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c-Met activation promotes extravasation of hepatocellular carcinoma cells into 3D-cultured hepatocyte cells in lab-on-a-chip device

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ABSTRACT

Activation of c-Met signaling is associated with an aggressive phenotype and poor prognosis in hepatocellular carcinoma (HCC); however, its contribution to organ preference in metastasis remains unclear. In this study, using a Lab on a Chip device, we defined the role of aberrant c-Met activation in regulating the extravasation and homing capacity of HCC cells. Our studies showed that (i) c-Met overexpression and activation direct HCC cells preferentially towards the hepatocytes-enriched microenvironment, and (ii) blockage of c-Met phosphorylation by a small molecule inhibitor attenuated extravasation and homing capacity of HCC cells. These results, thus, demonstrate the role of c-Met signaling in regulating the colonization of HCC cells preferentially in the liver.

Hepatocellular Carcinoma (HCC) accounts for nearly 90 % of all primary liver cancers and is the fourth leading cause of liver cancer-related deaths worldwide [1]. Hepatocyte growth factor (HGF) and its high-affinity receptor c-Met, have pivotal roles in normal liver development and regeneration; aberrant activation of HGF/c-Met signaling promotes invasion and metastasis in HCC by regulating epithelial-mesenchymal transition (EMT), cell survival, proliferation, differentiation, motility, invasion, and angiogenesis [1,2]. It is considered as one of the major signaling pathways targeted for therapeutic purposes against several cancers, including HCC [3]. High c-Met expression is associated with portal vein invasion and a decrease in the recurrence-free survival of HCC patients. Therefore, c-Met is suggested as a marker to predict recurrence in resected HCC patients [4]. However, the role of c-Met signaling on the organ preference of HCC cells needs to be better defined. Limited availability of 3D co-culture systems that mimic the heterogeneity and complexity of HCC tumor tissue impedes the revelation of nature of cancer and development of effective therapeutics. Conventional 2D cell cultures cannot recapitulate the complexity of the tumor microenvironment (TME). They lack cell-cell interactions between different cell populations and cell-extracellular matrix (ECM) interactions even though they are cost-effective and easy to handle. On the other hand, animal metastasis models are more expensive and time-consuming than *in vitro* models and ineffective in recapitulating organ-specific metastasis in HCC [5,6]. In this context, 3D Lab-on-a-chip (LOC) models have been becoming very convenient and feasible models for studying TME interactions and the mechanistic basis of organ-specific metastasis [7–9].

In this study, we used a LOC platform [8], EX-LOC, to quantitatively investigate the extravasation tendency of cancer cells towards different microenvironments enriched with organ-specific cell types and determined the role of c-Met signaling on the organ-specific extravasation preferences of HCC cells. The EX-LOC has three channels from top to bottom; medium, homing, and endothelial barrier channels (Fig. 1A, Day −7). In this system, HCC cells loaded in the endothelial barrier channel represent the circulating tumor cells, while HUVEC cells seeded in the same channel mimic the vascular endothelium that tumor cells must pass through to metastasize. In turn, liver epithelial cells, bone marrow fibroblasts, or lung epithelial cells seeded in the homing channel within the Matrigel represent the homing cells in the metastatic organs (Fig. 1A, Day −1). The medium channel is used to supply fresh medium to the system.

Pre-experimental processing of the EX-LOCs and culturing conditions of different cell types were previously described [8,10,11] and summarized in the Supplementary Methods file. Following preprocessing of EX-LOCs, tile scan images of mCherry-positive immortal liver epithelial cells (THLE-2), bone marrow stromal cells (HS-27A), and lung epithelial cells (BEAS-2B) embedded within Growth Factor Reduced Matrigel (GFR-Matrigel) were loaded into homing channels to mimic organ-specific microenvironments (Fig. 1A, day −1). GFR-Matrigel without homing cells was used as a negative control (Suppl. Fig.S1A and S1B). After polymerization of GFR-Matrigel with or without homing cells, HUVEC cells stained with a green cell tracker were loaded into the endothelial barrier channels (Fig. 1A, day (−1), bottom). EX-LOCs were incubated for one day to obtain an intact endothelial cell layer on the endothelial barrier channel-facing surface of the Matrigel and to allow liver, lung, and bone-homing cells to grow in the homing channel. Before loading HCC cells, formation of endothelial barrier and 3D growth of homing cells was confirmed by confocal *Z*-stack images. Subsequently, azurite-tagged HCC cells (SNU-449 or SNU-398) that vary in expression and/or activation levels of c-Met were loaded into the endothelial barrier channel to test the organ-specific extravasation potential of HCC cells (Fig. 1A, day (0), top). Tile scan images of EX-LOCs were collected using confocal microscopy (Fig. 1A, day (0), bottom) on days 2 and 5, and extravasated posts were determined to examine the extravasation capacity of HCC cells. On day 7, extravasation capacity of HCC cells was analyzed by counting the number of posts that were positive for azurite-tagged blue HCC cells extravasated through the endothelial barrier border (represented as white striped lines in Fig. 1A, day 7), into the m-Cherry tagged liver, lung, and bone marrow cells mimicking homing microenvironments. As shown in Fig. 1A, day (7),

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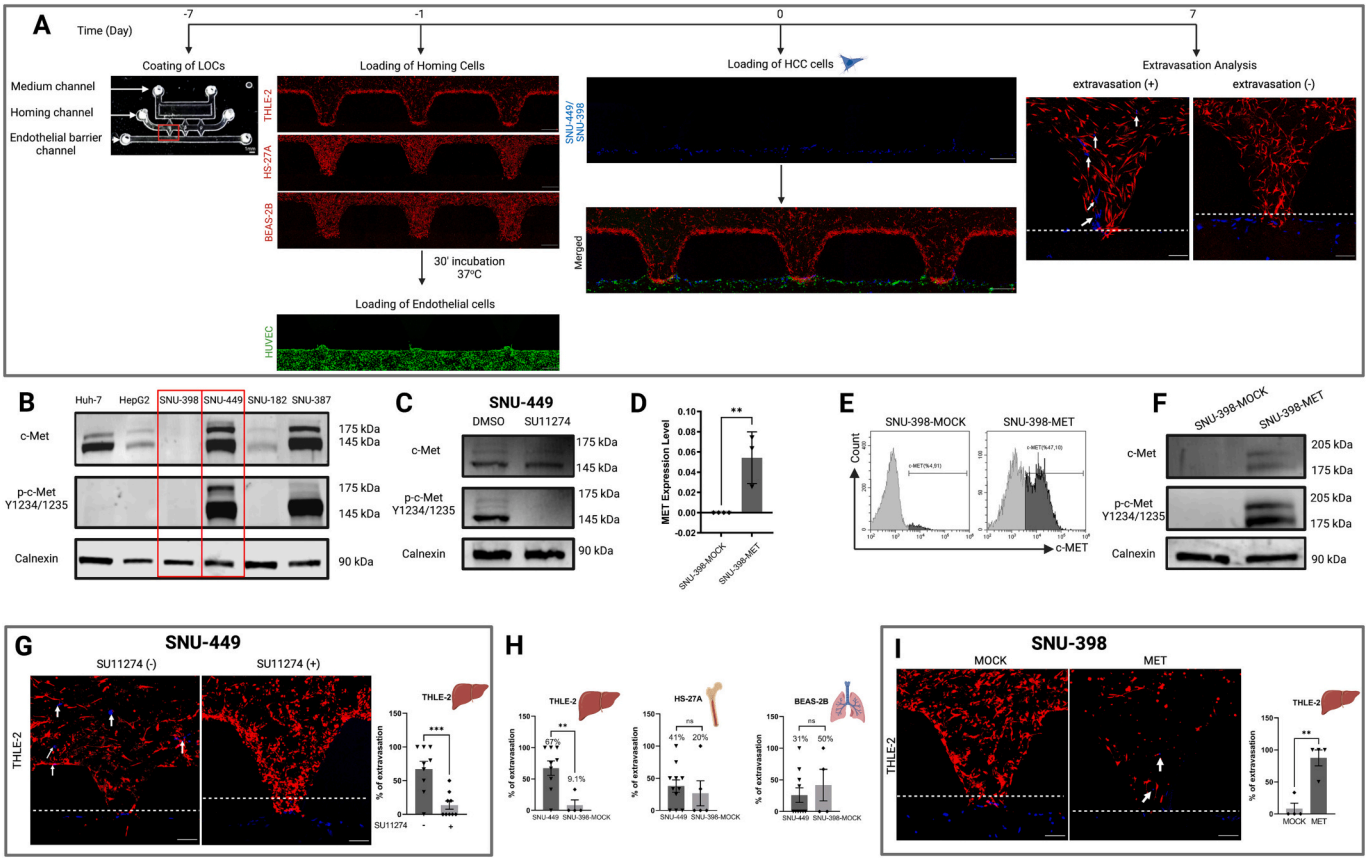

Fig. 1. c-Met activation regulates the extravasation capacity of HCC cells in a 3D lab-on-a-chip (EX-LOC) device. (A) Representative image of an EX-LOC and schematic diagram of the experimental workflow. The workflow starts with coating the EX-LOC and then loading the mCherry tagged homing cells (THLE-2/HS-27A/BEAS-2B) embedded in GFR-Matrigel to the homing channels for 30 min at 37 °C for matrigel polymerization. This step is followed by loading endothelial cells (HUVEC) into the chip and incubating for 24 h to create an intact endothelial barrier. Finally, azurite-tagged HCC cells (SNU-449 or SNU-398) were loaded, and then confocal imaging of the EX-LOC and extravasation analysis were performed based on extravasation (+) / (−) post numbers. White arrows represent extravasated HCC cells into homing microenvironments, and striped white lines represent endothelial barrier border (B) Western blot analysis of c-Met protein and its Y1234/1235 phosphorylation in six hepatocellular carcinoma cell lines. (C) Western blot analysis of c-Met and phospho-Met (Y1234/1235) protein levels of DMSO- or c-Met inhibitor SU11274- treated SNU-449 cell line. In western blot experiments, calnexin was used as a loading control. (D) RT- qPCR analysis of *MET* gene and (E) Flow cytometry analysis of c-Met expression in SNU-398-MET and SNU-398-MOCK cell lines. (F) Protein levels of c-Met and phospho-Met(Y1234/1235) in SNU-398-MOCK and SNU-398-MET cells determined by western blot analysis. (G) Extravasation preference of untreated or c-Met inhibitor SU11274 treated SNU-449 cells towards THLE-2 liver hepatocytes enriched microenvironments. (H) Extravasation rate of SNU-449 and SNU-398 cells into THLE-2, HS-27A, and BEAS-2B cells containing homing channels, respectively. (I) Extravasation rate of c-Met overexpressed SNU-398-MET and SNU-398-MOCK cells towards the THLE-2 liver hepatocytes enriched microenvironments. White arrows represent extravasated HCC cells through the endothelial barrier into homing microenvironments. White striped lines represent the endothelial barrier border. Each western blot and confocal microscopy data represents at least three independent experiments.

post having extravasated HCC cells (white arrows) into homing microenvironments was considered as "extravasation (+) post" while post with no detected extravasation was considered as "extravasation (-) post".

To investigate the role of c-Met in organ-specific extravasation capacity of HCC cells in EX-LOCs, we first determined the expression and/or activation levels of c-Met in six different HCC cell lines (HuH-7, HepG2, SNU-398, SNU-449, SNU-182, and SNU-387) by using western blotting. Densitometric analysis showed that the abundance of c-Met protein and its phosphorylation levels (Y1234/Y1235) were higher in SNU-449 cell lines than in other HCC cell lines tested (Fig. 1B). Conversely, c-Met expression/activation was not detected in SNU-398 cell lines (Fig. 1B).

c-Met overexpressing and c-Met constitutively active SNU-449 and c-Met null SNU-398 cells were selected for further investigation of the effect of c-Met activity on extravasation capacity by using two different approaches. Firstly, SNU-449 cells were treated with a small molecular inhibitor (SU11274, 2.5 μM) to inhibit c-Met activation, and inhibition of c-Met (Y1234/Y1235) phosphorylation by SU11274 treatment was validated by immunoblotting (Fig. 1C).

Secondly, c-*MET* gene was ectopically overexpressed in SNU-398 cell line, which lacks endogenous c-Met expression and activation. For this, SNU-398 cells were transduced with pLenti-Met-GFP and pLenti-GFP control vectorsexpression of c-Met in SNU-398-MET-GFP cells was validated by RT-qPCR (Fig. 1D), flow cytometry (Fig. 1E), and immunoblotting (Fig. 1F).

Then, the role of c-Met signaling on the extravasation tendency of cancer cells towards different microenvironments was investigated using the EX-LOC. In this regard, the effect of microenvironments enriched by organ-specific cells was first examined using homing cell-free GFR-Matrigel containing EX-LOCs. No extravasation of SNU-449 cells was observed through the HUVEC endothelial barrier in cell-free only matrigel EX-LOCs (Suppl. Fig. S1A). On the other hand, the presence of hepatocytes, lung epithelial cells, and bone marrow stromal cells in the homing channel of EX-LOCs induced the extravasation of SNU-449 cells through the endothelial barrier into all cell-enriched microenvironments (Suppl. Fig. S1C). The extravasation of SNU-449 cells treated with solvent (DMSO) was analyzed in 85 posts, and the overall extravasation ratio was determined as 47 % (Suppl. Table 1). The extravasation ratio was higher in the presence of liver hepatocytes (67 %) when

compared to bone (41 %) and lung (31 %) cells-enriched microenvironments (Suppl. Fig. S1C). The extravasation rate of HCC cells towards the microenvironment enriched with liver hepatocytes is significantly higher than that towards the lung epithelial cells-enriched microenvironment ($p = 0.0159$) (Suppl. Fig. S1C). c-Met inhibitor treatment significantly diminished extravasation of SNU-449 cells into the liver hepatocyte-containing ($p = 0.0008$) (Fig. 1G) and bone marrow stromal cell-containing homing channels ($p = 0.0195$) but not into the lung epithelial cells enriched homing channel ($p = 0.7516$) (Suppl. Fig. S1D). The most prominent effect of c-Met inhibitor treatment on extravasation ratio of SNU-449 cells was observed in a liver hepatocyte-enriched microenvironment containing EX-LOCs (Suppl. Table 1). The effect of the 2.5 μM c-Met inhibitor on the proliferation of SNU-449 cells was analyzed by MTT to test whether the decrease in the number of HCC cell-positive EX-LOC posts is a consequence of the antiproliferative effect of the inhibitor. We observed that c-Met inhibitor at the concentration used for extravasation experiments has no effect on proliferation rate (Suppl. Fig. S1E), but decreases colony formation capacity (Suppl. Fig. S1F) of SNU-449 cells. This data suggests that the inhibition of c-Met activation specifically decreased extravasation and clonogenic capacity of HCC cells towards liver hepatocyte-enriched microenvironments.

Similarly, the effect of organ-specific cell-enriched microenvironments on the extravasation capacity of control (SNU-398-MOCK) and c-Met overexpressing (SNU-398-MET) SNU-398 cells was determined. As expected, neither SNU-398-MOCK nor SNU-398-MET cells extravasated through the endothelial barrier in the absence of homing cells in only GFR-Matrigel containing EX-LOCs (Suppl. Fig. S1B). The extravasation ratio of c-Met null SNU-398-MOCK cells was significantly low (9.1 %) compared to that of c-Met active SNU-449 cells (67 %) towards the hepatocyte-enriched homing channels (Fig. 1H). Correspondingly, c-Met overexpression caused a 10-fold increase in extravasation of SNU-398-MET cells compared to MOCK cells through the endothelial barrier towards the THLE-2 hepatocyte-enriched microenvironment c-Met overexpressing SNU-398-MET cells exhibited a significantly higher extravasation ratio (90 %) through the endothelial barrier towards the hepatocyte enriched-EX-LOCs ($p = 0.0019$) (Fig. 1I) compared to lung epithelial cell (57 %) or bone marrow stromal cell (55 %) enriched EX-LOCs (Suppl. Table 2, Suppl. Fig. S1G). Furthermore, we performed a 2D colony formation assay to determine the effect of c-Met expression on colony formation capacity of SNU-398 cells. The area and intensity of colonies were significantly higher in SNU-398-MET cells than in SNU-398-MOCK cells (Suppl. Fig. S1H), suggesting that c-Met expression increases clonogenic capacity of HCC cells but not their short-term proliferation (Suppl. Fig. S1I).

Overall, this report showed that inhibition of c-Met activation using a small molecule inhibitor significantly diminished the extravasation of SNU-449 cells, specifically towards the homing channel enriched with liver hepatocytes. Likewise, overexpression of c-Met increased the extravasation of SNU-398 cells through the endothelial cell barrier towards the hepatocyte-enriched microenvironment.

Furthermore, our data showed that neither c-Met inhibitor treatment nor c-Met overexpression affects the proliferation of HCC cells. Interestingly, inhibition of c-Met activation significantly diminished the clonogenic potential of SNU449 cells, whereas c-Met overexpression in SNU398 cells significantly increased colony formation. Colony formation assay is designed to monitor the capacity of a single adherent cell to survive and produce a viable colony over time, whereas proliferation assays show the changes in the total number of a cell population within a sample over time after initial seeding. In this context, our data suggest that c-Met signaling is important in the clonogenic capacity of HCC cells and may play a role in the colonization of extravasated HCC cells in the homing environment.

In conclusion, this study shows for the first time that overexpression or constitutive activation of c-Met is crucial for extravasation of HCC cells into liver hepatocytes-enriched microenvironment using a 3D LOC model consisting of ECM, hepatocytes, endothelial cells, and HCC cells. Our data supports the idea that c-Met activation may have a strong potential to take a role in intrahepatic metastasis of HCC, and using c-Met inhibitors may prevent intrahepatic metastasis and/or recurrence in HCC patients. In contrast, c-Met activation did not affect the extravasation of HCC cells into bone- and lung-cell-enriched microenvironments representing the distant-organ microenvironments in HCC.

Most patients with HCC may experience recurrence or intrahepatic metastasis after curative resection [1,2]. In addition to intrahepatic metastasis, extrahepatic metastases may occur mainly in the lung (55 %) and regional lymph nodes (53 %) or bone (28 %) [12,13]. In this context, the findings of this study support that (i) LOC systems can be used to predict organ preference of metastatic cells, (ii) treatment with c-Met inhibitors after curative resection may prevent recurrence and intrahepatic metastasis in HCC patients with high c-Met expression and activation. However, further studies are needed to validate LOCs and test c-Met inhibitors' efficacy in preventing intrahepatic metastasis or recurrence of HCC.

CRediT authorship contribution statement

Gulsun Bağcı & Dehan Comez: Investigation, Formal analysis, Visualization, Writing -original draft & editing; **Ezgi Bagirsakci:** Investigation, Formal analysis, Visualization, Writing-review & editing; **Hande Topel & Yeliz Yilmaz**: Conceptualization, Methodology, Investigation, Formal analysis, Writing –review & editing; **Aysim Gunes:** Investigation, Formal analysis, Writing – review & editing; **Gizem B.Ayaz:** Investigation, Formal analysis, Visualization, Writing –review & editing, Fabrication of EX-LOCs. **Ismail Tahmaz & Muge Bilgen:** Investigation, Data Analysis, Fabrication of EX-LOCs; **Gulhas Solmaz:** Investigation, Formal analysis, Writing – review & editing; **Devrim P.Okvur:** Investigation, Methodology, Writing – review & editing, Supervision of the first design of LOC, LOC fabrication and optimization, Funding acquisition. **Nese Atabey:** Conceptualization, Investigation, Methodology, Writing-review & editing, Supervision, Funding acquisition, Project Management. All authors discussed the results and contributed to the final manuscript.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used BioRender in order to create Fig. 1G, 1H, 1I, and Suppl. Fig. 1C, 1G. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no competing interests except that D.PO was co-founder of Initio Biomedical Engineering (Izmir, TR).

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2023.119557.

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