A novel signaling pathway regulates colon cancer angiogenesis through Norrin

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Norrin binds to the frizzled-4 receptor, stimulating canonical Wnt signaling. We investigate here the role of colorectal cancer (CRC) produced Norrin in endothelial cell growth, motility, and blood vessel formation, as well as the expression of the Norrin signaling pathway components in the CRC tumor microenvironment. Norrin conditioned medium produced by CRC cell line CaCO2 transfected with Norrin expression construct increased endothelial cell motility. Blocking Norrin signaling reduced endothelial cell motility, branch point number (1/mm²), and the network length (mm/mm²) during in vitro angiogenesis. Colorectal tumors express Norrin protein. Endothelial cells in the colorectal tumor microenvironment contain all of the components of the Norrin signaling pathway needed to respond to Norrin protein. This study presents data that Norrin may play a role in the regulation of angiogenesis in the colorectal cancer tumor microenvironment.

Multiple signaling pathways regulate endothelial cells in the tumor microenvironment. While vascular endothelial growth factors (VEGF) and its family of receptors (VEGFR) are most well defined, there are multiple alternative angiogenic pathways involving fibroblast growth factor (FGF), Notch Delta-like ligand 4 (DLL4) and platelet-derived growth factors (PDGF). None of these pathways except VEGF are understood enough to allow for FDA approved therapy design.

Wnt signaling is also known to be an important regulator of angiogenesis and of other processes operative in the colorectal cancer tumor microenvironment. Wnt2 and Wnt5a are over expressed in colorectal tumors and the expression of frizzled (Fz) receptors is tightly regulated by secreted Wnt ligands. Wnt signaling contributes to tumor cell proliferation and inhibition of differentiation but is also a critical mediator of endothelial function. Wnt3a has been shown to induce endothelial cell proliferation and migration in the context of DVL3 phosphorylation. LEF1, a terminal component of the Wnt signaling cascade, directly influences endothelial cell invasion, and the pathway is basally activated in subconfluent endothelial cells.

Norrin, a non-Wnt ligand, binds selectively to Fz receptor subtype-4 (Fz4) and induces canonical Wnt signaling in conjunction with cell surface co-receptor LRP5. This ligand is involved in angiogenesis since overexpression in Norrin deficient mice induces the growth of ocular capillaries and inactivating mutations results in Norrie disease which is characterized by ocular vascular defects and impaired cochlear vascularization. Abnormalities in Fz4 or LRP5 result in defects in retinal vascular development and in a phenotypically similar condition, familial exudative vitreo-retinopathy (FEVR).

We have previously demonstrated the presence of Norrin, Fz4 and LRP5 mRNA in human tissue biopsies of colon cancer and speculated that Norrin signaling is involved in endothelial cell regulation in the tumor microenvironment. In this study, we demonstrate that Norrin is produced by human colorectal cancer cells, that it directly regulates endothelial cell proliferation and behavior and that all of the critical components necessary to respond to Norrin signals are expressed by endothelial cells in the tumor microenvironment.

Results

Effect of Norrin on endothelial cells in vitro. Utilizing an in vitro wound healing assay and EAhy926 cells, we demonstrate that Norrin blocking antibody significantly reduces endothelial cell migration, suggesting a basal dependence on Norrin signaling (Figure 1 A–C). The addition of supplemental Norrin promotes endothelial cell migration and motility (Figure 1 D–F). In the latter case, Norrin was provided by the addition of conditioned medium from CaCO2 colon cancer cells following transfection with a Norrin expression construct since this cell line is one of few colon cancer cell lines that does not basally produce Norrin. In an in vitro angiogenesis primary
endothelial cell culture model, exposure to Norrin blocking antibody reduces the branch point number (1/mm²) and reduces the endothelial cell network length (mm/mm²) during in vitro angiogenesis (Figure 2 and supplemental on-line video). These experiments are consistent with findings from reports that, in normal tissues, Norrin directly affects endothelial cell growth and function.11,12

Expression of Norrin protein in colorectal cancers and its blood vessels. Previously, we demonstrated that Norrin mRNA is expressed in colorectal tumors and the majority of CRC cell lines.14 It was shown in 2012 that Norrin is expressed in retina’s endothelial cells.16 The relatively low level of expression of the Norrin protein in most other tissues has made the detection of Norrin expression problematic. However, through the use of our sensitive double signal amplification system (see The Materials and Methods) we have been able to evaluate the expression of this protein in human colorectal tumors. Norrin protein is produced by colorectal tumors (Figure 3), and in the endothelial cells of tumor blood vessels (Figure 4, Row A). By this semi-quantitative technique, it appears that colorectal tumors express similar levels of Norrin protein as tumor blood vessel endothelial cells.

Expression of Norrin signaling pathway components in CRC endothelial cells. In order for endothelial cells in the tumor microenvironment to respond to Norrin, all functional receptor complex components must be expressed. The first component of Norrin signaling pathway is Frizzled receptor 4 (Fz4). This is the only Fz receptor to which Norrin protein binds, leading to activation, most prominently, of the canonical Wnt/beta-catenin pathway. Utilizing the same sensitive double signal amplification methodology used to detect Norrin expression, we demonstrate conclusively that CRC endothelial cells express Fz4 protein (Figure 4, row B). We previously demonstrated that the Lrp5 co-receptor was expressed in colorectal tumors, and cell lines, derived from the colorectal tumors.14 We demonstrate here that this co-receptor is expressed in colorectal tumor blood vessel endothelial cells (Figure 4, row C). A third component necessary for Norrin signaling in colorectal tumor blood vessels is TSPAN12 molecule. TSPAN12 participation in Norrin receptor complex significantly increases Norrin signaling through nuclear beta-catenin, but does not change Wnt ligand-dependent signaling.17 Thus, it appears that TSPAN12 is a specific Norrin signal amplifier.17 Using the sensitive double signal amplification methodology, we demonstrate that TSPAN12 protein is expressed in colorectal tumor blood vessel endothelial cells (Figure 4, row D).

Discussion
Parental primary human umbilical vein endothelial cells (HUVEC) are known to respond to Wnt3a treatment6,18,19 and we have previously defined the responsiveness of the endothelial cell line, EAhy926 to Wnt signals.2 This capability implies that endothelial cells in the tumor microenvironment respond to Wnt signals known to be operative there.2 While Norrin-associated Wnt signaling has
been linked to angiogenesis in the eye and ear11,12, its role in the tumor microenvironment, and specifically in colorectal cancer, has not been previously defined. We detected some time ago the influence of Wnt ligand on the expression of Norrin receptor Fz414. In this study, we describe that Norrin produced by colorectal tumor and endothelial cells14 directly promotes endothelial cell motility and other functions, and that blocking Norrin abrogates this effect. Specifically, blocking the Norrin signal in EAhy926 cells form vessel-like structures that can be maintained up to 120 hours until medium resources are depleted. Panel (B). In vitro angiogenesis at 40 hours in the presence of anti-Norrin antibody, showing markedly reduced vessel formation compared to control. Panel (C). Quantitative representation of the effect of anti-Norrin antibody on branch point number (1/mm²) over 160 hours. Anti-Norrin antibody reduces the branch point number during, in vitro angiogenesis. Panel (D). Quantitative representation of the effect of anti-Norrin antibody on network length (mm/mm²) over 160 hours. Anti-Norrin antibody reduces the network length during, in vitro angiogenesis.

In conclusion, our data demonstrate that Norrin, is produced by colorectal tumor, regulates endothelial cell motility and in vitro angiogenesis, and is expressed, along with the components of its receptor complex in the colorectal cancer tumor microenvironment. It may play an important role in regulating tumor angiogenesis in vivo in addition to other mediators such as vascular endothelial growth factor. The information produced by this study could be applied in the development of new therapeutic modalities for the colorectal tumors.

Methods

Constructs and cell lines. The mNorrin gene in pRK5 plasmid (generously provided by Dr. J. Nathans) was subcloned in pCMV Script prior to transfection into a norrin non-expressing colorectal cancer cell line, CaCO214. Stable clones were selected, and tested for expression by qRT-PCR. A mixture of norrin mRNA expressing clones was used to produce mNorrin conditioned medium in order to exclude the influence of the cloning artifacts related to the use of separate clones. The human endothelial-like immortalized cell line EaHy926, derived from the fusion of human umbilical vein endothelial cells (HUVEC) with the bronchial carcinoma cell line A549, and expressing an endothelial-like phenotype was a generous gift from Dr. CJ Edgell15. It was maintained in DMEM with 4.5 g/l glucose and 10% FBS.

Wound healing assay. The EaHy926 cells were plated at 50% confluency and grown overnight. After 24 hours, an innovative device WoundMaker from Essen Bioscience was applied to make a 740 um wound in the monolayer of the endothelial cells. Microscopic images of the wounds were collected every hour during 30 hours to assess the degree of endothelial cell migration into the denuded zone. The images were obtained and analyzed with the IncuCyte FLR System from Essen BioScience. All assays were performed in triplicate under the following conditions: 1) control, 2) addition of anti-Norrin blocking antibody, 3) addition of CaCO2-produced, Norrin-containing conditioned medium.

Angiogenesis assays. Network length (mm/mm²) (sum of the lengths of all of the networks in the image, normalized by the image area) and network branch points (1/mm²) (sum of the branch point count of all of the networks in the image) were estimated using StemKit from Essen Bioscience and IncuCyte ZOOM Angiogenesis Processing Overview, page 2. The CellPlayer Angiogenesis StemKit contains human Endothelial Colony Forming Cells (ECFC) and human Adipose Derived Stem Cells (ADSC). Separate, duplicate experiments were conducted each with 8 repeats for each condition. Blocking antibody studies were performed at two different concentrations (10 ug/ml and 20 ug/ml).

Specific methodology for treatment with blocking antibodies and Norrin conditioned medium. Exposure of the EAhy926 cells to Norrin blocking antibody (R&D Systems, Cat No. AF 3014) was accomplished according to the manufacturers'
recommended (The protocol is provided at: http://www.rndsystems.com/Products/a0014/Applications). The antibody at the final concentration of 12.5 μg/ml for the wound-healing assay and 10 μg/ml and 20 μg/ml for the angiogenesis assay were added directly to the cell culture medium. Cell monitoring began immediately after the addition of the blocking antibody and lasted to the end of the experiment. Matched IgG was included for the controls. Norrin conditioned medium from colorectal cancer cells CaCO2 stably transfected with Norrin/pCMV Script expression construct was prepared using the Nusse lab protocol for Wnt conditioned medium production (described on Wnt Homepage, http://www.stanford.edu/group/nusselab/cgi-bin/wnt/). CaCO2 cells stably transfected with the Norrin-expression construct were plated at 6–8% cell density and allowed to grow for 4 days. After 4 days the medium was collected and replaced with the fresh media. After 3 more days the medium was collected again, combined with the first batch, and filtered through 0.2 μm filter. This Norrin conditioned medium was diluted 1:1 with cell growth medium and used for the treatment of the cells for the wound healing assay.

Experiments evaluating the effect of Norrin conditioned medium in the StemKit angiogenesis assay system were not feasible because the concentrations required exceeded levels acceptable in conjunction with the proprietary StemKit medium.

Evaluation of Norrin pathway components in blood vessel endothelial cells by immunofluorescence staining. Fully de-identified colon cancer and normal colorectal mucosa tissue slides derived from paraffin-embedded tissue were obtained from archived specimens under a non-human subjects exemption by the institutional review board, in compliance with the Declaration of Helsinki. Samples from 10 individual and distinct patients were available for analysis and immunofluorescence staining. Evaluation of Norrin pathway components in blood vessel endothelial cells by immunofluorescence staining is depicted in Figure 4.

Figure 4 | Expression of Norrin signaling components, including Norrin, Frizzled 4, Lrp5 and TSPAN12 in endothelial cells in the CRC tumor microenvironment. In column 1, low power (5×) micrographs of the tumor microenvironment are depicted. The square demonstrates the region of higher (100×) magnification (columns 2–5). Green fluorescence (column 2) indicates expression of a specific Norrin signaling component (see row designation, below). Red fluorescence (column 3) marks endothelial cells (anti-CD31 positive) of colorectal tumor blood vessels. Blue fluorescence (column 4) depicts DAPI-stained nuclei. Merged images are depicted in column 5. Row (A). Expression of Norrin in colorectal tumor endothelial cells. Row (B). Expression of Frizzled 4 in colorectal tumor endothelial cells. Row (C). Expression of Lrp5 in colorectal tumor endothelial cells. Row (D). Expression of TSPAN12 in colorectal tumor endothelial cells.

 Statistical considerations. Expression of Norrin production in individual CaCO2 clones, in comparison to pCMV transfected control CaCO2 cells, was performed using a non-paired, two-tailed t-test with level of significance defined as p < 0.05. The wound and angiogenesis assays are semi-quantitative and therefore the quantitation graphs were not subjected for formal statistical analysis. Expression of antigens by confocal immunofluorescence was defined by the level of resolution attainable under the described experimental conditions.

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Acknowledgments
The authors thank Dr. J. Nathans for Norrin gene containing construct, Essen Bioscience for donating the time of IncuCyte FLR system, and Romana Huq, associate director of the shared Research Facilities at the Icahn School of Medicine at Mount Sinai for significant help and microscopy method development.

Author contributions
R.F.H. and K.P. conceived the study, and had oversight over experimental design and execution. M.P. and K.P. contributed performing tissue expression studies, microscopy, cell based assays, molecular studies, and data analysis. Each author contributed to manuscript preparation and all authors have reviewed and approved the final manuscript.

Additional information
Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors have no competing interests as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

How to cite this article: Planutis, K., Planutiene, M. & Holcombe, R.F. A novel signaling pathway regulates colon cancer angiogenesis through Norrin. Sci. Rep. 4, 5630; DOI:10.1038/srep05630 (2014).

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