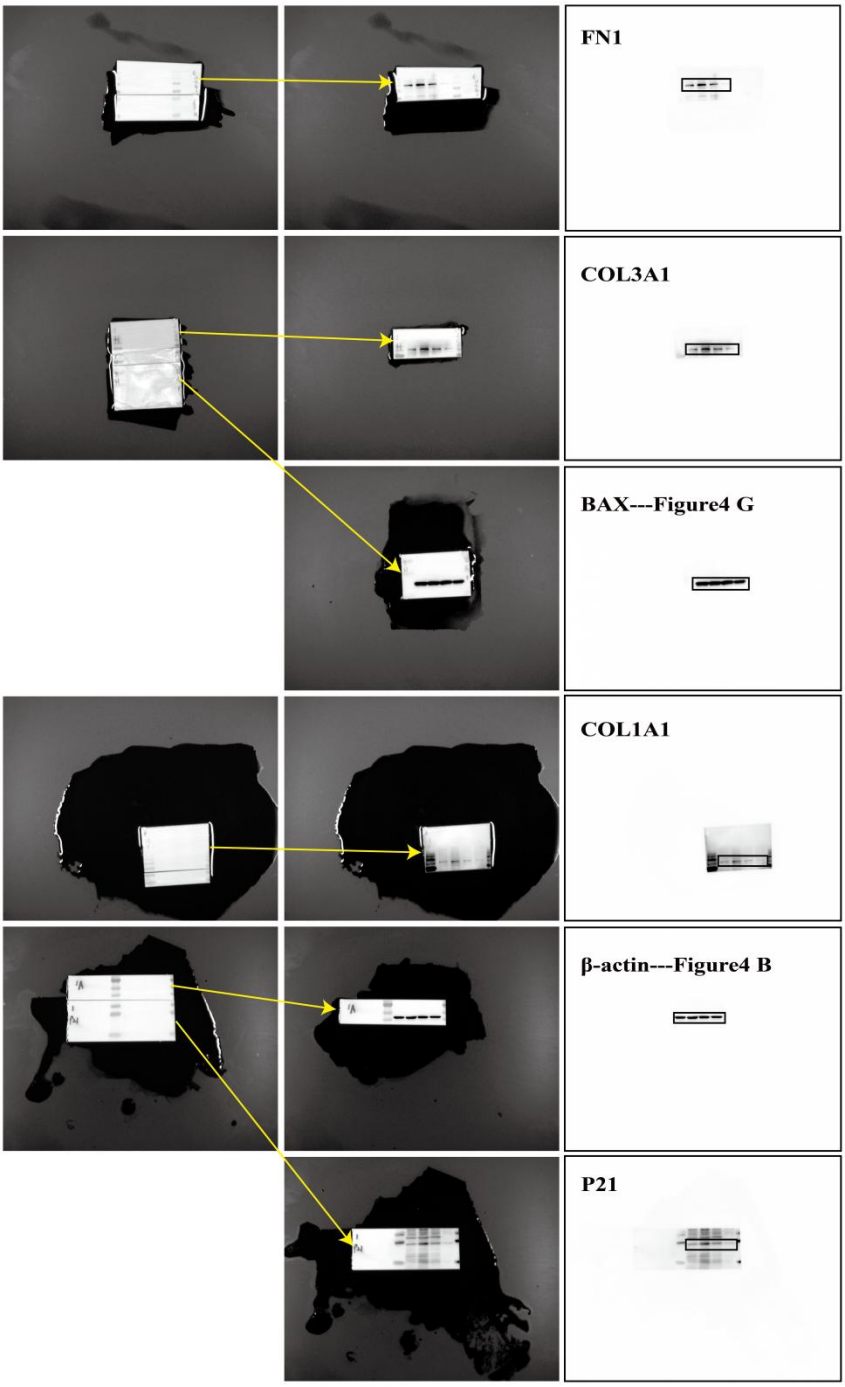
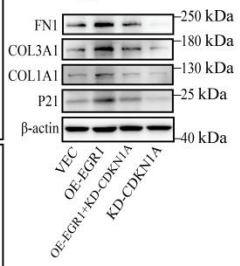

FN1
COL3A1
BAX---Figure4 G
COL1A1
β-actin---Figure4 B
P21

Figure4 B


FN1
COL3A1
COL1A1
P21
β-actin
250 kDa
180 kDa
130 kDa
25 kDa
40 kDa
VEC
OE-EGR1
OE-EGR1+KD-CDKN1A
KD-CDKN1A

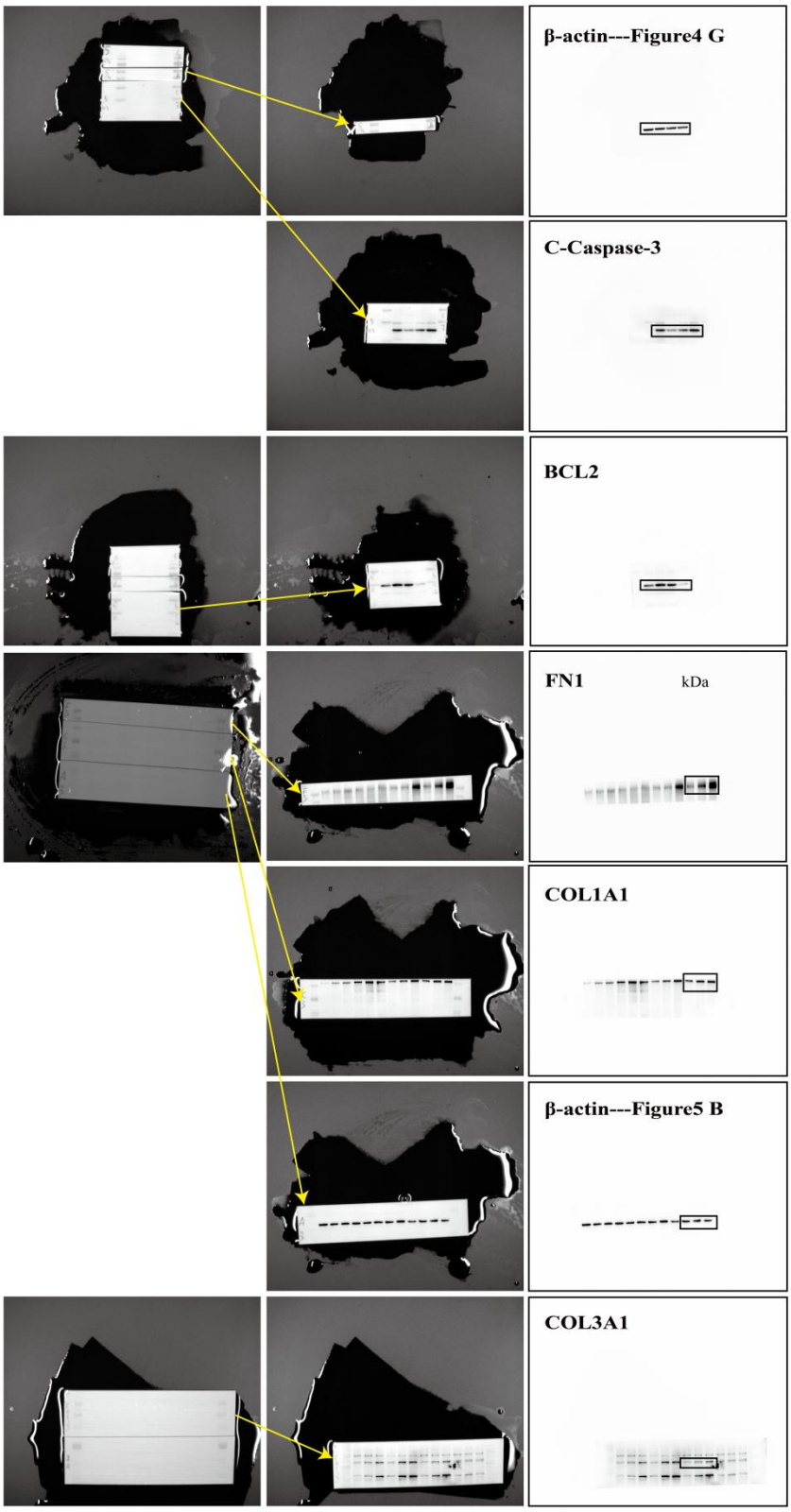


Figure4 G

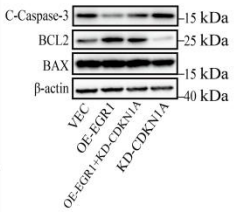
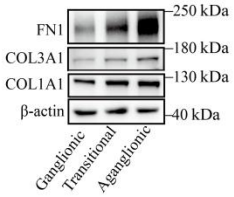


Figure5 B



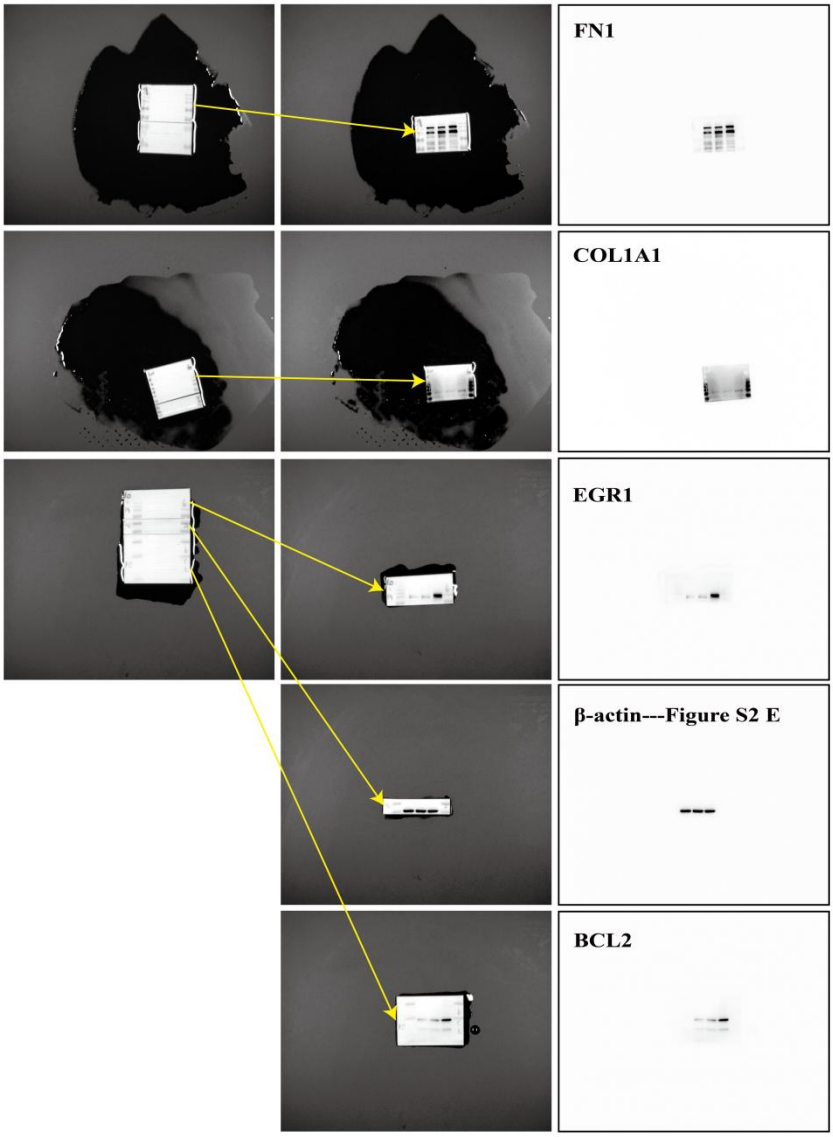
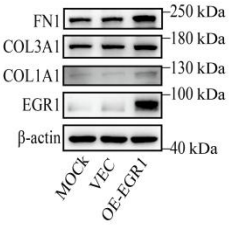
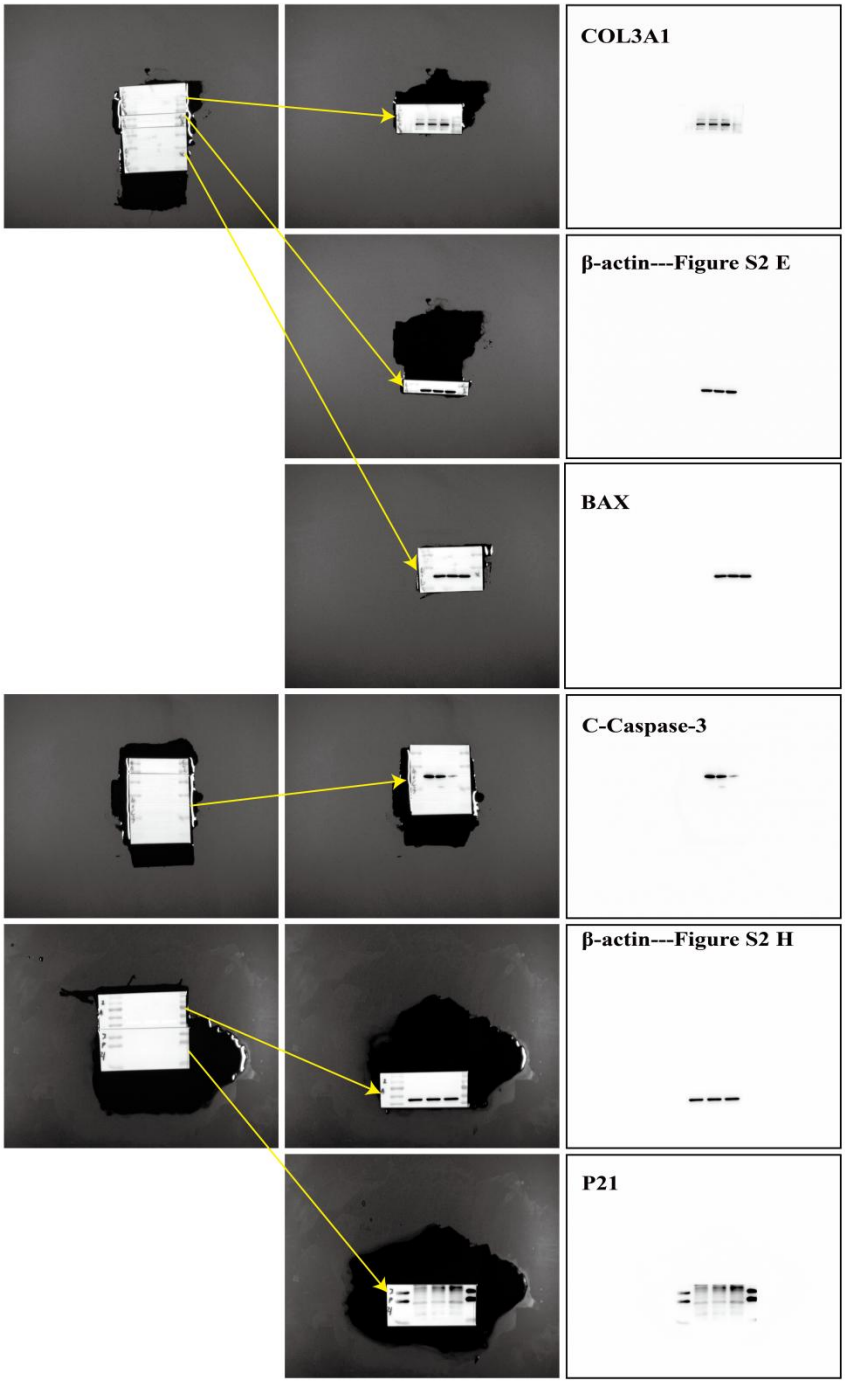
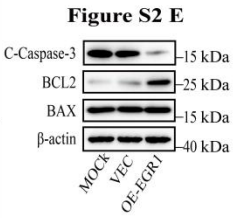
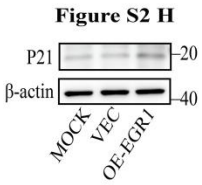

FN1
COL1A1
EGR1
β-actin---Figure S2 E
BCL2

Figure S2 B


FN1
COL3A1
COL1A1
EGR1
β-actin
250 kDa
180 kDa
130 kDa
100 kDa
40 kDa
MOCK
VEC
OE-EGR1


COL3A1
β-actin---Figure S2 E
BAX
C-Caspase-3
β-actin---Figure S2 H
P21


Figure S2 E
C-Caspase-3
BCL2
BAX
β-actin
15 kDa
25 kDa
15 kDa
40 kDa
MOCK
VEC
OE-EGR1


Figure S2 H
P21
β-actin
20
40
MOCK
VEC
OE-EGR1

Supplementary Table 1. Clinical and demographic information of HSCR patients enrolled in this study.

A. Individual patient data

Patient ID	Sex	Age at surgery (months)	HSCR subtype
1	F	12	Short-segment (S-HSCR)
2	M	6.8	Short-segment (S-HSCR)
3	M	12	Short-segment (S-HSCR)
4	M	8.5	Short-segment (S-HSCR)
5	F	14	Short-segment (S-HSCR)
6	M	4.2	Short-segment (S-HSCR)
7	F	18	Short-segment (S-HSCR)
8	F	1	Short-segment (S-HSCR)
9	M	19	Short-segment (S-HSCR)
10	F	11.7	Short-segment (S-HSCR)
11	M	4.3	Short-segment (S-HSCR)
12	M	11.5	Short-segment (S-HSCR)
13	M	2.2	Long-segment (L-HSCR)
14	M	7.2	Short-segment (S-HSCR)
15	M	4	Short-segment (S-HSCR)
16	M	1	Short-segment (S-HSCR)

B. Cohort summary

Variable	Value
Total enrolled patients (n)	16
Sex — Male, n (%)	11 (68.8%)
Sex — Female, n (%)	5 (31.2%)
Sex ratio (M : F)	11 : 5 ≈ 2.2 : 1
Age at surgery, months (mean ±	8.59 ± 5.70

SD)	
Age at surgery, months (median, range)	7.85 (1–19)
Short-segment HSCR (S-HSCR), n (%)	15 (93.8%)
Long-segment HSCR (L-HSCR), n (%)	1 (6.2%)
Diagnostic confirmation	Contrast enema + anorectal manometry + intraoperative histopathology
Surgical procedure	Trans-anal pull-through (Soave / Swenson / Duhamel approach)
Tissue sampling	Paired ganglionic (G), transitional (T), aganglionic (A) colonic segments
Patients with paired G/T/A tissues used in quantitative analyses	n = 16 (entire enrolled cohort; used for Fig 1B–C and Fig 5B Western-blot densitometry, and Sup Fig 2H qRT-PCR)
IRB / ethics approval	KYLL-2020(KS)-076, Qilu Hospital of Shandong University
Informed consent	Written, obtained from each legal guardian

Supplementary Table 2. siRNAs, viral constructs and plasmids used in this study.

Category	Item	Target insert	Sequence construct details	Vendor source	Working condition
siRNA	CDKN1A siRNA	Human CDKN1A	5'- GAUGGA ACUUCGACUUUGU- 3'; 5'- ACAAAGUCGAAGUUCCAUC -3'	boshang biotech jinan	50 nM, Lipofectamine 3000
siRNA	Nontargeting control (NC-siRNA)	—		boshang biotech jinan	50 nM
Adenovirus	Ad-Egr1	Mouse Egr1 (full length) + GFP	CMV promoter / backbone	Keyanyun biotech	MOI 500, 12 h
Adenovirus	Ad-GFP (control)	GFP only	CMV promoter / backbone	Keyanyun biotech	MOI 500, 12 h
Lentivirus	LV-EGR1	Human EGR1 (full length),	CMV transfer vector	Keyanyun biotech	MOI 20, 8 μg/mL polybrene, 12–

		puromycin cassette			16 h; puromycin 4 μg/mL × 3 d
Lentivirus	LV-vector (control)	Empty transfer vector, puromycin cassette	CMV	Keyanyun biotech	As above
Packaging plasmids	psPAX2; pMD2.G	—	Standard 2nd-generation packaging	Keyanyun biotech	Cotransfection in HEK293T
Expression plasmid	pcDNA3.1-EGR1	Human EGR1	CMV	Keyanyun biotech	Lipofectamine 3000
Expression plasmid	pcDNA3.1 (empty)	—	—	Keyanyun biotech	Lipofectamine 3000
Reporter	pGL3-CDKN1A-WT-Luc	Human CDKN1A promoter, −2,089 to −1	KpnI/NheI into pGL3-Basic; full sequence in Supplementary Methods	boshang biotech jinan	Sanger verified

Reporter	pGL3-CDKN1A-mut1-Luc	As above, GC-rich SP1/EGR1 site mutated	As pGL3-CDKN1A-WT-Luc, with the GC-rich region spanning nucleotides 1,894–1,970 of the cloned fragment mutated. Forty-seven nucleotides were substituted in three discontinuous blocks, converting the predicted SP1/EGR1 GC-box motifs to AT-rich sequence while leaving the intervening 30 nt and both flanks unchanged (GC content of the window 88% → 43%): block 1 (1,894–1,911) CCCCGGGGAGGGCGGTCC → AAAATTTTCTTTATTGAA; block 2 (1,938–1,951) GTCCCGCCTCCTTG → TGAAATAAGAAGGT; block 3 (1,956–1,970)	boshang biotech jinan	Sanger verified

			GGGCCCGGGCGGGGC → TTTAAATTTATTTTA. Constructed by seamless cloning on the wild-type backbone; the entire KpnI–NheI insert was Sanger-verified. Custom synthesis, Jinan Boshang Biotechnology Co., Ltd., project nos.G0393410-1 (WT) and G0393410-2 (MUT).		
Internal control	pRL-TK	Renilla luciferase	—	boshang biotech jinan	Cotransfected in every well

Supplementary Table 3 qRT-PCR primers used in this study.

Species	Gene	Description	Forward (5'→3')	Reverse (5'→3')
Human	EGR1	Early growth response 1	GGTCAGTGGCCTAGTGAGC	GTGCCGCTGAGTAAATGGGA
Human	FN1	Fibronectin 1	CGGTGGCTGTCAGTCAAAG	AAACCTCGGCTTCCTCCATAA
Human	COL1A1	Collagen type I, alpha 1	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Human	COL3A1	Collagen type III, alpha 1	GGAGCTGGCTACTTCTCGC	GGGAACATCCTCCTTCAACAG
Human	CDKN1A	Cyclin-dependent kinase inhibitor 1A	TGTCCGTCAGAACCCATGC	AAAGTCGAAGTTCCATCGCTC
Human	GLB1	Galactosidase beta 1	TATACTGGCTGGCTAGATCACTG	GGCAAAATTGGTCCCACCTATAA
Human	ACTB	Beta-actin (reference)	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
Mouse	Egr1	Early growth response 1	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTCGCTCGG

Mouse	Fn1	Fibronectin 1 (newly designed)	AAATCGTG CAGCCTCA ATCC	GGCTTGCTCTCGC AGTTAAA
Mouse	Col1a1	Collagen type I, alpha 1	TCCCTGGA ATGAAGGG ACAC	CTCTCCCTTAGGA CCAGCAG
Mouse	Col3a1	Collagen type III, alpha 1	CTGTAACA TGGAAACT GGGGAAA	CCATAGCTGAAC TGAAAACCACC
Mouse	Cdkn1a	Cyclin-dependent kinase inhibitor 1A	CCTGGTGA TGTCCGAC CTG	CCATGAGCGCAT CGCAATC
Mouse	Glb1	Galactosidase beta 1	GCACGGCA TCTATAAT GTCACC	GTATCGGAATGG CTGTCCATC
Mouse	Actb	Beta-actin (reference)	GTGACGTT GACATCCG TAAAGA	GCCGGACTCATC GTACTCC

Supplementary Table 4. Antibodies used in this study

Application	Antibody / Target	Host	Vendor	Catalog No.	Working dilution
WB	EGR1	Rabbit	Cell Signaling Technology	4154	1:5000
	COL1A1	Mouse	Proteintech	66761-1-Ig	1:5000
	COL3A1	Rabbit	Proteintech	22734-1-AP	1:5000
	P21	Rabbit	Affinity Biosciences	AF6290	1:1000
	Cleaved Caspase-3	Rabbit	Cell Signaling Technology	9661	1:1000
	BAX	Rabbit	Affinity Biosciences	AF6139	1:1000
	BCL-2	Rabbit	Affinity Biosciences	AF3498	1:1000
	HRP-anti-rabbit IgG	Donkey	Boster	BA1054	1:5,000
	HRP-anti-mouse IgG	Donkey	Boster	BA1051	1:5,000
	β-actin	Mouse	Proteintech	66009-1-Ig	1:10,000
IF / IHC	VIM	Rabbit	Proteintech	10366-1-AP	IF 1:200; IHC 1:200
	FN1	Mouse	Proteintech	66042-1-Ig	IF 1:200; IHC 1:200
	p75NTR (NGFR)	Rabbit	Abcam	ab52987	IF:1:200

	NESTIN	Rat	Abcam	ab81462	IF:1:100
	SOX10	Rabbit	Boster	BM4662	IF:1:200
	TUJ1	Mouse	Proteintech	66375-1-Ig	IF:1:200
	GFAP	Rabbit	Proteintech	16825-1-AP	IF:1:200
	Ki67	Mouse	Boster	M00254-1	IF:1:200
	COL1A1	Mouse	Proteintech	66761-1-Ig	IHC 1:200
	COL3A1	Rabbit	Proteintech	22734-1-AP	IHC 1:200
	EGR1	Rabbit	Proteintech	55117-1-AP	IF 1:200; IHC 1:200
IF	Alexa 488 anti-rabbit IgG	Goat	Proteintech	RGAR002	1:200
	Alexa 594 anti-rabbit IgG	Goat	Proteintech	RGAR004	1:200
	Alexa 488 anti-mouse IgG	Goat	Proteintech	RGAM002	1:200
	Alexa 594 anti-mouse IgG	Goat	Proteintech	RGAM004	1:200

	FITC anti-rat IgG	Goat	Proteintech	SA00003-11	1:100
IHC	HRP-anti-rabbit IgG	Goat	Proteintech	SA00001-2	1:500
	HRP-anti-mouse IgG	Goat	Proteintech	SA00001-1	1:500
CUT&RUN	EGR1	Rabbit	Cell Signaling Technology	55117-1-AP	1:50
CUT&RUN	Rabbit IgG	Rabbit	Proteintech	98136-1-RR	1:50

Supplementary Table 5 Experimental models, experimental units and replication for each assay.

Assay	Model	Groups compared	Experimental unit	Biological replicates (n)	Technical replicates	Read-out
Bulk RNA-seq (discovery)	HSCR patient colon	G / T / A	Patient (paired segments)	3 patients (9 samples)	1 library per sample	DEGs, paired DESeq2 (~ patient + segment)
qRT-PCR; Western blot	HSCR patient colon	G / T / A	Patient	16 paired patients	3	Relative mRNA / protein
IHC; SA-β-gal (tissue)	HSCR patient colon	G / T / A	Patient	3 patients	—	Qualitative evaluation of representative sections (no scoring)
qRT-PCR; Western blot; IF; EdU; Annexin V/PI; SA-β-gal	Primary mouse EMFs	MOCK / VEC / OE-EGR1	1 dam = 1 independent isolation	3 isolations (3 dams; 10 embryos per dam)	3 (wells / fields)	As per assay
Transwell migration	Primary mouse	MOCK / VEC / OE-EGR1	1 dam = 1 isolation (each	3	3 inserts × 3 fields	Migrated cells per field

	ENCCs + EMFs		cell type)			
CUT&RUN-qPCR	Primary mouse EMFs	IgG / VEC / OE-EGR1	1 dam = 1 isolation	3	3	Fold change over input
qRT-PCR; Western blot; EdU; Annexin V/PI; SA-β-gal	HFL-1	MOCK / VEC / OE-EGR1	Independent transduction	3	3	As per assay
RNA-seq	HFL-1	MOCK / VEC / OE-EGR1	Independent transduction	3	1 library per sample	DEGs, DESeq2 (~ group)
Transwell; wound healing	SH-SY5Y + HFL-1	MOCK / VEC / OE-EGR1	Independent transduction	3	3 inserts × 3 fields; 3 wounds	Migrated cells; % closure
CDKN1A knockdown assays	HFL-1	VEC / OE-EGR1 / OE-EGR1 + KD / KD	Independent transfection	3	3	As per assay
Dual-luciferase	HEK293FT	WT or MUT	Independent	3	3	Firefly / Renilla

reporter	reporter × VEC or EGR1	transfection

n denotes independent biological replicates. Each biological replicate was measured in technical triplicate and the mean of the technical measurements was used for statistical analysis; wells, inserts and fields were therefore not treated as independent observations. G, ganglionic; T, transitional; A, aganglionic; EMFs, embryonic intestinal fibroblasts; ENCCs, enteric neural crest cells; KD, CDKN1A knockdown; IF, immunofluorescence; IHC, immunohistochemistry; WT/MUT, wild-type / mutant CDKN1A promoter reporter.