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LETTER TO THE EDITOR

Tumor microenvironment-dependent epigenetic imprinting in the vasculature predicts colon cancer outcome

Dear Editor,

Tumor microenvironment (TME)-dependent stromal cell plasticity governs tumor development and therapy response. Tumor endothelial cells (TECs) are a major cellular component in this context [1]. In colorectal carcinoma (CRC), the stromal cell-dependent impact of the TME is illustrated by an improved survival depending on an interferon (IFN)-γ-dominated Th1-like TME associated with high T-cell density [2] and suppressed angiogenesis [3, 4]. Cellular transcriptional memory is reported in cell lines after repeated exposure to IFN-γ in vitro [5], suggesting that a Th1-like TME may also exert stable imprinting effects in vivo. Here, we investigated whether TME-dependent transcriptional imprinting in TECs from CRC patients can be exploited to retrieve clinically relevant signatures predicting outcomes.

TECs, corresponding normal endothelial cells (NECs), peripheral blood mononuclear cells (PBMCs) and tumor cells were isolated from CRC patients with a favorable Th1- or a worse Control-(non-Th-1)-TME and analyzed by multi-omics analyses to identify differentially expressed genes (DEGs) with parallel epigenetic and/or genomic alterations (methods given in Supplementary Material). Respective DEGs were validated, and their clinical impact was analyzed (Figure 1A). Guanylate-binding protein (GBP)-1, an established marker of an angiostatic Th1-TME in CRC [3, 4], was used to categorize Th1- and Control-CRC patients (Supplementary Figures S1 and S2A) based on a positive correlation with high IFN-γ levels and CD3 T cell density (Supplementary Figure S2B-C), that exhibited similar clinical characteristics (Supplementary Table S1). ECs were isolated using triple-positive-FACS-sorting [6] with a mean of 15,100 cells/sample (Figure 1A). EC purity (Supplementary Figures S2E and S3) and phenotype (Supplementary Figure S2F) were confirmed, and a population doubling time of up to 35 days was identified (Supplementary Figure S2G-H).

Cellular transcriptional memory in vitro is defined by an enhanced expression of marker genes upon repeated exposure to a stimulus (priming and re-stimulation). GBP-2/-3/-4/-5/-7, HLA-DRA/-DPB1/-DQB1, and -DQA1 are IFN-γ-dependent memory genes long-term activated by chromatin reorganization identified in HeLa cells [5]. The expression levels of these IFN-γ-memory markers were not different between Th1- *versus* Control-TECs, neither at basal untreated levels (Supplementary Figure S4A) nor after re-stimulation by IFN-γ (Supplementary Figure S4B). However, ECs could elicit IFN-γ-dependent cellular memory in vitro after repeated exposure to IFN-γ similar to HeLa (Supplementary Figure S4C). This suggested that Th1-TME-driven transcriptional imprinting of TECs in vivo is more complex and/or mechanistically different from solely IFN-γ-driven transcriptional memory in vitro.

To retrieve marker genes imprinted by a Th1-TME in vivo, the transcriptomes of Th1- versus Control-TECs were compared (Figure 1B). Multiple DEGs (n = 1,381, P < 0.05) were detected with 147 genes above fold change > 1.5 (Supplementary Table S2). Among these, the major upregulated DEGs were LIFR, LRMP and MERTK and among the downregulated they were CCDC80, DDR2, PDGFRB, STC2 and TGFB2 (Figure 1B, Supplementary Table S2). Increased SPARCL1 expression, previously detected in Th1-TECs [4], was also confirmed here (Figure 1B, Supplementary Figure S2D). A TME-dependent DEG pattern was absent in corresponding NECs (Supplementary Figure S5B). The relationship of the Th1-associated DEGs in

Abbreviations: 5-Aza, 5-Aza-2'-deoxycytidine; CCDC80, coiled-coil domain containing 80; COL12A1, collagen type XII alpha 1 chain; CRC, colorectal cancer; DDR2, discoidin domain receptor tyrosine kinase 2; DEGs, differenital expressed genes; DFS, disease-free survival; DNA, desoxyribonucleic acid; ECs, endothelial cells; GBP, guanylate binding protein; HLA-DRA/-DPB1/-DQB1/-DQA1, major histocompatibility complex, class II-DR alpha/DP beta 1/DQ beta 1/DQ alpha 1; IFN, interferon; LIFR, leukemia inhibitory factor receptor; LRMP, lymphoid restricted membrane protein; NECs, normal endothelial cells; MERTK, MER proto-oncogene, tyrosine kinase; Pat, patient; PBMCs, peripheral blood mononuclear cells; PDGFRB, platelet derived growth factor receptor beta; SNX10, sorting nexin 10; STC2, stanniocalcin 2; SPARCL1, secreted protein acidic and cysteine rich like 1; TECs, tumor endothelial cells; TGFB2, transforming growth factor beta 2; Th, T helper; TME, tumor microenvironment; VE-cadherin, vascular endothelial-cadherin.

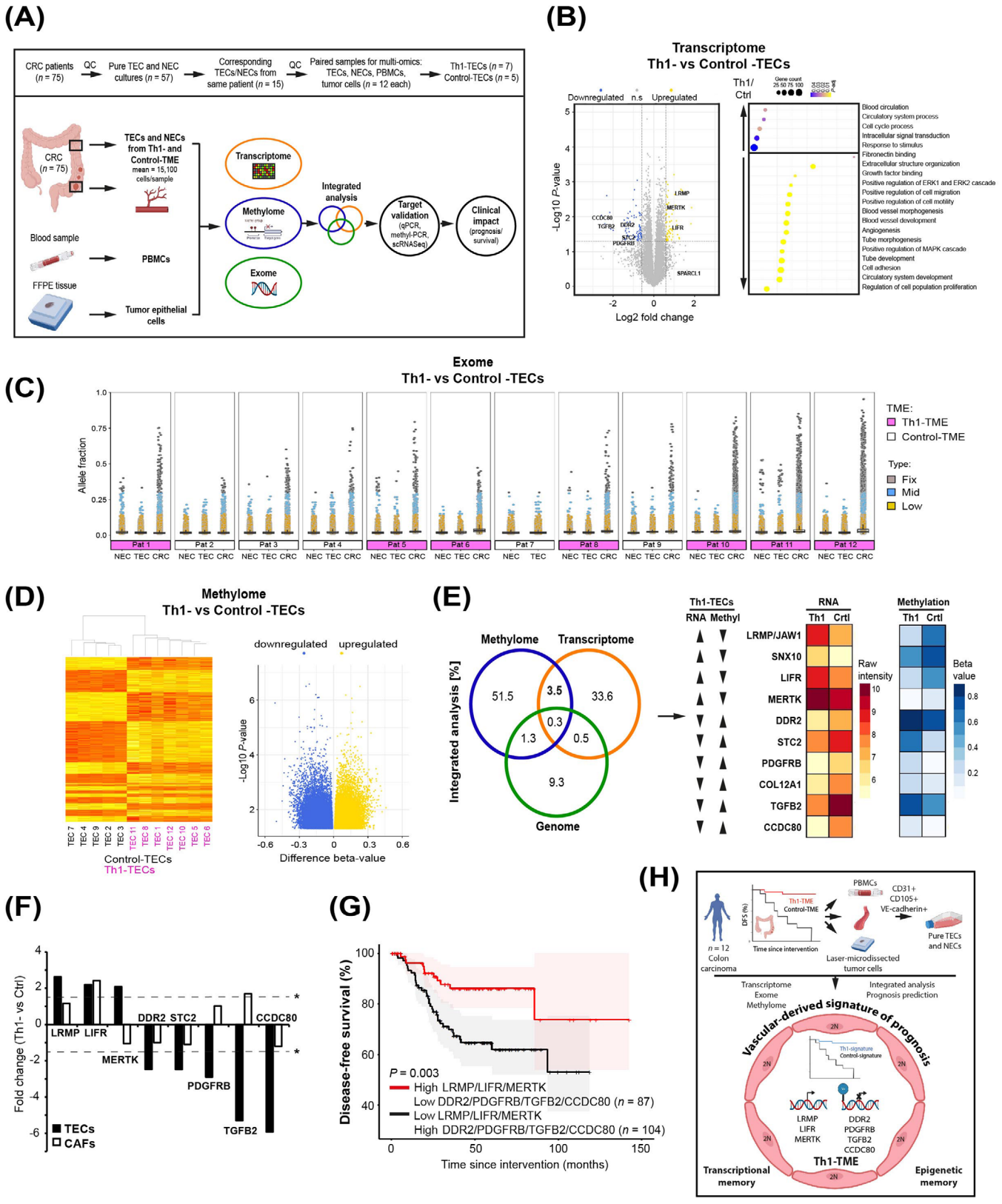

FIGURE 1 Tumor microenvironment-dependent epigenetic imprinting in the vasculature predicts colon cancer outcome. (A) Pure and viable tumor endothelial cells (TECs) and corresponding normal endothelial cells (NECs) were isolated from human CRC patients (mean 15,100 cells/sample) with different TMEs (Th1- vs Control-TME). To exploit tumor vessel-derived TME-dependent transcriptional imprinting and mechanisms of its manifestation, the cells were expanded in culture and compared by a multi-omics analysis of the transcriptome, methylome and exome. DNA extracted from fresh PBMCs and laser-microdissected tumor cells from FFPE-blocks of the same patients was

cultivated TECs with the immuno-angiostatic Th1-TME was supported by functional enrichment analysis identifying downregulated terms, including "*positive regulation of cell migration/motility*" and "*angiogenesis*" (Figure 1B). Consistent with published work [7], transcriptional profiling of all TECs versus NECs revealed DEGs ($n = 26$) (Supplementary Table S3) associated with metabolism such as "*prostaglandin metabolic process*" and "*benzo(a)pyrene metabolism*" (Supplementary Figure S5A).

Transcriptional imprinting in Th1-TECs may be manifested by genomic alterations [8] and/or epigenetic regulation [9]. Accordingly, ECs were subjected to exome sequencing compared to PBMCs and tumor cells as references to exclude patient- and to identify tumor-specific alterations. The tumor cells exhibited the highest number of somatic variants, while all ECs exhibited only a few and comparable variants independent of Th1- or Control-TME (Figure 1C, Supplementary Figure S6B, Supplementary Table S4). Patients 10-12 displayed increased somatic variants in tumor cells and were identified as microsatellite instable (Figure 1C), indicating that a higher tumor cell mutation frequency did not increase somatic variants in ECs per se. Only a few tumor cell-specific somatic variants were simultaneously present in ECs with no differences between TECs and NECs (Supplementary Figure S6A, Supplementary Table S5), rejecting the idea of genetic drift from tumor cells to ECs. This indicated that somatic variability is similarly low in TECs and NECs, irrespective of the TME, demonstrating that transcriptional imprinting is not manifested at the level of genomic somatic variants.

Next, the methylome was compared between Th1- and Control-TECs and identified to be different (Figure 1D). All Th1-TECs clustered together (Figure 1D), including multiple hits at single genes (Supplementary Table S6). Clustering was less stringent between TECs and NECs, indicated by a lower log2-FC range and two TEC samples clustering with NECs (Supplementary Figure S7A) in contrast to the analysis between Th1- and Control-TECs. This suggested that differential methylomes between Th1- and Control-TECs may contribute to the regulation of TEC transcriptional imprinting.

Next, the potential of genomic versus methylation alterations to regulate TME-dependent transcriptional imprinting was analyzed. Changes in genome structure occurred at 0.5%, and methylation changes occurred 7-fold more frequently at 3.5% (Figure 1E), suggesting a superior role of methylation. Methylation is usually associated with suppression of gene expression and its absence with activation. Analyses of an inverse relation of methylation and gene expression identified the genes LIFR, LRMP/JAW1, MERTK, and SNX10 with increased transcription and reduced methylation, as well as CCDC80, COL12A1, DDR2, PDGFRB, STC2 and TGFB2 in the opposite direction in Th1-TEC DEGs (Figure 1E, Supplementary Table S6). Differential RNA expression could be independently validated for 8 of the 10 genes (Supplementary Figure S5C) and the differential methylation pattern for all genes except STC2 (Supplementary Figure S7B-C). Treatment of Th1-TECs with the DNA methylation inhibitor decitabine restored the expression of methylated targets such as TGFB2 or CCDC80 (Supplementary Figure S7D), confirming their regulation by methylation. Accordingly, the multi-omics analyses finally retrieved a seven-gene imprinting signature with inverse relation of transcription and methylation.

This signature was identified to be EC-specific due to lacking coherent expression in cancer-associated fibroblasts (CAFs) from Th1- vs Control-CRC for all genes, except

used as a control in the exome analysis. The obtained results were subjected to integrated bioinformatical analyses and independently experimentally validated. The prognostic value of the extracted signature was analyzed in CRC patients. (B) Transcriptome analyses of Th1- vs Control-TECs to identify DEGs (left, $P < 0.05$, log2FC > 0.585/FC > 1.5-fold) and functional differences (right). n.s. = not significant. (C) Exome analysis of Th1- vs Control-TECs as depicted by scatter plots of the PBMC-corrected allele fractions with somatic variants in corresponding TECs, NECs, and tumor cells (CRC) for each patient depicted individually. Detailed subgroup analyses are shown in Supplementary Figure S6B. (D) Methylome analysis of Th1- vs Control-TECs conducted by EPIC methylation chip analyses and depicted after hierarchical clustering (left, y-axis corresponds to individual probe sets) and by a volcano plot (right, $P < 0.05$). (E) Integrated analysis (gene overlap percentage) of the transcriptome, methylome and genome reveals an increased methylome/transcriptome overlap as compared to the genome/transcriptome (left). The top 10 DEGs in Th1-TECs and Control-TECs with inverse relation of transcription and methylation are given (right). (F) TEC imprinting genes show a different expression pattern in cancer associated fibroblasts (CAFs) isolated from a Th1- vs. Control-TME ($n = 3$ each) compared to TECs (FC > 1.5, $^*P < 0.05$ is indicated by dashed lines). (G) Survival analysis as depicted by a Kaplan-Meier curve of the 7 independently validated vascular imprinting genes associated with a Th1-TME (high LRMP, LIFR and MERTK expression in combination with low DDR2, PDGFRB, TGFB2 and CCDC80 expression) predicted significantly improved disease-free survival in CRC patients (GEO database GSE161158). (H) In summary, ultrapure viable TECs from 12 human CRC patients were isolated by FACS (CD31-, CD105-, and VE-cadherin-positive cells) and systematically compared at the genomic, transcriptional and epigenomic levels with corresponding normal endothelial cells (NECs), PBMCs and laser-microdissected tumor cells. These analyses identified TME-imprinted transcriptional imprinting in TECs maintained in culture by epigenetic mechanisms rather than somatic variants. Integrative bioinformatics retrieved a seven gene imprinting signature capable to predict patient's prognosis. Illustrations created using BioRender.com.

LIFR (Figure 1F). TEC-associated imprinting was validated using an independent scRNAseq data set in CRC. ECs were composed of lymphatic, proliferative, stalk-like and tip-like ECs, with proliferative EC only present in TECs (Supplementary Figure S8A). All imprinted genes were expressed in at least one EC population (Supplementary Figure S8C). Consistent with our previous findings, SPARCL1, a marker of ECs in an angiostatic Th1-TME [4], was more highly expressed in NECs compared to TECs (Supplementary Figure S8C) and, within these, most highly in quiescent, nonproliferating stalk-like TECs (Supplementary Figure S8B). In the Th1-TEC population, 5/7 marker genes were coregulated in the expected direction (Supplementary Figure S8C), confirming TEC-associated expression of the imprinted genes. Moreover, expression of the DEGs in tumor vessels was also confirmed by IHC, for example, for DDR2, MERTK and PDGFRB (Supplementary Figure S8D).

The clinical impact of the Th1-TEC imprinting signature was analyzed in an independent CRC cohort (n = 191). Analyses of the seven individual genes revealed a prognostic value for DDR2, PDGFRB and MERTK only (Supplementary Figure S9). Remarkably, the combined imprinting signature was found to be associated with an overall improved disease-free survival rate in accordance with the respective TME (Figure 1G), confirming its clinical relevance.

In conclusion, TME-dependent transcriptional imprinting specifically induced in TECs and stably maintained by epigenetic DNA methylation was identified. A corresponding imprinting signature allowed tumor vessel-based prognoses prediction (Figure 1H). Considering known epigenomic subtypes of CRC [10], this provides novel perspectives for corresponding targeted therapies and highlights the contribution of vascular cells within the TME.

AUTHOR CONTRIBUTIONS

Elisabeth Naschberger and Michael Stürzl designed the experiments. Elisabeth Naschberger, Richard Demmler, Yanmin Lyu and Christian Flierl contributed to data acquisition. Katja Petter and Tobias Gass helped in conducting the experiments. Maximilian Fuchs, Nicholas Dickel, Meik Kunz, Charles Gwellem Anchang, Bernd Popp and Steffen Uebe contributed to bioinformatical and statistical analysis. Elisabeth Naschberger, Richard Demmler, Arif Bülent Ekici, Carol Immanuel Geppert, Andreas Ramming, Claudia Günther, Susanne Merkel, Vera Simone Schellerer, Simon Völkl, Michael Scharl and Michael Stürzl interpreted and analyzed the data; Vera Simone Schellerer, Carol Immanuel Geppert and Arndt Hartmann provided key reagents; Elisabeth Naschberger and Michael Stürzl wrote the manuscript. All authors reviewed and edited the manuscript. All authors read and approved the final manuscript. The corresponding author is responsible for all aspects of the work.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the ethics committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg (AZ 159_15B, TuMiC Study), and all patients provided written informed consent.

CONSENT FOR PUBLICATION

Not applicable.

DATA AVAILABILITY STATEMENT

Access to the multi-omics data sets is available through ArrayExpress (https://www.ebi.ac.uk/arrayexpress/) using the accession codes: E-MTAB-10465 (transcriptome), E-MTAB-10521 (exome) and E-MTAB-10467 (methylome). All other data presented in this study are available on request from the corresponding authors.

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REFERENCES

1. Goveia J, Rohlenova K, Taverna F, Treps L, Conradi LC, Pircher A, et al. An integrated gene expression landscape profiling approach to identify lung tumor endothelial cell heterogeneity and angiogenic candidates. Cancer Cell. 2020;37(3):421.
2. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science. 2006;313(5795):1960–64.
3. Naschberger E, Croner RS, Merkel S, Dimmler A, Tripal P, Amann KU, et al. Angiostatic immune reaction in colorectal carcinoma: Impact on survival and perspectives for antiangiogenic therapy. Int J Cancer. 2008;123(9):2120–29.
4. Naschberger E, Liebl A, Schellerer VS, Schütz M, Britzen-Laurent N, Kölbel P, et al. Matricellular protein SPARCL1 regulates tumor microenvironment-dependent endothelial cell heterogeneity in colorectal carcinoma. J Clin Invest. 2016;126(11):4187–204.
5. Siwek W, Tehrani SSH, Mata JF, Jansen LET. Activation of clustered ifngamma target genes drives cohesin-controlled transcriptional memory. Mol Cell. 2020;80(3):396–409 e6.
6. Naschberger E, Regensburger D, Tenkerian C, Langheinrich M, Engel FB, Geppert C, et al. Isolation of human endothelial cells from normal colon and colorectal carcinoma - An improved protocol. J Vis Exp. 2018(134):57400.
7. Zecchin A, Kalucka J, Dubois C, Carmeliet P. How endothelial cells adapt their metabolism to form vessels in tumors. Front Immunol. 2017;8:1750.
8. Streubel B, Chott A, Huber D, Exner M, Jager U, Wagner O, et al. Lymphoma-specific genetic aberrations in microvascular endothelial cells in B-cell lymphomas. N Engl J Med. 2004;351(3):250–59.
9. Nunez JK, Chen J, Pommier GC, Cogan JZ, Replogle JM, Adriaens C, et al. Genome-wide programmable

transcriptional memory by CRISPR-based epigenome editing. Cell. 2021;184(9):2503–19 e17.
10. Orouji E, Raman AT, Singh AK, Sorokin A, Arslan E, Ghosh AK, et al. Chromatin state dynamics confers specific therapeutic strategies in enhancer subtypes of colorectal cancer. Gut. 2022;71(5):938–49.

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