Blocking endothelin-1-receptor/β-catenin circuit sensitizes to chemotherapy in colorectal cancer

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Colorectal cancer (CRC) is one of the main causes of tumor-related mortality worldwide and its therapy mainly relies on the use of conventional chemotherapeutic drugs. 1 Most CRC carry mutations leading to overactivation of the β-catenin pathway. 2 CRC stem-like cells (CRC-SC) have been shown to be responsible for tumor propagation, metastasis and resistance to conventional anticancer drugs. 3–12 An accurate understanding of the cross-talk between signaling in CRC-SC could allow the development and the clinical use of effective therapies to enhance CRC drug sensitivity. In CRC-SC β-catenin signaling pathway promotes tumor growth, and progression by sustaining stem cell expansion. 11–14 A key property of CRC-SC with activated β-catenin signaling is the protection against conventional chemotherapeutics. 2,15 The molecular mechanisms responsible of the fine-tuning of β-catenin-mediated stemness and drug response remain largely unexplored but represent a potentially promising area for novel therapeutic interventions. Therefore, drugging upstream signaling molecules endowed with functions that regulate β-catenin activity may represent a novel approach to sensitize to chemotherapy. In this context, endothelin-1 (ET-1) elicits pleiotropic effects in tumor cells and in the host microenvironment, modulating the epithelial-to-mesenchymal transition (EMT), the expansion of vascular network and immune response. 16 ET-1 acts through autocrine and paracrine signaling by binding two distinct receptors: endothelin A (ET AR) and B (ET BR), which belong to the G-protein-coupled receptor family. The activation of ET-1/ET AR axis is recognized as a common mechanism underlying the progression of various solid tumors, including CRC. 16–18 In CRC, the components of the ET-1 system are expressed not only by tumor cells, but also by microenvironmental elements, such as fibroblasts, endothelial cells and macrophages. 17–26

Increased levels of ET-1 have been detected in plasma and tissue samples from patients with CRC. 19,20 Moreover, in CRC the ET-1 gene regulation is directly upregulated by β-catenin. 27 Microarray molecular profiling and real-time PCR on CD133 + fractions of CRC lines revealed that ET-1 transcripts are highly expressed compared with CD133 − counterparts. 28 A preclinical study in CRC demonstrated that zibotentan, a selective ET AR antagonist, suppressed tumor growth and progression indicating a potential role of this antagonist as adjuvant therapy. 29 More recently in CRC cells, it has been demonstrated that overexpression of the ET-1 gene is a determinant of acquired resistance to MEK and PI3K inhibitors. 30 However
the molecular mechanism of ET-1 axis to protect against drugs 
treatment in CRC remains elusive.

In our recent studies, we reported that ET-1/ET_{\alpha}R axis,
through the contribution of the scaffold protein β-arrestin1
(β-arr1), appears to be critical in the signaling cross-talk,
providing a mechanism of escape to a new less-adverse 
niche, in which evasion of drug-induced apoptosis ensures cell 
survival required for tumor progression.\textsuperscript{16,31–35} In this regard,
ET-1 has been recently identified as a key component that 
sustains maintenance and clonal expansion of cardiovascular 
stem cells population.\textsuperscript{36} Moreover, a recent report suggests 
that β-arr1 has an important protective role, reducing the 
chemotherapy-induced intestinal stem cell apoptosis.\textsuperscript{37} To 
dissect the intricate interplay between ET-1R/β-arr1 and 
β-catenin, here we report a ET-1R/β-arr1-mediated epigenetic 
mechanism in regulating β-catenin signaling to promote EMT 
and protection against chemotherapy. Understanding whether 
targeting ET-1R/β-arr1 connected with β-catenin pathway 
in CRC-SC can restore sensitivity to chemotherapy, is thus 
essential to develop more effective strategies in this 
malignancy.

Results

Expression of ET-1R/β-arr1 axis in CRC-SC. We first 
evaluated the expression of ET-1 axis in CRC-SC features, 
by using a panel of patient-derived CRC-SC isolated from 
human tumor samples (CC09, CSC5, CSC2 and CSC1), all 
carrying APC gene mutations.\textsuperscript{3,10,12} The mRNA and protein 
expression analysis showed that all CRC-SC expressed ET-1 
and its receptors, ET_{\alpha}R and ET_{\beta}R, and released high levels 
of ET-1, within the physiologically range needed for the 
activation of ET-1R in an autocrine manner\textsuperscript{38} (Figures 1a–c).
To explore the role of β-arr1 to create intracellular signaling 
cross-talk upon ET-1R activation, we first evaluated β-arr1 
expression in CRC-SC. These cells expressed β-arr1 both at 
mRNA and protein levels (Figures 1a and b). After ET-1 
stimulation, we observed an increase of CRC-SC viability, 
which was reduced after treatment with macitentan, a potent 
ET_{\alpha}R antagonist with significant affinity for ET_{\beta}R. The same 
effect was observed after β-arr1 silencing (Figure 1d and 
Supplementary Figure S1a and S2). When cultured in stem 
cell medium, CRC-SC grow in large round, unattached 
floating spheroid colonies (sphere). As shown in Figure 1e, 
whereas the ET-1 addition enhanced the CRC-SC sphere 
formation ability, increasing not only the sphere number but 
also the size, macitentan treatment negatively affected the 
stemness property of CRC-SC, indicating that the ET-1R/β- 
arr1 axis sustains CRC-SC features.

ET-1R/β-arr1-driven EMT and invasive behavior in 
CRC-SC. Next, we analyzed whether the features of 
CRC-SC driven by ET_{\alpha}R/β-arr1 were associated 
with molecular changes consistent with EMT.\textsuperscript{39–44} To this end 
we examined the expression of the epithelial marker 
E-cadherin and its transcriptional repressors, Twist and Snail, 
as well as the expression of mesenchymal markers, such as 
N-cadherin and vimentin. Upon ET-1 stimulus, we observed 
an increase of N-cadherin, vimentin, Snail and Twist 
expression levels, associated with a concomitant decrease 
in E-cadherin expression (Figures 2a and b). In addition, as a 
result of macitentan treatment or β-arr1 silencing, restored 
E-cadherin expression and inhibited N-cadherin, Snail and 
Twist expression were observed (Figure 2b). Concordantly, 
the analysis of mRNA levels upon ET-1 stimulation showed 
the increased expression levels of Snail and N-cadherin and 
the decrease of E-cadherin that were reverted following 
macitentan treatment (Figure 2c). Remarkably, macitentan, 
or the β-arr1 knockdown, inhibited the ET-1-induced suppres-

dition of E-cadherin promoter activity (Figure 2d) and induction 
of Snail promoter activity (Figure 2e). Next, we evaluated 
whether the ET_{\alpha}R/β-arr1-driven acquisition of EMT pheno-
type correlates with expression and activation of the 
proteolytic enzymes, matrix metalloproteases (MMP), and 
with an increase of invasive potential of CRC-SC. As shown 
in Figures 2f and g, the ET-1-induced MMP-2 and -9 secretion 
and activation were inhibited by macitentan treatment, as well 
as β-arr1 silencing, as determined by immunoblotting and 
zymography. In this context, as a result of enhanced 
proteolytic activity of CRC-SC upon ET_{\alpha}R/β-arr1 signaling 
activation, when CRC-SC were silenced for β-arr1 or were 
treated with macitentan, the ET-1-induced cell invasion was 
significantly impaired compared with control cells (Figure 2h), 
providing evidence that ET_{\alpha}R/β-arr1 axis activation may be a 
critical event to drive EMT and invasive behavior in CRC-SC.

ET-1R/β-arr1 axis links β-catenin pathway in CRC- 
SC. The β-catenin signaling pathway represents an hallmark 
of CRC-SC governing the maintenance of stemness.\textsuperscript{2,11–15} We 
observed that ET-1 stimulation induced the β-catenin 
nuclear translocation, an effect that was inhibited upon 
macitentan treatment (Figure 3a), enhancing the levels of 
serine/threonine non-phosphorylated β-catenin active form 
(Supplementary Figure S3a). We analyzed the nucleocyto-
plasmic shuttling of both β-arr1 and β-catenin in the cytosolic 
and nuclear extracts of CRC-SC upon different times of ET-1 
stimulation. ET-1 induced a time-dependent nuclear translo-
cation of both proteins with a peak after 30 and 60 min of ET-1 
challenge (Figure 3b). Of relevance, this effect was strongly 
reduced upon macitentan treatment and β-arr1 silencing 
(Figure 3c). Moreover, we analyzed β-arr1 and β-catenin 
potential physical interaction in the nucleus, by using co-
immunoprecipitation (IP) assays. β-arr1 bound to β-catenin 
in the nuclei of ET-1-treated cells (Figure 3d), and this 
interaction was inhibited by macitentan, suggesting that 
ET-1R/β-arr1 links β-catenin in the nucleus of CRC-SC that 
is hampered by ET-1R blockade. Consistent with these 
results, the transcriptional activity of β-catenin, induced by 
ET-1 in CRC-SC, was inhibited after treatment with maci-
tentan, or by β-arr1 silencing (Figure 3e and Supplementary 
Figure S3b), indicating that ET-1/β-arr1/β-catenin interaction 
is necessary for inducing β-catenin/T cell factor 4 (TCF4) 
transcriptional activity. Given that ET-1 is also a downstream 
target gene of β-catenin/TCF4,\textsuperscript{27,31,32} by using a reporter 
plasmid with ET-1 promoter sequence, containing a functional 
TCF binding element (TBE), we demonstrated that ET-1 
promoter activity was significantly upregulated after ET-1 
stimulation and inhibited when the cells were treated with 
macitentan, or silenced for β-arr1 (Figure 3f). Next, through
ET-1 was accompanied with phosphorylation of both p42/44MAPK and Akt, and macitentan treatment, or the specific β-arr1 silencing, significantly decreased the ET-1-mediated MAPK and Akt activation (Figure 4a and Supplementary Figure S4a). Because a typical property of CRC-SC is the resistance to treatment with standard chemotherapeutic agents, as oxaliplatin (OX) and 5-fluorouracil (5-FU), we evaluated the response of CRC-SC following exposure to OX and 5-FU at clinically relevant doses,10 and found that these cells were poorly responsive to chemotherapeutic drugs-induced apoptosis even at the highest concentration used (Figure 4b). The treatment with macitentan or the loss of β-arr1 in a combination regimen with OX or 5-FU, induced a more effective reduction of cell vitality (Figure 4c and Supplementary Figure S4b). Furthermore, CRC-SC that overexpressed exogenous β-arr1-FLAG showed a greater poor sensitivity to OX or 5-FU, compared with parental cells (Figure 4d and Supplementary Figure S1b), suggesting that ETAR/β-arr1 blockade might sensitize CRC-SC to OX and 5-FU-induced apoptosis. Mechanistically, we found that the combined treatment of macitentan with chemotherapeutic agents, resulted in an enhanced expression of the poly ADP-ribose polymerase (PARP) cleaved form (Figure 4e and Supplementary Figure S4c). In addition, we found that ET-1 increased the expression of the prosurvival factor Bcl-XL, induced apoptosis even at the highest concentration used (Figure 4f). This implies that the activation of ET-1/β-arr1 survival signaling is involved in the protection of CRC-SC against chemotherapeutics, suggesting that

Macitentan hampers ET-1/β-arr1/β-catenin axis in CRC-SC. ETAR/ETBR and β-arr1 survival signaling is involved in the protection of CRC-SC against chemotherapeutics, suggesting that

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Figure 2  ET-1R/β-arr1 axis drives EMT process in CRC-SC. (a) Lysates from CC09 cells were analyzed by IB for the expression of epithelial (E-cadherin) and mesenchymal (N-cadherin, Snail, and vimentin) markers after ET-1 (100 nM) stimulation for 24 h. Tubulin was used as loading control. (b) Lysates from CC09 cells transfected with SCR or si-β-arr1 and treated for 24 h with ET-1 (100 nM) and/or MAC (1 μM) were analyzed by IB for the expression of E-cadherin, N-cadherin Snail and Twist. Tubulin was used as loading control. (c) Snail, E-cadherin, and N-cadherin expression in CC09 cells upon ET-1 (100 nM) and/or MAC (1 μM) treatment evaluated by qPCR, normalized using endogenous cyclophilin-A. Values are shown as mean ± S.D. from three independent experiments repeated in triplicates (*P < 0.002 versus CTR; **P < 0.005 versus ET-1). (d) E-cadherin promoter activity and Snail promoter activity (e) evaluated in CC09 cells transfected with SCR or si-β-arr1 and treated for 24 h with ET-1 (100 nM) and/or MAC (1 μM). Values are shown as mean ± S.D. from three independent experiments repeated in triplicates (*P < 0.01 versus CTR; **P < 0.001 versus ET-1 in SCR-transfected cells). (f) Lysates from CC09 cells transfected with SCR or si-β-arr1 and exposed to ET-1 (100 nM) and/or MAC (1 μM) for 24 h were analyzed for MMP-2 and -9 by IB. Tubulin was used as loading control. (g) Conditioned media collected from CC09 treated as in (f) were used to determine the secretion and activity of MMP-2 and -9 by gelatin zymography. (h) Cell invasion assay of CC09 cells transfected with SCR or si-β-arr1 and exposed to ET-1 (100 nM) and/or MAC (1 μM) for 24 h. Values are shown as mean ± S.D. from three independent experiments repeated in triplicates (*P < 0.002 versus CTR; **P < 0.001 versus ET-1 in SCR-transfected cells). Representative images of invading cells were shown in the right panel.

molecular targeting of ET-1R in CRC-SC might improve the efficacy of chemotherapeutic regimens.

ET-1R blockade inhibits tumor growth and restores sensitivity to chemotherapeutic drugs in CRC-SC patient-derived xenografts. To verify whether ET-1R blockade by macitentan would also affect tumor growth in vivo, patient-derived CC09 and CSC5 CRC-SC were injected into the flank of mice and were allowed to grow until they reached a detectable size. The xenografts obtained were confirmed as colorectal adenocarcinoma, as showed by hematoxylin–eosin staining in the representative sections from tumor xenografts (Figure 5a and Supplementary Figure S5a). Next, we tested the effect of macitentan alone or in combination with chemotherapy in CRC-SC xenografts. After the appearance of palpable tumors (day 14 for CC09 xenografts, and day 42 for CSC5 xenografts), mice were randomized into different groups of ten mice undergoing the following treatments for 4 weeks: (i) vehicle (control), (ii) macitentan (30 mg/Kg/oral daily), (iii) OX (0.25 mg/Kg/i.p. once a week) or 5-FU (15 mg/Kg/i.p. daily), (iv) macitentan plus OX or 5-FU. These treatments, were generally well tolerated without any loss of weight or detectable signs of acute or delayed toxicity. At the end of 4 weeks of treatment, tumor size of mice treated with macitentan significantly decreased compared with vehicle-treated mice (~60% for CC09 xenografts; 55% for CSC5 xenografts, P < 0.02), demonstrating the therapeutic potential of macitentan in controlling tumor growth. Most importantly, a synergistic growth-inhibitory effect, as calculated by Chou and Talalay method, was observed when macitentan was used in combination with OX, compared with macitentan- or OX-treated mice (90 versus 68% or 12% respectively, for CC09; 84 versus 55% or 49% respectively, for CSC5 xenografts; P < 0.05) or in combination with 5-FU compared with macitentan- or 5-FU-treated mice (91 versus 50% or 10% respectively, for CC09 xenografts; P < 0.05, Figure 6), thus proving the ability of macitentan to sensitize CRC-SC xenografts to different chemotherapeutic drugs. Moreover, the therapeutic effects of macitentan were long-lasting, both in monotherapy or combination with chemotherapeutic drugs, for up to 2/3 weeks after termination of treatments. Indeed, we still found a persisted tumor growth reduction even during the treatment-free period in mice treated with macitentan compared with vehicle-treated mice (46% at day 63, for CC09 xenografts, Figure 5a; 40% at day 55 for CC09 xenografts, Figure 6; 61% at day 77 for CSC5 xenografts, Supplementary Figure S5a; P < 0.02), and a persistent synergistic inhibitory effect in combinatorial regimes (91 versus 46% for macitentan or 37% for OX, for CC09 xenografts; 89 versus 40% for...
macitentan or 20% for 5-FU, for CC09 xenografts, and 83 versus 61% for OX, for CSC5 xenografts; *P<0.05). In parallel, immunoblotting analysis of tumor xenografts of mice co-treated with macitentan and OX showed a marked effect in reducing MAPK and Akt activation, and a reversion of EMT effectors compared with controls (Figures 5b and c and Supplementary Figures S5b and c). These findings indicate that blockade of ET-1R with macitentan, in combination with chemotherapeutic drugs, controls EMT aggressive phenotype of CRC-SC, thus increasing sensitivity to the chemotherapy and promoting tumor regression.

Discussion

The presence of stem cell-like cells has been recognized as the main cause of failure in the treatment of several malignancies.5,6 It is therefore evident that a therapeutic approach to target CSC pool could be more effective to eradicate tumor. Drug sensitivity is frequently associated with dysregulation of a signaling network rather than of a single pathway. In the present study, we provide evidence that ET-1R/β-arr1 links β-catenin signaling to sustain CRC-SC features, also through the amplification of ET-1 autocrine loop, outlining a model in which ET-1 interlinks β-catenin signaling to support progression and recurrence of CRC. The findings presented here reveal that ET-1R/β-arr1 axis has a critical role in CRC-SC signaling and chemoprotection. Notably, ET-1R blockade by macitentan markedly affected the signaling cross-talk mediated by β-arr1 involved in the maintenance of CRC-SC and drug response. Specifically, ET-1R blockade results in a strong decrease of survival Akt/MAPK signaling pathways,45,46 with the consequent reduction of cell growth.45,46
**Figure 4** ET-1R blockade sensitizes CRC-SC to standard chemotherapeutic drugs. (a) Lysates from CC09 cells treated for 1 h with ET-1 (100 nM) and/or MAC (1 μM) and transfected with SCR or with si-β-ar1 were immunoblotted with anti-pp42/44MAPK, anti-p42/44MAPK, anti-pAkt and anti-Akt. (b) Effect of exposure to different concentrations of oxaliplatin (OX) and 5-fluorouracil (5-FU) after 24 h on cell vitality of CC09 cells. (c) Time-dependent effect of treatment with MAC (1 μM) or OX (100 μM) or 5-FU (50 μg/ml) alone and combination, for 24 h on cell growth of CC09 cells transfected with SCR or with si-β-ar1. Values are shown as mean ± S.D. from three independent experiments repeated in triplicates (*P<0.001 versus CTR; **P<0.001 versus OX; ***P<0.002 versus 5-FU). (d) Effect of treatment with OX (100 μM) or 5-FU (50 μg/ml), for 24 h on cell growth of CC09 cells transfected with empty vector (Mock) or with β-ar1-FLAG. Values are shown as mean ± S.D. from three independent experiments repeated in triplicates (*P<0.001 versus CTR; **P<0.002 versus chemotherapy-treated cells). (e) IB analysis of PARP cleaved form (cl PARP) in CC09 cells treated for 24 h with MAC (1 μM) or OX (100 μM) or 5-FU (50 μg/ml) alone and combination. Tubulin was used as loading control. (f) IB analysis of Bcl-XL in CC09 cells treated for 24 h with ET-1 (100 nM) or MAC (1 μM) or OX (100 μM) alone and combination. Tubulin was used as loading control.

**Figure 5** ET-1R blockade by macitentan inhibits tumor growth and restores sensitivity to oxaliplatin in CRC-SC xenografts. (a) CC09 cells (5 x 10^5) were injected s.c. into the flank of nude mice. When tumors were detected, mice were treated with vehicle (CTR), or MAC (30 mg/Kg/oral daily), or OX (0.25 mg/Kg/i.p. once a week), or MAC (30 mg/Kg/oral daily) with OX (0.25 mg/Kg/i.p. once a week) combination for 4 weeks. The comparison of the time course of tumor growth curves by two-way ANOVA with group-by-time interaction for tumor growth was statistically significant (P<0.02). Data points, averages ± S.D. The upper panels represented the hematoxylin–eosin staining of transplanted tumor xenografts (scale bar, 50 μm) or the images of tumors from each treatment group. (b) Expression of pp42/44MAPK, p42/44MAPK, pAkt and Akt as evaluated by IB on total extracts from tumors of CC09 xenografts. (c) E-cadherin, N-cadherin, Snail and vimentin, evaluated by IB on total extracts from tumors of CC09 xenografts.
Figure 6 Macitentan inhibits tumor growth and restores sensitivity to 5-FU in CRC-SC xenografts. CC09 cells (5 x 10^5) were injected s.c. into the flank of nude mice. When tumors were detected, mice were treated with vehicle (CTR), or MAC (30 mg/Kg/oral daily), or 5-FU (15 mg/Kg/l.p daily), or MAC + 5-FU (15 mg/Kg/oral daily) for 4 weeks. The comparison of the time course of tumor growth curves by two-way ANOVA with group-by-time interaction for tumor growth was statistically significant (P < 0.05). Data points, averages ± S.D.

Materials and Methods
Materials. ET-1 was (Bachem, Bubendorf, Torrance, Switzerland) and it was used at 100 nM and incubated with the cells for the indicated times. Macitentan (MAC), also N-(5-[4-bromophenyl]-6-[2-[5-bromopyrimidin-2-yl]thiophen-2-yl]pyrimidin-4-yl)-N-propylsulfamide, (Actelion Pharmaceuticals, Allschwil, Switzerland). Pre-treatment of cells with MAC was used at the concentration of 1 μM for 30 min prior to the addition of ET-1. Oxaliplatin (SUN PHARMA, Goregaon (E), Mumbai) used at 100 μM and Fluorouracil (5-FU) (TEVA, Petach Tikva, Israel), used at 50 μg/ml.

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Cell culture. The patient-derived CRC-SC enriched cultures (CC09, CSC5, CSC2 and CSC1), isolated from human tumor samples and capable to reproduce a histological copy of the original patient tumor when inoculated in immunocompromised mice, were kindly provided by the Istituto Superiore di Sanità (ISS) CSC biobank.3,10,12 Cells were cultured in ultra-low attachment plates and maintained in stem cell medium (DMEM/F12, supplemented as reported in ref. 10) and monthly tested for mycoplasma contamination. Genomic analysis of CRC-SC showed that CC09 carried mutant BRAF, PIK3CA, APC, TP53 and SMAD4; CSC5 carried mutant KRAS, PIK3CA, APC and SMAD4; CSC2 carried mutant KRAS, APC, TP53 and SMAD4; CSC1carriedmutant PIK3CA, APC and SMAD4 (ref. 3) and unpublished results.

Immunoblotting analysis. Whole-cell lysates of CRC-SC were prepared using a ice-cold modified RIPA buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing a mixture of protease and phosphatase inhibitors or NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific, Waltham, USA) to separate cytoplasmic and nuclear fractions. Protein content of the extracts was determined using protein assay kit (Bio-Rad, Hercules, CA, USA) and resolved by SDS-PAGE. Immunoblotting of Abs specific for β-arr1 (K-16: sc-9182), β-Catenin (E-5: sc-7963), tubulin (DM1A: sc-32293), MMP-2 (K-20: sc-8835), MMP-9 (M-17: sc-6841), Snail (E-18: sc-10432), vimentin (C-20: sc-7557), Histone H3 (C-16: sc-6854), PCNA (F-2: sc-25280), (Santa Cruz Biotechnology Dallas, TX, USA), HSP-70 (Enzo Life Sciences, New York, USA, ADI-SPA-812), Twist (ab50581), ETαR (ab178454), ETβR (ab39960) (Abcam, Cambridge, UK), AKT (9227) pAKT (Ser-473) (9271), p44/42MAPK (9102), ppp2/4MAPK (4377), cleaved-PARP (9541), non-phospho (active) β- Catenin (Ser33/37/Thr41) (4270) (Cell Signaling, Beverly, MA, USA), N-cadherin (610920), E-cadherin (610181) (BD Biosciences, Franklin Lakes, NJ, USA) were detected using HRP-conjugated anti-mouse or anti-rabbit Abs (Pierce, 31460) and visualized by enhanced chemiluminescence detection system (ECL, Bio-Rad).

Immunoprecipitation analysis. Nuclear extracts were immunoprecipitated as previously described,21 using β-arr1, or non-specific immunoglobulin G (IgG) (Santa Cruz Biotechnology) and protein G-agarose beads (Thermo Fisher Scientific) at 4 °C overnight. Immunoprecipitates were resolved by SDS-PAGE and the proteins were detected by IB with the following Abs: β-Catenin, β-arr1 and Histone 3 (Santa Cruz Biotechnology). To obtain clean and specific IB signals of β-arr1 which run very close to heavy chain of IgG, we used HRP-conjugated protein A (Thermo Scientific, Waltham, MA, USA) instead of HRP-conjugated secondary Ab.

RNA silencing and transfection. For the silencing of β-arr1, the CRC-SC were transiently transfected with ON-TARGET plus Human ARRB1 siRNA-SMART pool, containing four different siRNAs targeting β-arr1 (Dharmacon, Lafayette, CO, USA). The ON-TARGET plus Control Non-targeting siRNA (Dharmacon) was used as negative control. For exogenous expression of β-arr1 we used pcDNA3-β-arr1 (Invitrogen, CA, USA), E-cadherin (610181) (BD Biosciences, Franklin Lakes, NJ, USA) were transiently co-transfected with ET-1 promoter reporter sequence, spanning −1300 to +230 bp surrounding the transcriptional initiation site and containing a functional TBE located at −73 to −67 bp, kindly provided by Dr. Z. Zhang (University of California San Diego School of Medicine, La Jolla, CA, USA). The release of ET-1 in media of CRC-SC was measured in triplicate on 96-well microtiter plates by using an ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to manufacturer’s instructions, with pGL3-SNA (−869/+59) construct (kindly provided by Dr. A. Garcia de Herreros, Instituto Municipal d’Investigació Mèdica, Universitat Pompeu Fabra, Barcelona, Spain) or with pGL2-Emc3 construct (kindly provided by Dr. E.R. Fearon, University of Michigan, Ann Arbor, MI, USA), respectively containing luciferase gene under the control of the human Snail and E-cadherin promoter, and with pMV-β-galactosidase vector (Promega, Madison, WI, USA). Transcriptional activity of β-catenin/TCF was evaluated by transient transfection of CRC-SC with TOP/Flash luciferase reporter, containing multiple TCF4 binding sites for β-catenin and FOP/Flash luciferase reporter (negative control) (results were expressed as the ratio of TOP/Flash over FOP/Flash activity) and pCMV-β- galactosidase vector. To measure the ET-1 promoter activity, the human CRC-SC, were transiently co-transfected with ET-1 promoter reporter sequence, spanning −1300 to +230 bp surrounding the transcriptional initiation site and containing a functional TBE located at −73 to −67 bp, kindly provided by Dr. Z. Zhang (University of California San Diego School of Medicine, La Jolla, CA, USA), and pCMV-β- galactosidase vector. Reporter activity was measured using the Luciferase assay system (Promega) and normalized to β-galactosidase activity.

Gelatin zymography. To detect MMP-9 and -2 activity in conditioned media, CRC-SC transiently transfected with si-β-arr1 or with negative control, were stimulated with ET-1 or MAC, alone or in combination for 24 h. Conditioned media was collected and concentrated by using Spin-X UF concentrator columns (Corning, New York, NY, USA), conditioned medium was separated by 9% SDS-PAGE gels containing 1 mg/ml gelatin. The gels were washed for 30 min at 22 °C in 2.5% Triton X-100 and then incubated in 50 mM Tris (pH 7.6), 1 mM ZnCl2, and 5 mM CaCl2 for 18 h at 37 °C. After incubation the gels were stained with 0.2% Coomassie Blue. Enzyme-digested regions were identified as white bands on a blue background and quantified by computerized image analysis of the band. Molecular sizes were determined from the mobility, using gelatin zymography standards (Bio-Rad Laboratories, Richmond, CA, USA).

Invasion assay. Chemoinvasion assays were carried out using modified Boyden Chambers consisting of transwell membrane filter inserts with 8 μm size polycarbonate membrane precoated with polymerized collagen placed in a 24-well plate (BD Biosciences). The human CRC-SC clones transiently transfected with si-β-arr1 or with negative control, were stimulated with ET-1 or MAC, alone or in combination with ET-1, added to the lower chamber. The cells were left to migrate for 24 h at 37 °C. Cells on the upper part of the membrane were scraped using a cotton swab and the migrated cells were stained using Diff-Quick kit (Merz-Dade). The experiment was performed in triplicates for all conditions described. From every transwell, several images were taken under a phase-contrast microscope at ×10 magnification and two broad fields were considered for quantification. The results of the analysis of the individual photos are depicted as dots in the various graphs, normalized to control and shown as fold of control.

ELISA. The release of ET-1 in media of CRC-SC was measured in triplicates on microtiter plate by using an ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA, USA) according to manufacturer's instructions.

Cell viability analysis. The human CRC-SC were seeded in triplicates, in 24-well plates. The cells were transiently transfected with si-β-arr1 or β-arr1-FLAG with or without specific negative control and treated with ET-1, MAC, OX and 5-FU, alone or in combination. After 24 or 48 or 72 h cell viability was determined by counting the
cells, for each time point, using a Neubauer-counting chamber and a bright field microscope. The trypan blue dye exclusion method was used to evaluate the percentage of viable cells.

Clonogenic assay. The CRC-SC tumor spheres were mechanically dissociated and the resulting cells were seeded at low densities (1000 cells/ml) in 12-well low adhesion plates at 1 ml per well, in stemness medium with or without ET-1 or MAC, alone or in combination. After 7 days of growth, the tumor spheres obtained were analyzed and quantified by a phase-contrast microscope at ×10 magnification.

CRC-SC xenografts. Athymic (nu/nu) mice, 5- to 6-week-old of age (Charles River Laboratories, Milan, Italy), were subcutaneously injected with 5x10⁵ viable CRC-SC, in 100 μl PBS/Matrigel (BD Biosciences) into the flank, following the guidelines of the Italian Ministry of Health. For all the CRC-SC xenografts were detectable within 2–6 weeks. Tumor xenografts were extracted, formalin-fixed, and paraffin-embedded. Haematoxylin-eosin-stained sections were subsequently evaluated by a pathologist in comparison with human tumors. For drug testing, 5x10⁵ viable CRC-SC, in 100 μl PBS/Matrigel, were subcutaneously injected into the flank of mice and were allowed to grow until they reached a detectable size. Drug treatments were started when tumors were detected, mice were randomized into different groups of 10 mice undergoing the following treatments for 4 weeks: (i) vehicle (control), (ii) macitentan (30 mg/Kg/oral daily), (iii) ET-1 or MAC, alone or in combination. After 7 days of growth, the tumor spheres in 12-well low adhesion plates at 1 ml per well, in stemness medium with or without Clonogenic assay. The percentage of viable cells, for each time point, using a Neubauer-counting chamber and a bright field microscope.

Statistical analysis. Statistical analysis was performed using Student’s t-test and Fisher’s exact test to compare in vitro experiments. The time course of tumor growth was compared across the groups using two-way ANOVA, with group and time as variables. All statistical tests were carried out using SPSS software (SPSS 11, SPSS Inc. Chicago, IL, USA). A two-sided probability value of <0.05 was considered statistically significant. The Wincoxon rank-sum test was used to analyze the gene expression obtained from TCGA of colon adenocarcinoma samples.

Conflict of Interest
The authors declare no conflict of interest.

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