

LETTER TO THE EDITOR

Low cholesterol biosynthesis favors epithelial-to-mesenchymal transition maintenance and influences tumor molecular subtyping and disease-free survival in colon cancer patients

Dear Editor,

To metastasize, epithelial cancer cells undergo an epithelial-to-mesenchymal transition (EMT) during migration, whereupon they activate a mesenchymal-to-epithelial transition (MET) when colonizing the new niche [1]. Targeting EMT or MET inducers might thus impair the metastatic process [2, 3]. However, the identification of genes involved in epithelium-mesenchyme switches (EMS) is difficult in complex samples such as human tumors. To overcome this problem, we used spheroids of a colorectal cancer (CRC) cell line, HT29, to mimic EMS in a controlled environment. HT29 spheroids were grown without any treatment (baseline condition), exposed to StemXVivo® EMT Inducing Media Supplement, an EMT inducer, for up to 9 days (EMT condition), or exposed to the EMT inducer for 5 days then grown for 4 more days without treatment to mimic EMT followed by MET (EMT-MET condition) [4] (Figure 1A). Detailed experimental procedures are available as Supplementary Materials. In baseline condition, cells grew in dense spheroids; in EMT condition, the spheroids became loose with visible peripheral rounded cells; in EMT-MET condition, the cells grew back in dense spheroids (Figure 1B). Variation of key EMS proteins paralleled the macroscopic changes: pro-mesenchymal proteins (Fibronectin and Vimentin) were increased, while the epithelial marker Occludin was decreased in the EMT condition compared to baseline and EMT-MET conditions (Supplementary Figure S1A).

To identify biological pathways activated during EMS, we performed DNA microarray analyses. Supervised anal-

ysis found 3,131 genes differentially expressed (Supplementary Figure S1B). We surmised that gene expression signature of each condition should overlap with the consensus molecular subtypes (CMS), which distinguishes CRC based on the tumor's prominent biological profiles [5]. As expected, the gene expression signature of the baseline condition was enriched in CMS3 transcripts (i.e., metabolic epithelial) [6]. Compared to the baseline condition, the EMT condition showed an increase in CMS4 transcripts (mesenchymal-like), while the EMT-MET condition displayed a decrease in CMS4 transcripts and an increase in CMS2 transcripts (canonical epithelial; Supplementary Figure S1C). Spheroids transiently exposed to the EMT inducer activated a reversible gene expression program, coherent with CRC CMS classes.

To identify pathways important for EMS, we analyzed the 3,131 genes with the Gene Set Enrichment Analysis (GSEA) computational method, with respect to the 50-hallmark gene sets from the Molecular Signatures Database. We focused on the three gene sets that were inversely regulated in the EMT *versus* baseline conditions and in the EMT-MET *versus* EMT conditions (Figure 1C). We retained the “EMT” ($q < 0.001$), “Bile acid metabolism” ($q < 0.009$), and “Cholesterol homeostasis” gene sets ($q < 0.001$). The expression of the “EMT” gene set was opposite of the other gene sets. Using different strategies, models and pathologies, similar regulation of the “EMT” and “Cholesterol” pathways have also been reported [7, 8].

The “Cholesterol homeostasis” and the “Bile acid metabolism” pathways can be targeted by statins (β -hydroxy β -methylglutaryl-CoA [HMG-CoA] reductase inhibitors). We thus used two statins, simvastatin and lovastatin, to evaluate their impact on EMS. Statins alone (Supplementary Figure S2A) or in co-treatment with the EMT inducer (Supplementary Figure S2B) had no effect on spheroid growth. Addition of statins during the EMT-MET

List of abbreviations: CMS, consensus molecular subtype; CRC, color rectal cancer; EMS, epithelium/mesenchyme switches; EMT, epithelial-to-mesenchymal transition; GO, Gene Ontology; GSEA, Gene Set Enrichment Analysis; MET, mesenchymal-to-epithelial transition; RFS, recurrence-free survival; TGF- β , transforming growth factor-beta.

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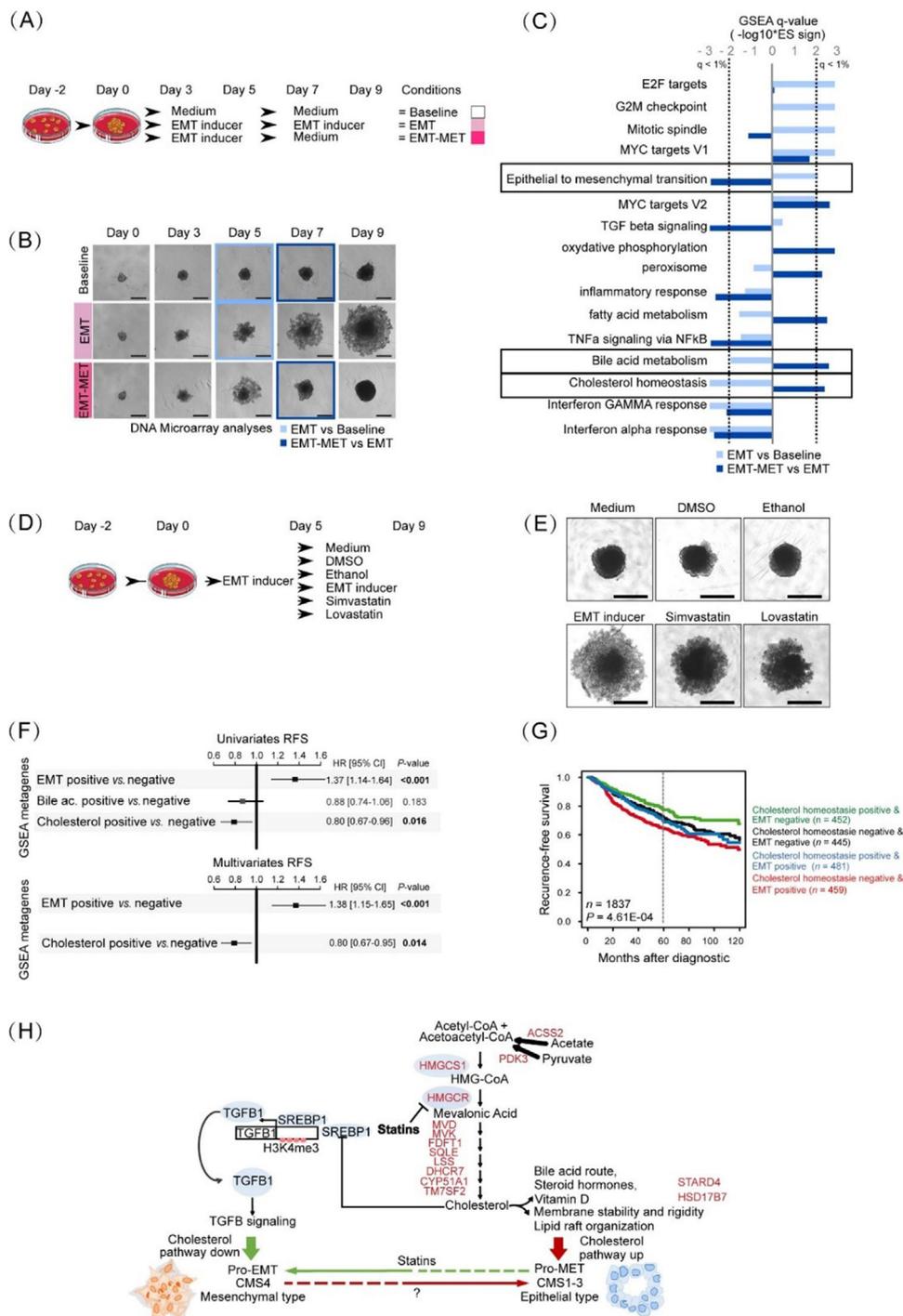


FIGURE 1 Induction of EMS program in HT29 cells grown as spheroid regulates the cholesterol pathway and influences disease-free survival in colon cancer patients. (A-C) Experimental setting used for the establishment of EMS conditions: HT29 cells were grown in 3D with ULA plate for 2 days (Day-2). At day 0, spheroids were treated with the EMT-inducing cocktail (StemXvivo™ EMT Inducing Media, Biotechne®) for at least 5 days. For EMT-MET samples, spheroids were washed off the EMT-inducing cocktail at day 5 and grown for 4 more days in regular medium (A). Representative images of spheroids observed with the Baseline, EMT and EMT-MET conditions, from days 0 to day 9 (B). Framed images correspond to sample collection time points for microarray analysis. The light blue boxes identify samples used for the EMT vs Baseline analysis, and the dark blue boxes identify samples used for the EMT-MET vs EMT analysis (B). Gene Set Enrichment Analysis (GSEA) of the 3,311 genes differentially regulated between Baseline, EMT and EMT-MET conditions. Sixteen gene-sets from the MSigDB Molecular Signatures Database were differentially regulated in at least one comparison (q-value<1%): Baseline vs. EMT (light blue boxes) or EMT vs. EMT-MET (dark blue boxes). The three gene sets inversely regulated between EMT vs. Baseline and EMT-MET vs. EMT are framed in black boxes (C). (D) Experimental setting used to study statins' impact on EMS: Cells were grown as spheroids for 2 days (Day-2). From day 0 to day 5, spheroids were treated with the EMT inducer. Then, the EMT inducer was washed off and replaced with medium, DMSO

condition allowed to maintain a loose EMT-like phenotype (Figure 1D-E, Supplementary Figure S2C). This effect was visible for high doses (Supplementary Figure S2D), with no impact on cell viability (Supplementary Figure S2E). Inhibiting the “cholesterol synthesis” pathway after EMT induction interfered with the completion of the MET program.

To look at the clinical relevance of this finding, we selected the core genes that were reversibly regulated within each gene set (14 genes for the “EMT”, 17 for the “Bile acid metabolism”, and 21 for the “Cholesterol homeostasis” gene sets) and built three independent metagenes (Supplementary Table S1), robust enough to distinguish each condition (Supplementary Figure S3A). We then assessed the prognostic value of each metagene (positive vs. negative) in our gene expression database of 2,239 clinically annotated primary CRCs, including 1,837 samples with available recurrence-free survival (RFS) data. Only the positive “EMT” metagene (HR [hazard ratio] = 1.37; 95% CI [confidence interval] = 1.14%-1.64%) and the negative “Cholesterol homeostasis” metagene (HR = 1.25; 95% CI = 1.04%-1.49%) were associated with shorter RFS (Supplementary Figure S3B) and showed independent prognostic value in univariate and multivariate analyses (Figure 1F). We thus combined the “EMT” and the “Cholesterol homeostasis” metagenes in a multi-metagene model (Figure 1G). The complementarity of the two metagenes was confirmed with the likelihood ratio (LR) test (Supplementary Figure S3C). The multi-metagene model separated the CRCs of the training set ($n = 450$ randomly split samples) into a “low-risk” and a “high-risk” classes with significant difference in 5-year RFS rate (75% [95% CI = 69%-83%] vs. 66% [95% CI = 59%-74%]) (Supplementary Figure S3D). This difference was maintained in the validation set ($n = 1,837$ remaining samples; “low-risk” class: 74% [95% CI = 70%-78%] vs. “high-risk” class: 68%

[95% CI = 64%-72%]; Supplementary Figure S3E). With this model, the “high-risk” class was associated with higher proportions of female ($P = 0.002$), pathological stage 3 ($P < 0.001$), and CMS4 patients ($P < 0.001$) than the “low-risk” class (Supplementary Table S2). We then compared the prognostic value of our multi-metagene model with other clinicopathological factors in univariate and multivariate analyses. The other variables significant in univariate analysis were the pathological stage ($P < 0.001$) and the CMS classification ($P = 0.002$). In multivariate analysis, only the pathological stage and our multi-metagene model ($P = 0.040$) remained significant, indicating that our multi-metagene model is a robust and independent prognostic factor for RFS in CRC (Supplementary Table S3). By combining a 3D culture model (i.e., cell-cell interaction relevant), a wide-spectrum EMT inducer and a dynamic dimension to the analysis, we identified two sets of genes reversibly and inversely regulated during EMS: EMT-related genes and genes from the cholesterol pathway. Some or a combination of these “new” EMT genes might be used as biomarkers to identify CRC patients at risk of relapse [9]. The genes of the cholesterol metagene encode most of the enzymes responsible for cholesterol synthesis (Figure 1H, in red). A link between cholesterol accumulation and gastrointestinal cancer occurrence exists. Also, exogenous cholesterol inhibited squalene epoxidase, a rate-limiting enzyme in cholesterol biosynthesis, to induce EMT [8]. Here, we showed that all components of the endogenous cholesterol synthesis pathway were down-regulated in response to external signals to maintain a pro-mesenchymal phenotype. Showing that cholesterol synthesis is affected by different mechanisms suggests a key role of this pathway in the regulation of EMS, and subsequently in metastatic seeding. Furthermore, the effects of statins in our in vitro model indicate that this regulation might be time-dependent to prevent the execution

(0.5%), Ethanol (0.5%), EMT inducer (StemXvivo), Simvastatin (50 μ M) or Lovastatin (50 μ M) for 4 days. (E) Representative images of the spheroids obtained with each condition, showing that statins prevented EMT-MET program completion. (F) Forest plots showing the hazard ratio for survival events of the 3 selected GSEA metagene and recurrence-free survival in CRC patients in univariate and multivariate analyses. A ratio greater than 1.0 indicates poor prognosis and a ratio lower than 1.0 indicates good prognosis. The horizontal lines for each condition defines the 95% confidence interval. Statistical values were obtained with a Wald test. (G) Kaplan-Meier curves for RFS according to the four groups used to build the multi-metagene model: “EMT”-negative/“Cholesterol homeostasis”-positive group (green, $n = 452$), “EMT”-negative/“Cholesterol homeostasis”-negative group (black, $n = 445$), “EMT”-positive/“Cholesterol homeostasis”-positive group (blue, $n = 481$), and “EMT”-positive/“Cholesterol homeostasis”-negative group (red, $n = 459$). Statistical values were obtained with a log-rank test. (H) Scheme of the cholesterol biosynthesis pathway and hypothetical impact on tumors molecular differentiation. Cholesterol is involved in multiple biological homeostatic metabolic processes (bile acids, steroid hormones and vitamin D precursors, membrane stability and rigidity, lipid raft organization). Cholesterol inhibits the synthesis of SREBP1, a transcription factor for the TGF β 1 signaling. TGF β 1 signaling is a key feature of the CMS4 mesenchymal class. Our data suggest that low cholesterol in a pro-EMT environment may favor the shift towards CMS4 mesenchymal tumors, while high cholesterol may favor more epithelial-like differentiated tumors. Genes downregulated in the Cholesterol homeostasis metagene are labelled in red. Statins are inhibitors of the HMGCR enzyme and are labelled in green. Abbreviations: 3D, three dimensions; ULA, ultra-low adhesion; EMT, epithelial-to-mesenchymal transition; MET, mesenchymal-to-epithelial transition; SD, standard deviation; GSEA, Gene Set Enrichment Analysis; DMSO, Dimethylsulfoxide (vehicle); RFS, recurrence-free survival.

of the EMT-MET program. Accordingly, a recent study showed that statins not only increased metastatic seeding in a murine model of pancreatic adenocarcinoma but also reduced metastatic formation and growth by blocking the MET [10]. Finally, this regulation could also impact the primary tumor. Our multi-metagenome model indeed showed an enrichment in CMS4 tumors (*i.e.*, associated with worse survival), in the “high-risk” class (36% vs. 1%). This is also supported by the fact that inhibition of the cholesterol pathway induced transforming growth factor- β (TGF- β) signaling to promote EMT and tumor aggressiveness in murine pancreatic adenocarcinoma [10].

Altogether, these data suggest that a time-dependent regulation of EMS and cholesterol pathways might drive tumor aggressiveness during CRC progression. Importantly, this process may translate in any carcinoma with basal/squamous/mesenchymal differentiation and impact tumor dissemination capacities and prognosis.

DECLARATIONS

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COMPETING INTERESTS

The authors declare that they have no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

AUTHOR CONTRIBUTIONS

Anaïs Aulas, François Bertucci, Daniel Birnbaum and Emilie Mamessier designed the study. Anaïs Aulas, Maria Lucia Liberatoscioli, Lucas Usclade, and Olivier Cabaud performed the experiments. Pascal Finetti and François Bertucci performed the biocomputational analyses. David J. Birnbaum provided the clinical rationale. François Bertucci, David J. Birnbaum, Daniel Birnbaum proofed the manuscript. Anaïs Aulas, Maria Lucia Liberatoscioli, and Emilie Mamessier designed the figures and wrote the manuscript. All authors read and approved the final manuscript.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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