

Deconstructing Gastric Cancer Colonization: The interplay between immune cells, gastric cancer cells and extracellular vesicles

Sara Jorge Moreira da Rocha

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2020



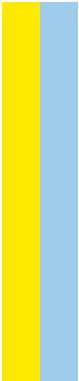
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Tese de Candidatura ao grau de Doutor em Biotecnologia
Molecular e Celular Aplicada às Ciências da Saúde;
Programa Doutoral da Universidade do Porto
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Ao Joãozinho

*A todos os que lutam,
e aos que estão ao seu lado*

Funding | Financiamento

PhD Fellowships (PD/BI/113971/2015 & PD/BD/128406/2017) provided by the Portuguese Foundation for Science and Technology (FCT) of the Portuguese Ministry of Science, Technology and Higher Education.

Bolsa de doutoramento (PD/BI/113971/2015 & PD/BD/128406/2017) concedida pela Fundação Portuguesa para a Ciência e a Tecnologia (FCT) do Ministério da Ciência, Tecnologia e Ensino Superior.



Publications | Publicações

This thesis is in compliance with the terms of the *n.º 2, alínea a) do artigo 31º do Decreto-Lei n.º 115/2013 de 7 de Agosto*, as the work developed and hereby presented is published in peer-reviewed scientific journals, submitted or in preparation for publication, as following detailed:

PAPER I

Original research – Published

Sara Rocha, Sara P. Teles, Mafalda Azevedo, Patrícia Oliveira, Joana Carvalho & Carla Oliveira (2019) Gastric Cancer Extracellular Vesicles Tune the Migration and Invasion of Epithelial and Mesenchymal Cells in a Histotype-Dependent Manner. *International Journal of Molecular Sciences*; **20**(11). pii: E2608. doi: 10.3390/ijms20112608.

PAPER II

Original research – Published

Sara Rocha*, Joana Carvalho*, Patrícia Oliveira, Maren Voglstaetter, Domitille Schvartz, Andreas R. Thomsen, Nadia Walter, Richa Khanduri, Jean-Charles Sanchez, Andreas Keller, Carla Oliveira# & Irina Nazarenko# (2018) 3D Cellular Architecture Affects MicroRNA and Protein Cargo of Extracellular Vesicles. *Advanced Science (Weinh)*; **6**(4):1800948. doi: 10.1002/advs.201800948. eCollection 2019 Feb 20.

PAPER III

Original research – Manuscript in preparation

Sara Rocha, Mafalda Santos, Irina Amorim, Sara P. Teles, Patrícia Oliveira, Joaquin J. Maqueda, Diana Martins, Nuno Mendes, Joana Carvalho & Carla Oliveira. Extracellular vesicles promote gastric cancer metastasis to brown adipose tissues.

PAPER IV

Original research – Manuscript submitted for publication

Sara Rocha, Afonso Basto, Marieke E. Ijsselsteijn, Sara P. Teles, Maria M. Azevedo, Gilza Gonçalves, Irene Gullo, Gabriela M. Almeida, Marta Oliveira, Fátima Carneiro, João T. Barata, Luís Graça, Noel F. C. C. de Miranda, Joana Carvalho & Carla Oliveira. Regulatory T cells fuel de novo expression of ILR2 α and proliferation of intestinal-type gastric cancer.

COMPLEMENTARY PUBLICATIONS IN RELATED FIELDS

Patrícia Oliveira, Joana Carvalho, **Sara Rocha**, Mafalda Azevedo, Inês Reis, Vânia Camilo, Bárbara Sousa, Sofia Valente, Joana Paredes, Raquel Almeida, David Huntsman & Carla Oliveira (2016) *Dies1/VISTA* expression loss is a recurrent event in gastric cancer due to epigenetic regulation. *Scientific Reports*; **6**:34860. doi: 10.1038/srep34860

Sílvia J. Bidarra, Patrícia Oliveira, **Sara Rocha**, Diana P. Saraiva, Carla Oliveira & Cristina C. Barrias (2016) A 3D *in vitro* model to explore the inter-conversion between epithelial and mesenchymal states during EMT and its reversion. *Scientific Reports*; **6**:27072. doi: 10.1038/srep27072.

Barbara Korsak, Gabriela M. Almeida, **Sara Rocha**, Carla Pereira, Nuno Mendes, Hugo Osório, Patrícia M. R. Pereira, João M. M. Rodrigues, Rudolf J. Schneider, Bruno Sarmiento, João P. C. Tomé & Carla Oliveira (2017) Porphyrin modified trastuzumab improves efficacy of HER2 targeted photodynamic therapy of gastric cancer. *International Journal of Cancer*; **141**:1478-89. doi: 10.1002/ijc.30844.

PUBLICATIONS IN CONFERENCE PROCEEDINGS

Sara Rocha, Joana Carvalho, Patrícia Oliveira, Maren Voglstaetter, Domitille Schvartz, Andreas Thomsen, Nadia Walter, Richa Khanduri, Jean-Charles Sanchez, Andreas Keller, Carla Oliveira & Irina Nazarenko. ISEV2018 abstract book. The impact of 3D cellular architecture in the microRNA and protein content of extracellular vesicles. (2018). *Journal of Extracellular Vesicles*, 7(Suppl 1), 1461450. <https://doi.org/10.1080/20013078.2018.1461450>

Joana Carvalho, **Sara Rocha**, Patrícia Oliveira & Carla Oliveira. The Fifth International Meeting of ISEV, ISEV2016, Rotterdam, The Netherlands, 4 - 7 May, 2016. Gastric cancer cell-derived extracellular vesicles modulate E-cadherin expression, invasion and migration of normal cells from the tumour microenvironment. (2016). *Journal of extracellular vesicles*, 5, 31552. <https://doi.org/10.3402/jev.v5.31552>

Acknowledgements | Agradecimentos

Life is never flat!

Foram várias as vezes em que usei esta frase para me referir aos modelos 3D, e só agora percebo o quão bem descreve estes últimos anos, repletos de momentos altos e uns tantos...um pouco menos altos. Este é o momento para agradecer a todos os que sorriam comigo, e que nunca me deixaram sozinha nesta aventura. As palavras nunca serão suficientes para agradecer a vossa dedicação, preocupação e amizade!

À minha orientadora, Carla, e co-orientadores, Joana, João e Marta! Obrigada por terem aceite guiar-me neste desafio, por me terem ajudado sempre que precisei, e por estarem sempre disponíveis e entusiasmados com este trabalho.

Carla, muito obrigada por ter acreditado em mim, e por me ter dado a oportunidade de fazer o doutoramento num ambiente tão desafiante. Obrigada por me ter concedido a liberdade para experimentar, e falhar, e voltar a repetir...e às vezes falhar novamente! Obrigada pela preocupação constante, não só com a estudante, mas com a pessoa.

Joana, obrigada por me dificultares a perceção de onde termina a orientação, para começar a amizade. Obrigada por teres partilhado tudo o que sabias comigo, e por me teres ajudado a descobrir quando também para ti era uma novidade. Obrigada por teres estado sempre comigo, para comemorar ou para me limpar as lágrimas quando a coisa se complicava. *Quando for grande quero ser como tu!*

A todos que passaram pelo grupo ERiC ao longo destes anos. Obrigada pela entreeajuda, pelas discussões produtivas, e por terem tornado todas estas horas, dentro ou fora do laboratório, bem mais divertidas! Aprendi com todos vocês e espero também ter conseguido contribuir de alguma forma.

Patrícia, obrigada por me teres mostrado o significado de fazer parte da família Ipatimup. Obrigada por seres autêntica, e pelas tantas vezes em que ficares até mais tarde só para fazer companhia, ou para me ajudar quando não era a tua obrigação. Obrigada pelo carinho e amizade, pelas cervejas e jantares, por me ouvires e nunca calares.

Korsak, our time at the lab was short but good enough to start an amazing friendship. Thank you for being so kind and supportive. I truly admire you my polish pónei!

Sarinha, obrigada não me deixares ficar maluquinha! Obrigada por me ouvires e teres sempre algo a dizer. Continuo à espera da minha carta de Cambridge!

Carlita, foi uma jornada longa e dura, mas conseguimos!! Obrigada por seres a minha companheira nesta aventura e pelo teu sorriso contagiante todos os dias.

Mafalda, obrigada pelas chamadas para a tua avó! Estava a brincar (será?). Obrigada por teres sido sempre tão positiva e tão segura quando eu começava a panicar! Nunca mais vou esquecer as nossas aventuras naquele edifício! xD

Diana, obrigada por seres a personificação da alegria e por encheres o ambiente de positividade sempre que entras no laboratório. Obrigada pelos conselhos, pela ajuda, e pelos constantes “sucesso”!

Anabela, obrigada por seres a nossa “mãezinha”. Obrigada pelo carinho e preocupação, e por estares sempre disponível para fazer o que fosse necessário para me ajudar!

Ao *Professor Luís* e a todos os membros do seu grupo, me terem acolhido tão bem na capital.

Afonso, obrigada pela paciência e por teres sido incansável deste o primeiro dia. Obrigada pelo tanto que me ensinaste, num período tão curto. Obrigada pelo teu entusiasmo e positivismo, e por não me deixares desistir naqueles dias tão complicados.

To *Noel* and the Immunogenomics group. Thank you all for the fruitful discussions, and for the amazing experience in Leiden. Thank you for the lunches, the happy hours, and all the celebrations. It was really good to share this experience with you.

Marieke, thank you for being so kind and so patient with me.

Joana e Catarina, obrigada por me terem integrado tão bem, dentro e fora do laboratório. Obrigada por todos os bons momentos, pela vossa preocupação, e por toda a ajuda ainda antes de ter ido do Porto. Obrigada por terem feito com que Portugal estivesse “mais perto”.

À *Irina*, obrigada por todas as horas no microscópio e por tratares do nosso trabalho com tanta dedicação e entusiasmo.

To all national and international collaborators. Thank you for your great contributions, this may be *my* thesis, but it is definitely, *our* work.

A todos os “*facilitadores*” que fazem do Ipatimup e do i3S, um lugar especial para trabalhar. Obrigada Nuno, Maria Lázaro, Maria Azevedo, Catarina, Emília, Patrícia, Rob, Mafalda, Hugo, Rossana, Rui, André,... Obrigada por toda a ajuda, compreensão e amizade!

À *verdadeira família Ipatimup*, ao gangue do almoço, das cervejas, dos jantares, das house parties ou zooms...O que importa é estarmos juntos a dizer disparates! Obrigada

por todas as conversas em que não aprendemos nada, por todos os momentos à chuva e ao vento no cantinho do Ipatimup, e por me terem aceitado de volta! Obrigada por me manterem sã, dentro da minha própria insanidade! *Pestana, Joaninha, Rui, Babi, Tito, Ritinha, Monique, Inês, Joaninha...* Obrigada por toda a vossa ajuda no dia-a-dia e nos momentos mais difíceis...Lamento, mas um bocadinho de vocês agora vem comigo! ☺

À minha twin! *Joana*, obrigada por estares sempre aqui, sempre preocupada e sempre a tentar ajudar. Obrigada por partilhares os bons e os maus momentos, por me conheceres tão bem que já não é preciso dizer nada para adivinhares.

À *Ana Margarida*, à *Ana Dias*, ao *Henrique*, à *Sara* e à *Tânia*, que partilharam comigo esta aventura que é o Biotech.

Aos meninos do GEDY. Obrigada por terem tornado as minhas horas na sala de culturas bem mais agradáveis.

Ao *Sr. Oliveira*, ao *Sr. Mendes*, ao *Sr. Carlos*, à *Zezinha*, ao *Sr. Mário*. Sem vocês o Ipatimup/i3S não seria a mesma coisa! Obrigada pelos carinhosos “bom dia” e “até amanhã”, e pela constante brincadeira que alegra os nossos dias!

À *Sara Silva*, ao *Francisco Coelho* e à *Patrícia Simões*. Daquele rol de amigos que podemos não ver/ou falar durante meses e continua tudo igual! Obrigada por me acompanharem sempre nas minhas aventuras, e pela alegria com que partilham as minhas conquistas!

À minha família, que sempre me apoiou em todos os momentos. Obrigada por estarem sempre interessados e preocupados. Obrigada por estarem sempre a ajudar e disponíveis para o que for preciso. Obrigada pelos momentos de alegria e descompressão, pelos ajuntamentos que acabam com qualquer dieta! Obrigada *Ana, Paulo, Joaninha, Tiago, D. Dorinda, Carlos, Cláudia, Nuno, Cristina, Fátima, Nati, Isidro*,...e aos miúdos que querem os trigémeos também, vá! ;)

À minha *Maggie*, que me mostrou o que é um amor incondicional. Obrigada pela tua companhia, pelas pequenas asneiras, pelas inúmeras brincadeiras. Obrigada por tomares conta de mim, quando eu é que devia tomar conta de ti.

Aos meus avós, que me criaram e ajudaram a construir um futuro que fosse melhor. Obrigada pelo orgulho que sentem sempre que passo mais uma etapa, por continuarem a ver-me crescer e por acompanharem cada momento. Avó, obrigada por todos os abraços doces, pelos carinhos sempre no momento certo, e pelas palavras cheias de ternura que só tu sabes dizer.

Às minhas irmãs, *Sofia* e *Sílvia*, as minhas duas melhores amigas. *Nunca, Nada, nem Ninguém*, nos separará! A minha admiração por vocês cresce a cada dia que passa. Sinto-me orgulhosa por tudo aquilo que já alcançaram e muito, mas muito, feliz por fazer parte das vossas aventuras e conquistas. Esta tese é também vossa. Por tudo aquilo que tiveram de aturar, pela vossa ajuda, pelo vosso apoio, pela vossa preocupação. Se tivesse de escolher não teria escolhido melhor! Obrigada!

Aos meus *pais*, para quem as palavras não chegam, nem nunca chegarão. É impossível exprimir em palavras tudo aquilo que vocês fizeram por mim. Tudo aquilo que tiveram de abdicar por nós, e para que pudéssemos ter aquilo que vocês não tiveram. Obrigada por fazerem de nós a vossa prioridade, sempre! Obrigada por todos os beijos, e abraços, que nunca são suficientes. Por todas as palavras e por todos os silêncios. Obrigada por terem sido sempre o meu porto de abrigo, e os meus guias em todas as decisões. Obrigada pelo vosso esforço, mas acima de tudo obrigada pelo vosso amor.

Ao *Pedro*. Obrigada por despertares o melhor que há em mim, e me deixares ver coisas que sozinha nunca conseguiria. Obrigada por continuares a querer partilhar todos os momentos comigo. Somos a combinação improvável, que sobreviveu à distância, ao tempo, e agora também à escrita de uma tese de doutoramento em pleno confinamento... Obrigada pelo teu apoio incondicional, pela tua compreensão e paciência, e por continuares sempre a querer mais e melhor!

Ao *Joãozinho*! Pensavas que me tinha esquecido de ti? És o último porque *tu* foste a razão pela qual tudo isto começou. Obrigada por me mostrares onde ir buscar força quando achamos que já não há mais. Obrigada por me fazeres dar valor àquilo que é realmente importante. Obrigada por seres uma inspiração nos bons e nos maus momentos.

A todos, muito obrigada.

Sem vocês nunca teria tido a oportunidade de escrever estas palavras!

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Abstract

The successful development of solid tumours is determined by the composition of the tumour microenvironment and the dynamic communication established between malignant and non-malignant cells. Extracellular vesicles (EVs) stand-out, amongst the mechanisms of tumour intercellular communication, as stable carriers of bioactive molecules that sustain cell-cell communication. In gastric cancer (GC), accumulating data advocates that EVs play a crucial role in tumour development, however whether and how EVs participate in GC distant dissemination remains scantily explored. Furthermore, the intrinsic and environmental factors that modulate the cargo and effects of GC-EVs have not yet been unveiled.

GC thrives upon tight immunosurveillance that demands the establishment of effective strategies for tumour immune evasion. Indeed, GC patients display increased numbers of regulatory T cells (Tregs) both in peripheral blood and among tumour-infiltrating lymphocytes. Nevertheless, the contribution of Tregs to the development and progression of GC remains poorly defined, and their prognostic value highly controversial.

To clarify the role of EVs and Tregs in GC development and progression, we hypothesized that Tregs modulate GC cell phenotype, which consequently alters GC communication mediated by EVs. This thesis addressed: 1) the impact of the tumour histological type and 3D cell architecture in the cargo and function of GC-EVs; 2) the role of EVs in the *in vivo* dissemination of GC cells, and; 3) the interactions between Tregs and GC cells, and how that influences GC progression.

In Chapter 1, we dissected the effect of EVs released by GC cells, representing the two main histological subtypes of GC, on the behaviour of non-malignant and isogenic epithelial and mesenchymal cells (*Paper I*). We found that EVs from diffuse-type GC hampered the migration of recipient cells, mainly if these were in a mesenchymal state. Contrariwise, EVs from intestinal-type GC stimulated invasion of epithelial cells, which was not observed for mesenchymal recipient cells. This study demonstrated that GC-EVs conveyed messages that were tumour histotype dependent, and differentially perceived by recipient cells with distinct differentiation phenotypes.

Thereafter, we studied the impact of 3D cellular architecture on the content and function of GC-EVs (*Paper II*). A comparative analysis of biochemical features, small RNA and proteomic profiles of EVs, evidenced an upregulation of microRNAs and downregulation of proteins in 3D EVs. Moreover, ARF6 signalling pathway was found to be downregulated in 3D EVs. In diffuse-type GC, the observed differences resulted in enhanced invasion of recipient epithelial non-malignant cells by 3D EVs, as compared to the 2D counterparts.

Overall, this study suggested that changes in cell architecture modulated the EV-associated microRNA and protein content, thus modifying the EV functional effect on recipient cells.

To further explore the impact of cellular architecture and EV-conveyed messages, in Chapter 2, we performed *in vivo* metastatic assays in which mice were primed with 2D or 3D GC-EVs, before injection with 2D or 3D cells (*Paper III – in preparation*). Intestinal-type GC cells, either cultured in 2D or 3D conditions, disseminated to the lung. Interestingly, initial priming with 2D/3D GC-EVs, reduced the metastatic burden of 3D cells, which was not observed for 2D cells. As for diffuse-type GC, mice injected with 3D cells had reduced disease-free survival, as compared with 2D counterparts. 3D diffuse-type GC cells displayed tropism towards brown adipocyte depots, which was potentiated by EVs, mainly by those retrieved from 3D cultures. Comparative analysis of the content of 2D and 3D EVs, unravelled a high prevalence of microRNAs involved in adipocyte differentiation, conversion of white to brown adipocytes, and activation of brown adipocytes, in 3D EVs produced by diffuse-type GC cells. Altogether these results demonstrated that the intrinsic histology and 3D cellular architecture of GC cells modulate the cargo and the role of EVs in GC progression and dissemination.

Lastly, in Chapter 3, we disclosed the effect of Tregs in tumour cell behaviour and GC development (*Paper IV*). A comprehensive immunophenotyping of 82 human GC cases demonstrated a higher prevalence of regs in intestinal-type GCs, as compared to diffuse-type GCs. Making use of the 3D model previously established for GC cells, we developed direct co-cultures between Tregs and GC cells. By monitoring of these cultures, we observed that Tregs preferentially infiltrate intestinal-type GC spheroids. Strikingly, Tregs induced membranous ILR2 α expression, specifically in intestinal-type GC cells, which associated with activation of MAPK signalling pathway and spheroid growth. These findings are consistent with the identification of a new tumour promoting role of Tregs in GC. This discovery provides insightful implications for therapeutic strategies targeting intestinal-type GCs and other tumours with similar immune context.

Overall, this thesis highlighted cellular architecture and tumour histology as key factors modulating EV content, unveiled novel mechanisms underlying GC dissemination, and disclosed a new and unforeseen role of Tregs in GC development. Further experiments consolidating these findings may provide new candidates for diagnosis and prognosis biomarkers and inspire innovative therapeutic strategies in GC.

Resumo

A capacidade com que os tumores sólidos se desenvolvem é determinada pela composição do microambiente tumoral e pela comunicação dinâmica estabelecida entre células malignas e não malignas. As vesículas extracelulares (EVs) destacam-se, entre os mecanismos de comunicação intercelular tumoral, por proporcionarem o transporte estável de moléculas bioativas que mantêm a comunicação entre células. Em cancro gástrico (GC), foi já demonstrado que as EVs desempenham um papel fundamental no desenvolvimento do tumor. No entanto, o modo como estas EVs contribuem para a disseminação de GC à distância encontra-se ainda pouco explorada. Adicionalmente, os fatores intrínsecos e ambientais que modulam a carga e o efeito das GC-EVs permanecem ainda desconhecidos.

O GC prospera sob uma rigorosa vigilância imunológica que requer o desenvolvimento de estratégias eficazes de evasão do tumor. De facto, doentes com GC apresentam valores elevados de células T reguladoras (Tregs), tanto no sangue periférico como entre os linfócitos que infiltram o tumor. Todavia, a contribuição das Tregs para o desenvolvimento e progressão do GC permanece mal definida e seu valor prognóstico bastante controverso.

De modo a clarificar o papel das EVs e das Tregs no desenvolvimento e progressão do GC, desenvolvemos a hipótese de que as Tregs modulam o fenótipo das células de GC, consequentemente alterando a comunicação via EVs estabelecida pelo tumor. Esta tese versou: 1) o impacto do tipo histológico do tumor e da arquitetura celular 3D na carga e função das GC-EVs; 2) o papel das EVs na disseminação *in vivo* de células de GC e; 3) as interações entre Tregs e células de GC, bem como a sua influência na progressão do GC.

No capítulo 1, dissecamos o efeito das EVs produzidas pelas células de GC, representativas dos dois principais subtipos histológicos de GC, no comportamento de células epiteliais e mesenquimais, não malignas e isogénicas (*Publicação I*). Neste estudo, descobrimos que as GC-EVs do tipo difuso reduzem a migração de células recipientes, principalmente das células mesenquimais. Por outro lado, as GC-EVs do tipo intestinal promoveram a invasão de células epiteliais, mas não das células mesenquimais. Este estudo demonstrou que a mensagem transmitida pelas GC-EVs depende do tipo histológico do tumor, e tem efeitos diferentes de acordo com o estado de diferenciação das células recipientes.

Posteriormente, procedemos ao estudo do impacto da arquitetura celular 3D no conteúdo e na função das GC-EVs (*Publicação II*). A análise comparativa das características bioquímicas e dos perfis de pequenos RNAs e de proteínas das EVs, evidenciou uma regulação positiva de microRNAs e negativa de proteínas nas EVs produzidas em culturas 3D. Adicionalmente, verificou-se que a arquitetura celular 3D tem um efeito negativo

na regulação da via de sinalização da ARF6 em EVs. As diferenças observadas foram particularmente relevantes no caso das GC-EVs produzidas por células do tipo difuso, uma vez que as EVs produzidas em culturas 3D potenciaram a invasão de células epiteliais não malignas, o que não se verificou com as EVs derivadas de culturas 2D. Este estudo postulou que a alteração da arquitetura celular conduz à modulação do conteúdo em microRNAs e proteínas nas EVs, e à consequente modificação do seu efeito funcional nas células recetoras.

De forma a compreender melhor o impacto da arquitetura celular e das mensagens transmitidas pelas EVs, no Capítulo 2, desenvolvemos ensaios metastáticos *in vivo*, nos quais tratamos os ratinhos com GC-EVs isoladas de culturas 2D ou 3D, antes de serem injetados com células cultivadas em 2D ou 3D (*Publicação III - em preparação*). Células de GC do tipo intestinal, cultivadas em condições 2D ou 3D, disseminaram para o pulmão. Curiosamente, o tratamento prévio com 2D/3D GC-EVs, reduziu a formação de metástases apenas na condição em que foram injetadas células 3D. Relativamente ao GC do tipo difuso, ratinhos em que foram injetadas células 3D apresentaram diminuição da sobrevivência livre de doença, comparativamente àqueles que foram tratados com células 2D. As células 3D do tipo difuso desenvolveram tropismo para o tecido adiposo pardo, tropismo este que foi potenciado pelas EVs, particularmente as que foram obtidas das culturas 3D. A análise comparativa entre o conteúdo das EVs 2D e 3D, demonstrou um predomínio de microRNAs envolvidos na diferenciação de adipócitos, conversão de adipócitos brancos em adipócitos castanhos, e ativação de adipócitos castanhos, em GC-EVs produzidas por células do tipo difuso em 3D. Estes resultados demonstraram que a histologia e a arquitetura celular 3D das células de GC modulam o teor e o efeito das EVs na progressão do GC.

Por fim, no Capítulo 3, analisamos o efeito das Tregs no comportamento das células tumorais e no desenvolvimento do GC (*Publicação IV*). A imuno-fenotipagem de 82 tumores de doentes com GC demonstrou uma elevada prevalência de Tregs em GC do tipo intestinal, por comparação ao tipo difuso. Utilizando o modelo 3D previamente estabelecido para as células de GC, desenvolvemos co-culturas diretas entre Tregs e células tumorais. Através da monitorização destas culturas, verificamos que as Tregs infiltram preferencialmente os esferoides de GC do tipo intestinal. As Tregs induziram a expressão de ILR2 α na membrana das células tumorais do tipo intestinal, bem como ativação da via de sinalização da MAPK e crescimento dos esferoides. Estas observações são consistentes com a identificação de uma nova função de promoção tumoral de GC pelas Tregs. Esta descoberta poderá ter implicações relevantes em estratégias terapêuticas direcionadas ao GC do tipo intestinal, ou ainda para outros tumores que apresentem um contexto imunológico semelhante.

Esta tese demonstrou o papel fundamental da arquitetura celular e da histologia do tumor na modulação do conteúdo e função das EVs, expôs novos mecanismos envolvidos na disseminação do GC, e revelou uma nova e inesperada função das Tregs no desenvolvimento de GC. A realização de estudos adicionais para consolidação destes resultados, poderá conduzir à identificação de novos candidatos a biomarcadores de diagnóstico e prognóstico, bem como inspirar estratégias terapêuticas inovadoras em GC.

Abbreviations

2D Two-dimensional

3D Three-dimensional

ACRG Asian Cancer Research Group

BMP Bone morphogenic protein

CA Carbohydrate antigen

CAFs Cancer-associated fibroblasts

CCL C-C Chemokine Ligand

CCR C-C chemokine receptor

CEA Carcinoembryonic antigen

CIN Chromosomal instability

circRNA Circular RNA

CTLA-4 Cytotoxic T-lymphocyte-associated protein 4

DDR Discoidin domain receptor

EBV Epstein-Barr virus

ECM Extracellular matrix

EGF Epidermal growth factor v

EGFR Epidermal growth factor receptor

EMT Epithelial-mesenchymal transition

EVs Extracellular vesicles

FIGC Familial intestinal gastric cancer

FoxP3 Forkhead Box P3

G-DIF Genomic diffuse

G-INT Genomic intestinal

GAPPS Gastric adenocarcinoma and proximal polyposis of the stomach

GC Gastric Cancer

GITR Glucocorticoid-induced tumour necrosis factor receptor-related protein

GKN Gastrokine

GS Genomically stable

H. pylory *Helicobacter pylori*

HDGC Hereditary diffuse gastric cancer

HGF Hepatocyte growth factor

HMGB Mobility group box

IDO Indole amine 2,3-dioxygenase

IFN- γ Interferon- γ

IL Interleukine

IL2R IL-2 receptor

JAK2 Janus-associated kinase 2

LGR5 Leucine-rich repeat-containing G protein-coupled receptor 5

LncRNA Long non-coding RNA

MAPK Mitogen-activated protein kinase

MHC Major histocompatibility complex

MIF Migration inhibitory factor

MSI Microsatellite instability

MSS Microsatellite stable

MUC Mucin

NF Nuclear factor

PD Programmed cell death

PD-L Programmed cell death ligand

PDXs Patient-derived tumour xenografts

PI3K Phosphatidylinositol-3-kinase

PTEN Phosphatase and tensin homolog

RANK receptor activator of nuclear factor- κ B

RTK Receptor tyrosine kinases

STAT Signal transducer and activator of transcription

SUFU Suppressor of fused protein

TCGA The Cancer Genome Atlas

TCR T cell receptor

TFF Trefoil Factor

TGF Transforming growth factor

TILs Tumour-infiltrating lymphocytes

TLR Toll-like receptor

Tregs Regulatory T cells

TRIM Tripartite motif containing

Prologue

Integrative studies of the tumour and its microenvironment are of utmost importance, and developing complex, yet tuneable, models to study the tumour-microenvironment dynamics is an unmet clinical need. Due to the high heterogeneity of GCs, the 'one-size-fits-all' is not a good strategy to follow even in preclinical *in vitro* experiments. GC models should rather be tailored to recapitulate tumour properties as much as possible, meaning that a bottom-up strategy, in which models are progressively upgraded, could be a robust avenue to improve the translation of preclinical evidences into clinical results.

The present thesis was designed under this conceptualization: to deconstruct GC into specific components that can be studied individually and, thereafter, assembled to achieve a solid understanding of GC biology. My great expectation is that the data resulting from this work could provide insightful knowledge and foster innovative approaches to improve the diagnosis and treatment of GC patients.

In this thesis, General Introduction, details crucial aspects regarding the developed work, by reviewing the current scenario of GC disease and exploring state-of-the-art knowledge and trends related with 3D preclinical tumour models, extracellular vesicles and regulatory T cells in GC. Chapters 1, 2, and 3 describe and discuss the work performed under the scope of this thesis and resulting scientific discoveries. Within these chapters, specific introduction, material and methods, results and discussion sections were included. At last, transversal findings and final observations are presented in the Concluding Remarks section.

General Introduction

1. The Disease Spectrum Called Gastric Cancer

1.1. The knowns of gastric cancer pathogenesis

Gastric cancer (GC) comprises a spectrum of diseases that develop upon a malignant transformation of cells located at the stomach. Nowadays, more than 1 000 000 new cases of GC are diagnosed per year, making this cancer the fifth most prevalent worldwide.¹ From these patients, 60% are expected to develop locally recurrent or metastatic disease, which negatively impacts their prognosis and likelihood of survival. As so, every year, GC causes 783 000 deaths, ranking as the third-leading cause of cancer-related deaths worldwide.¹

GCs are mainly sporadic, with only 10% presenting familial history, from which hereditary forms account for 1-3% (Box 1).^{2,3} Most GCs arise from epithelial cells that compose the glands of the mucosa, and so named adenocarcinomas; only 10% of GCs have origin in other cell types, giving rise to lymphomas, sarcomas, and carcinoids.⁴ Adenocarcinomas often initiate from premalignant lesions of the mucosa *via* the “Correa cascade”.⁵ Briefly, environmental (*e.g.* *Helicobacter pylori* (*H. pylori*) infection, diet, smoking) and genetic factors (*e.g.* polymorphisms, genetic instability) may predispose to chronic and atrophic gastritis, characterized by the loss of glandular elements and replacement by metaplastic cells or fibrosis. Atrophic gastritis may progress to a more severe presentation in which the gastric mucosa is transformed into an intestinal-like phenotype, known as intestinal metaplasia. Through the combination of genetic events and the interaction with the host microenvironment, intestinal metaplasia may give rise to dysplasia and, ultimately, to carcinoma.

Box 1 | Familial forms of GC

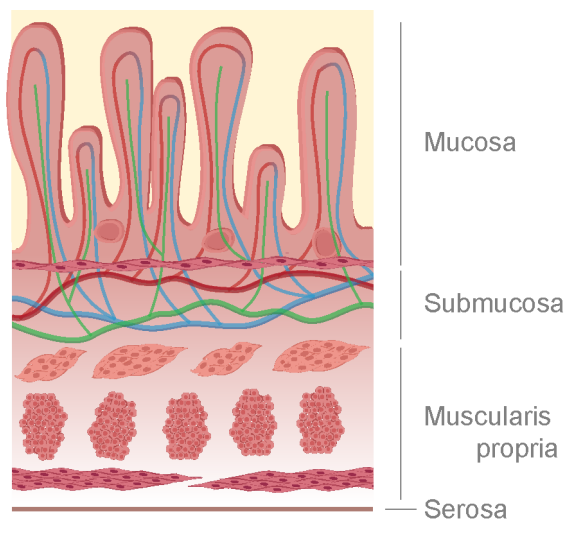
Hereditary GC is represented by three main syndromes: hereditary diffuse gastric cancer (HDGC), gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), and familial intestinal gastric cancer (FIGC). HDGC, the first hereditary syndrome identified in GC, is mainly caused by heterozygous germline mutations of E-cadherin (*CDH1*) or E-catenin (*CTNNA1*) genes.^{6,7} Nevertheless, a reasonable proportion of HDGC cases have unknown inherited cause, which also occurs for non-APC related GAPPS and for all FIGC.⁸

This transformation cascade has been well defined along the years. However, it is not sufficient to explain the pathogenesis of all GC cases. Indeed, GCs that poorly associated with environmental factors are thought to arise from genetic alterations. At the somatic level, GCs display a wide range of molecular alterations,⁹ that include: gene mutations, predominantly of *TP53* gene (~50% of GCs); copy number alterations, namely amplification of *ERBB2* gene (~15% of GCs); gene fusions; epigenetic modifications, alternative splicing; and, non-coding RNA mechanisms, particularly mediated by microRNAs. Such genetic events are at the basis of the malignant transformation of epithelial cells, allowing them to acquire advantages that sustain their local and systemic dissemination.

To successfully disseminate, GC cells often acquire proliferative or mobility skills that enable them to grow or spread locally and gradually along the stomach wall (Box 2, Figure 1).¹⁰ Once tumour cells reach the submucosa, they may intravasate into blood or lymphatic vessels and spread systemically. Dissemination through the lymphatic vasculature results in lymph node metastasis, while haematogenous dissemination typically culminates in liver and lung metastasis. Alternatively, tumour cells that perform a complete invasion of the gastric wall, may directly invade the organs that are adjacent to the stomach, *i.e.*, spleen, pancreas, colon and liver, or disseminate through the peritoneum. In women, intraperitoneal dissemination may lead to ovary metastasis, giving rise to Krukenberg tumours.¹¹ In either case, tumour cells initiate a metastasis cascade that requires survival in the hostile environments of vasculature and intraperitoneal fluid circulation; extravasation, and colonization into other organs. Metastasis, particularly in distant organs, is a demanding and extremely harsh process, to which not all tumour cells are equipped for.¹² Thus, metastasis is a highly inefficient process that may take several years to occur. Unfortunately, this process is thought to be initiated during the early steps of GC progression, where the disease is asymptomatic.¹³ Altogether, these factors give enough time for a successful metastatic event to occur, thus culminating in a high prevalence of metastasis, the most frequent cause of GC patients' death.

Box 2 | Layers of the stomach wall

The stomach wall is structured into four main tissue layers that enable the chemical and mechanic functions of the stomach. The mucosa is a highly specialized glandular layer encompassing surface mucous cells, gastric pits and glands, lamina propria and muscularis mucosa. This layer is followed by the submucosa, a connective tissue containing blood and lymphatic vessels, the muscularis propria, built by three sublayers of muscle, and; the serosa, the fibrous membrane that covers the outside of the stomach.



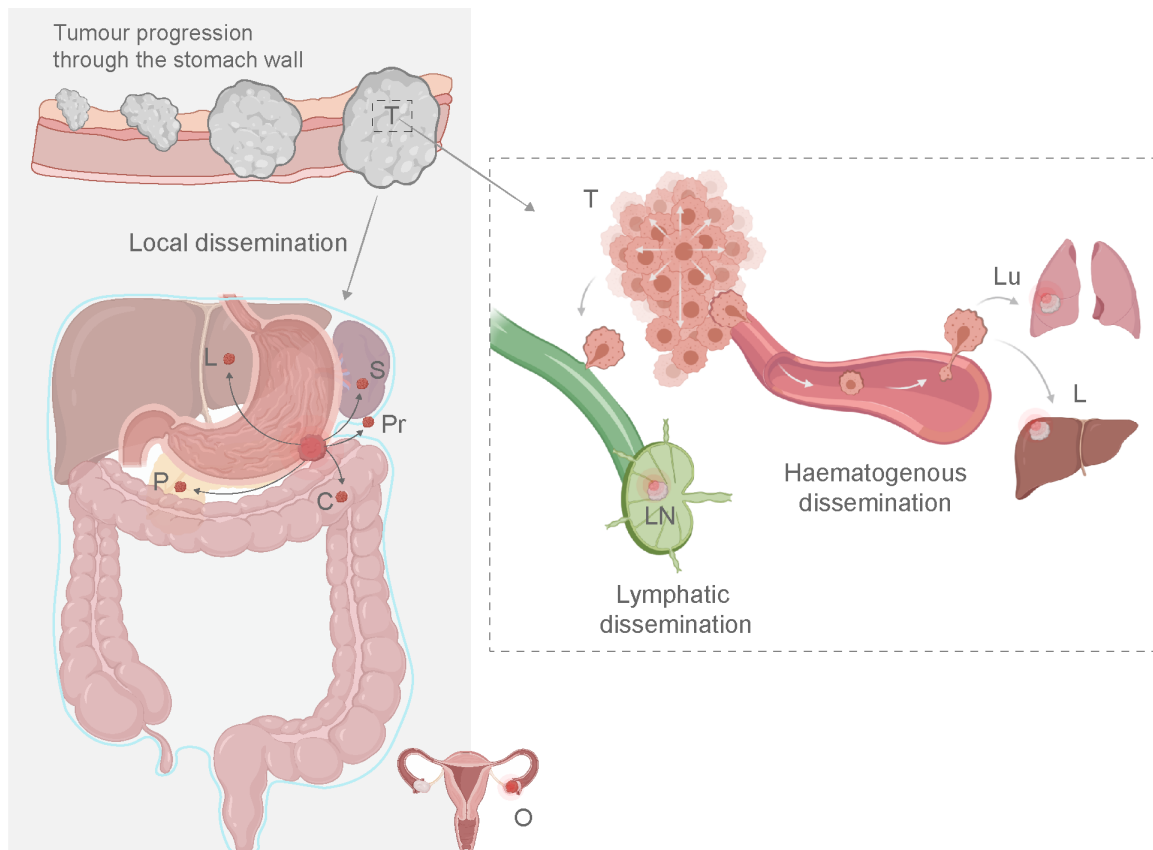


Figure 1. GC local and systemic dissemination. Tumours (T) may disseminate locally, through the complete invasion of the wall, to organs surrounding the stomach, e.g. liver (L), spleen (S), pancreas (P), colon (C) and peritoneum (Pr). Peritoneal dissemination may cause ovary metastasis (O) in women. Tumour cells may also enter lymphatic (green) and blood (red) vessels, and eventually originate metastasis in local and regional lymph nodes (LN), lung (Lu) and liver (L).

1.2. Histological and molecular classification of GC

The intrinsic cellular and environmental factors, along with the location of the GCs may strongly conditionate their growth and behaviour, leading to a wide variety of histological patterns. Adenocarcinomas have been traditionally classified into intestinal and diffuse types, according to Laurén classification (Figure 2).¹⁴ Intestinal GCs, commonly found in elderly people, present glandular structures that grow through the stomach wall, and often disseminate haematogenous to distant organs. Intestinal GCs behave less aggressively than the diffuse GCs,¹⁵ which display poorly cohesive and infiltrative cells that disseminate to lymph nodes and to the peritoneum, inducing peritoneal carcinomatosis. Diffuse GCs are prevalent in young people, particularly in females, and may present a hereditary component. Although both subtypes share similar environmental risk factors, these seem to be more associated with intestinal GCs. Consequently, intestinal GCs are commonly diagnosed in high-risk regions and its incidence is steadily decreasing over time. Although dating 55 years, Laurén's classification continues to be widely accepted and used, due to the clinical relevance of the intestinal and diffuse subtypes. In some patients, tumours simultaneous present intestinal and diffuse growth patterns.¹⁶ These tumours, classified as mixed GCs, associate with larger tumours, enhanced local and lymphatic invasion, and lymph

node metastases, leading to a worst prognosis as compared to ‘pure’ intestinal or diffuse GCs.¹⁶ Nevertheless, some patients present tumours with neither intestinal, nor diffuse features, that are classified as indeterminate GCs. This last group includes a particularly wide range of histological patterns, which fostered the need of more detailed histological classifications, as proposed by the Japanese Gastric Cancer Association and the World Health Organization.¹⁷⁻¹⁹ Although extremely informative, these classifications do not show significant associations with epidemiological, clinicopathological or prognostic features.

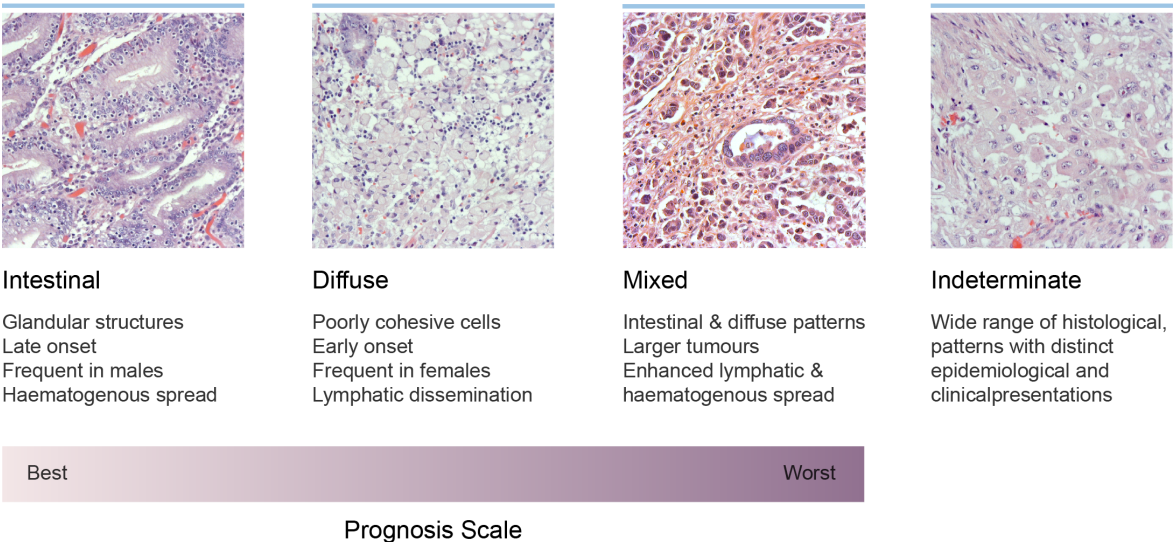


Figure 2. Histological classification of GCs according to the Laurén classification. Representative images of the morphological patterns and summary of the main features for each class of tumours.

Recently, the advent of next-generation sequencing approaches in the study of large series of GCs, guided the identification of new GC classifications based on tumour molecular features. The first classification of GCs was based on gene expression signatures and proposed by Tan and colleagues, in 2011.²⁰ Their study showed that a panel of 37 GC cell lines could be divided into two subtypes: genomic intestinal (G-INT) and genomic diffuse (G-DIF). While G-INT displayed up-regulation of carbohydrate and protein metabolism, and cell adhesion, G-DIF were enriched for cell proliferation and fatty acid metabolism. These subtypes were further validated in 521 patients from four independent cohorts, which showed that patients with G-DIF had poor prognosis, as compared to the ones with G-INT. The overall concordance between their genomic classification and Laurén classification was 64%, which could be potentially explained by the contribution of mixed and indeterminate GCs.

Through the combination of tumour biological features and their response to therapy, Lei and colleagues classified GCs as proliferative, metabolic, and mesenchymal GCs.²¹ These subtypes were respectively associated with an enrichment of gene sets related to cell cycle, metabolism and cell adhesion/extracellular matrix interactions. Both metabolic and mesenchymal GC subtypes had low number of *TP53* mutations and seemed to respond better to treatments, whereas proliferative GCs had high number of *TP53* mutations and shorter disease-free survival.

The efforts made by The Cancer Genome Atlas (TCGA) network further expanded the molecular GC characterization through the simultaneous analysis of somatic copy-number variation, whole-exome sequencing, DNA methylation, mRNA and microRNA sequencing, and protein expression in 295 primary gastric adenocarcinomas.²² This comprehensive analysis culminated in the identification of four GC subtypes: Epstein-Barr virus (EBV)-positive GC, GC with microsatellite instability (MSI), genomically stable (GS) GC, and GC with chromosomal instability (CIN). EBV-positive GCs had pronounced DNA hypermethylation, exhibited recurrent mutations of *PIK3CA*, *ARID1A* and *BCOR*, and amplification of *JAK2* and *ERBB2* genes. This profile indicates that EBV-positive GCs may respond well to targeted therapies based on phosphatidylinositol-3-kinase (PI3K), and Janus-associated kinase 2 (JAK2) inhibitors. MSI GCs, caused by the transcriptional silencing of the DNA mismatch repair gene *MLH1*, harboured high mutation rates and recurrent mutations of *PIK3CA*, and *ERBB3* genes. Infection by EBV, in the first group, and the high mutation load observed in MSI GCs elicit strong anti-tumour immune responses. To counteract this, tumour cells acquire overexpression of programmed cell death ligand (PD-L)-1/2, which binds to programmed cell death (PD)-1 receptors on activated T cells, causing their inhibition.²³ Therefore, EBV-positive and MSI GCs with overexpression of PD-L1/2 are excellent candidates for immunotherapy based on immune checkpoint antagonists. CIN GCs featured marked aneuploidy, and recurrent amplification of receptor tyrosine kinases (RTK) genes, e.g. *ERBB2*. Lastly, tumours without aneuploidy, hypermethylation, or elevated mutation rates were included in the GS subtype. GS tumours had *CDH1* and *RHOA* mutations, and *CLDN18-ARHGAP* rearrangements. Such genetic alterations are known to disrupt cell adhesion and promote cell migration/invasion. While EBV-positive, MSI, and CIN GCs were more associated with intestinal histology, the GS subtype was enriched in tumours histologically classified as diffuse.

Overall, the TCGA study provided crucial information for GC patient stratification and therapeutic targets for distinct patient groups. Nevertheless, no association with clinical outcome or prognosis was found in this cohort. This gap was filled with the study of the Asian Cancer Research Group (ACRG), which analysed targeted sequencing, genome-wide copy number and gene-expression data of 300 primary GCs to identify clinically relevant subtypes of GC.²⁴ Four subtypes of GC were identified based on the clinical outcomes and prognosis: MSI-high GC, microsatellite stable/epithelial-mesenchymal transition (MSS/EMT) GC, MSS/*TP53*⁺ GC, and MSS/*TP53*⁻ GC. MSI-high GC was the group with best overall prognosis and lowest recurrence frequency (22%). The majority of MSI-high tumours had intestinal histology and were diagnosed at early stages. MSI-high GC associated with hypermutation of *ARID1A*, *MTOR*, *KRAS*, *PIK3CA*, *ALK* and, *PTEN* genes. Similar to the MSI group described by the TCGA study, these tumours also displayed overexpression of PD-L1, and had abundant T cell infiltration. At the opposite side of the spectrum, MSS/EMT GCs had the worst prognosis and the highest frequency of recurrence (63%). MSS/EMT GCs were mostly of diffuse-type histology, diagnosed at advanced stages, and at earlier age. Moreover, peritoneal dissemination was frequently observed in patients carrying MSS/EMT tumours. Despite the low mutation load, MSS/EMT GCs often display loss of *CDH1*

expression, which associated with loss of cell adhesion, angiogenesis and motility. The subtypes that had intermediate prognosis and recurrence rates, MSS/*TP53*⁺ GC and MSS/*TP53*⁻ GC, generally displayed intestinal histology and occurred in males. MSS/*TP53*⁺ GCs were characterized by frequent EBV infection and mutations of *ARID1A*, *PIK3CA*, *SMAD4*, and *APC* genes, whereas MSS/*TP53*⁻ GCs featured high *TP53* mutation rates.

Comparing the last two GC classifications, the EBV⁺, MSI, CIN, and GS subtypes defined in TCGA cohort generally match the MSS/*TP53*⁺, MSI, MSS/*TP53*⁻, and MSS/EMT groups proposed by the ACRG. The integrative data provided by these studies paved the way for a better patient stratification, and for the development of tailored therapies and immunotherapies in GC.

1.3. Diagnosis and treatment of GC

GC is asymptomatic during the initial steps of disease progression, and even at late stages symptoms are not specific, which hampers an accurate GC diagnosis. Furthermore, clinically relevant GC biomarkers fail to detect tumors at early stages. Carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)19-9, the most widely used GC serological biomarkers, are increased in advanced stage GC and are predictive of relapse. However, none of these biomarkers, or their combination, are GC specific or present sufficient sensitivity to detect GC at early stages.²⁵ For these reasons, GC patients are often diagnosed at advanced forms of disease progression.

GC screening is usually done by endoscopy, that allows for imaging analysis of the lumen of the stomach and collection of tissue from suspected lesions (biopsies).²⁶ When combined with ultrasounds, endoscopy may also provide information on tumour penetration depth or invasion of adjacent structures. Endoscopy may be complemented with computed tomography scan or magnetic resonance imaging of the chest, abdomen, and pelvis, to evaluate metastasis. The stage, *i.e.*, the extent of cancer, is crucial to define patient prognosis and to design adequate therapeutic strategies. Thus, patients diagnosed with GC undergo multiple tests, allowing the establishment of a clinical stage (cTNM) that guides initial therapeutic decisions.²⁷ Given the benefits observed from preoperative (neoadjuvant) therapy in patients with localized GC, the number of patients that receive this therapy has been rising. Neoadjuvant therapy may reduce tumour burden and metastasis, which affects patient staging. Thus, before continuing the therapeutic course, patients may be re-staged (ypTNM) to potentially adjust therapy. When patients are eligible for surgery, the pathological assessment of the invaded structures will enable the refinement of the staging (pTNM). The TNM staging system for GC describes tumours according to the depth of invasion of the stomach wall (T), the regional lymph nodes that were infiltrated by tumour cells (N), and the presence of distant metastasis (M).

Patients with local or locoregional disease are eligible for surgery, the only potentially curative treatment currently available.²⁸ GC resection surgery may require total, subtotal

or distal gastrectomy, depending on tumour depth, as well as nodal dissection, to remove infiltrated lymph nodes.²⁸ Patients that present inoperable locally advanced or metastatic tumours (Stage IV) have the worst prognosis. If patients are fit enough, they are guided to palliative chemotherapy that shows improved survival and quality of life, as compared to best supportive care alone. Currently, the 5-year survival rate significantly decreases from 57% to 5%, for patients with clinical stage I and IV, respectively (Table 1).²⁷

Table 1. Clinical stage and 5-year overall survival in GC patients. Adapted from the AJCC Cancer Staging Manual²⁷

Clinical stage group	5-year survival (%)	Patients (n)
Stage I	56,7	1418
Stage II	37,4	1079
Stage III	25,9	1427
Stage IV	5,0	3382

The molecular characterization of GC has provided new therapeutic targets and identified patients that may benefit from these therapies, as well as from immunotherapy. Indeed, some of these have already been included in GC therapeutic strategies, mainly for patients with advanced disease. Unfortunately, so far, these therapies have only minimally improved patient prognosis.²⁹

Currently, GC management entails two main problems: the late diagnosis and the limited success of the available therapeutic options for advanced disease. Both problems seem to be partially due to the high tumour heterogeneity, that reflects not only the distinct histological and molecular features observed interpatient, but also the intratumoral heterogeneity, *i.e.*, variations within the same tumour.³⁰ As aforementioned, GC diagnosis is established based on biopsies, reflecting tumour pathology in specific sites of the tumour and at a single timepoint, *i.e.*, the moment of diagnosis. Thus, therapeutic decision is often made upon a static and limited sample analysis, that may not fully represent the tumour. Moreover, biopsies are invasive methods, which hampers the dynamic follow-up of disease progression.

For these reasons, current efforts to find early GC biomarkers are focusing on liquid biopsies (Box 3).³¹ This minimally invasive collection of tumour-derived material would facilitate the dynamic and more frequent monitoring of the disease. Additionally, these samples would include the contribution of all tumour cells, which may reduce the sampling problem associated with tissue biopsies. Given the known pathogenesis of GC, the most promising bodily fluids for liquid biopsies include

Box 3 | Liquid biopsies

Liquid biopsies are samples obtained from body fluids, *e.g.*, blood, saliva, or urine, containing tumour genetic material or bioproducts resulting from its activity. Circulating tumour cells, cell-free nucleic acids (*e.g.*, DNA, microRNAs or long non-coding RNAs) and extracellular vesicles (EVs) have been extensively studied in the demand for novel diagnostic and therapeutic biomarkers from liquid biopsies.

the blood, peritoneal fluid, gastric juice, and lymphatic fluid.²⁵ This strategy has provided a high number of biomarker candidates with good sensitivity and specificity; however, none of these has been validated for clinical use.²⁵

To resume, GC is a highly heterogeneous disease, both genetically and biologically. Accumulating knowledge on the environmental factors that cause GC has been translated into a gradual reduction in GC incidence; however, GC pathogenesis remains largely unknown. Consequently, GC patients are often diagnosed at advanced stages, presenting local recurrent or metastatic disease. For these patients, available treatment options are largely palliative and the 5-year survival extremely reduced. Recent efforts have provided valuable information for GC patient diagnosis, stratification, treatment, and follow-up, but their clinical value is yet to be confirmed. Making use of the great amount of molecular information currently available, prospective studies should focus on addressing specific aspects of GC biology using adequate research models.

2. 3D (Re)Modelling of Gastric Cancer

2.1. Modelling cancer research

Tumours are carved in a complex three-dimensional (3D) microenvironment, where biochemical and biophysical stimuli interact and are integrated to determine cell fate. The high density of cellular and extracellular factors involved in tumour development and progression, is a major hurdle for the development of faithful pre-clinical cancer models.³² Consequently, 90% of the drugs that perform well in pre-clinical studies fail in human clinical trials, leading to a challenging translation of promising scientific knowledge into effective therapies.^{33,34}

Despite the acknowledged importance of 3D cell architecture, as well as cell-cell and cell-matrix interactions, cancer research continues to conventionally use two-dimensional (2D) models that poorly recapitulate tumour features. These 2D cultures are largely performed in flat plastic or glass surfaces, and represent a simple, convenient, and cost-effective option. Moreover, cell monolayers allow an easy handling and integration with a wide range of techniques.³⁵ Nevertheless, these *in vitro* cultures do not reproduce *in vivo* cell architecture, polarization, or growth; hence, their physiological relevance is highly limited.³⁶

In contrast, animal models provide the *in vivo* complexity and dynamics of tumour processes. These models have been widely used to gain insight on the basics of cancer, and for the establishment of adequate therapeutic strategies, before translation into clinical trials. Patient-derived tumour xenografts (PDXs) are one of most common *in vivo* tumour models, and closely mimic the biological tumour features.³⁷ PDXs are generated by subcutaneous or orthotopic transplantation of fresh patient-derived samples into immunodeficient mice. These models are expensive, time- and resource-consuming approaches, and their success may be limited by the efficiency of engraftment, and by the availability of patient-derived

samples in some tumour types. Of notice, as PDXs may undergo mouse-specific tumour evolution, the output provided by these models may diverge from that observed in patients, thus making these models less representative of human tumours.³⁸ Moreover, animal models may not faithfully recapitulate the genetic heterogeneity, histological complexity, immune interactions, and anatomical human contexts.³⁹ Hence, differences between species, may foster distinct responses in animal models and in humans, namely regarding toxicity.⁴⁰ Lastly, animal experimentation raises important ethical considerations, and their use should be restricted as much as possible.

Under this scenario, 3D models have been proposed to improve the output of *in vitro* studies and to reduce the use of animal models. Since the first report of multicellular spheroids in cancer research, in 1971,⁴¹ the field has evolved to better recapitulate tumour specifications and heterogeneity, leading to a complex myriad of 3D *in vitro* cancer models. This path has shown that the composition and architecture of 3D cultures may deeply affect cell behaviour, drug sensitivity, and toxicity.⁴² Thus, to select the most appropriate 3D model, researchers have to consider the pros and cons (Figure 3), the ability to mimic specific biological contexts, and the predictive value of each method.

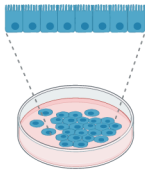
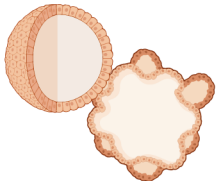
	2D monolayers	Spheroids/organoids	Animal models
PROS	 Well established and inexpensive, easy manipulation and downstream analysis.	 Physiologically relevant, allow a more realistic representation of cellular interactions and <i>in vivo</i> conditions.	 Provide patho/physiological context and dynamis.
CONS	Lack of predictivity and reduced representation of the real cellular environment.	Some models contain undesirable elements or may be difficult to apply in large-scale studies.	Genetic differences may hamper mouse-human comparisons; Technically and ethically challenging.

Figure 3. Pros and cons of the 2D *in vitro* (monolayers), 3D *in vitro* (spheroids and organoids) and animal models.

Spheroids have been the most widely used 3D model in cancer research. These spherical and heterogeneous cell aggregates are generally composed by a layered structure, with rapidly proliferating cells surrounding a more quiescent and hypoxic core.⁴³ Spheroids maintain the 3D architecture and tissue-specific functions, either growing in suspension or embedded in relevant matrices.⁴⁴ This model offers a highly controlled and relevant context to assess cell-cell and cell-matrix interactions underlying tumour growth, progression, and therapeutic response.⁴⁵ Spheroid arrangement generates gradients of nutrients, metabolites, and oxygen that recapitulate those observed in tumours. Interestingly, when spheroids grow

over 400–600 μm , the central core becomes necrotic, as observed in poorly vascularized tumours.⁴⁶ To date, several methods have been proposed to create multicellular spheroids, commonly using immortalized cell lines, which may no longer replicate the genetic heterogeneity of the original sample. To avoid this caveat of *in vitro* models, the use of primary cells should be further optimized.

Spheroids have been widely used to study stem cell properties; this particular subtype of spheroids is generated by seeding low numbers of cells into a non-attachment environment, and under supplementation with specific stemness-associated growth factors. Cells that survive and proliferate, leading to spheroid formation, are thought to have stemness properties. This method has been useful to study and identify adult stem cells in tissues and tumours, which paved the way for the development of organoids.

Organoids are differentiated and self-organized organotypic structures, that can be derived from adult or induced pluripotent stem cells.⁴⁷ This model was first established in 2009 by Sato and colleagues,⁴⁸ who showed that single intestinal stem cells could generate highly polarized epithelial structures, with proliferative crypts and differentiated villus. In this study, cells expressing leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) were embedded in Matrigel and cultured under serum-free medium, containing stem cell niche- and tissue-specific growth factors: R-spondin, a Wnt agonist; epidermal growth factor (EGF), and; noggin, an inhibitor of bone morphogenetic protein. Alternatively, organoids can also be produced using an air-liquid interface, with growth factors being supplied by stromal cells.⁴⁹

Currently, organoids can be derived from several healthy organs, such as intestine, brain, and stomach, but also from tumour tissues.⁴⁷ Organoid cultures can be stably maintained for long periods, and genetically engineered to closely replicate tumour genomic alterations. This methodology holds crucial implications in prospective cancer research, as it enables the parallel study of tumour and healthy tissue-derived organoids, to better evaluate therapeutic efficacy and toxicity. Nonetheless, organoid cultures also display limitations like the lack of blood vessels.⁵⁰ Moreover, their *ex vivo* culture requires the supplementation with growth factors and embedding in an appropriate extracellular matrix (ECM), which may influence or even hamper the study of certain biological questions. An additional concern is the maintenance of their clonal heterogeneity, as organoids containing more than one tumour clone may undergo selective pressure along passages. Thus, during a long-term culture, the overall genotype and phenotype of organoids may be altered, which may induce reproducibility issues.⁵¹

Both spheroids and organoids may be embedded in natural or synthetic ECM structures (Box 4), that provide important environmental cues for the modulation of intercellular signalling, and consequently, cell behaviour.^{52,53} Tissue specific decellularized matrices have been presented as a good option to specifically reproduce the *in vivo* extracellular signals involved in tumour pathogenesis.⁶¹⁻⁶³ The decellularization process involves the removal of cellular components from native or regenerated tissues, maintaining the ECM composition

Box 4 | Synthetic and natural ECM

Synthetic materials, like polyethylene glycol, provide a great control over the physical and biochemical experimental conditions, but they do not represent the *in vivo* matrix composition and they are not bioactive, *i.e.*, they lack interaction with cells. To improve their bioactivity, synthetic materials can be modified to include well-known ECM signals, such as cell adhesion peptides (Arginine-Glycine-Aspartic acid), metalloproteinase sensitive sites, and growth factors. Due to their highly regulated composition, these scaffolds can be easily produced and used for large-scale studies.

Natural matrices, such as collagen or reconstituted basement membrane (*e.g.*, Matrigel), are easy to use, bioactive, and capable of reconstituting the ECM in which tumours arise; nevertheless, their composition is not constant, which may be particularly important regarding their content in growth factors. Moreover, the composition of the ECM varies among tissues. Hence, using mouse sarcoma basement membrane (Matrigel) may not be the best option to recapitulate the ECM of human gastric adenocarcinoma, for instance. To address this problem, several studies have already proposed Matrigel substitutes for organoid cultures.^{54,60}

and architecture.⁶⁴ Experiments using decellularized ECMs may be difficult to implement in large scale due to their limited source. Additionally, differences among individuals, along with intra- and intertumour heterogeneity may hamper the reproducibility of *in vitro* experiments. Further studies, on tissue-specific composition and arrangement of these matrices may allow for its *in vitro* production using, for instance, 3D bioprinting techniques.

Indeed, microfabrication techniques allow the establishment of *in vitro* co-cultures with well-defined localization and composition of ECM and cellular subtypes.⁶⁵ Therefore, complex cultures can now be generated to reconstitute several *in vivo* environments and niches. Combination of the aforementioned techniques with microfluidics further allows the study of diffusion of substrates and nutrients, as well as the effect of mechanical factors.^{66,67} Microfluidics is known to provide a higher control and predictability of the spatio-temporal dynamics of the cell microenvironment. These dynamic models have particularly improved the study of the metastatic cascade, as they enable the follow-up of tumour microenvironment communication, cell migration and invasion, intravasation/extravasation, and colonization.⁶⁸ Notably, the dynamic mechanical forces provided by microfluidics can replicate forces encountered in the body, such as the shear stress in blood circulation.^{69,70} Microfluidic experiments offer the representation of different microenvironments and cell populations confined in independent, albeit connected, compartments. These organ-on-a-chip models are the ultimate *in vitro* representation of physiological and pathological *in vivo* scenarios.

2.2.3D *in vitro* models in gastric cancer

The history of 3D models in GC research (Figure 4) is substantially shorter as compared to other fields. Indeed, the value of these models to better understand GC molecular



Figure 4. Major breakthroughs in GC research using 3D *in vitro* models. Studies using spheroids are represented in black and organoid based studies in grey.

pathogenesis and treatment response is only now being unveiled.

The first 3D models of GC were developed in 2001, when Mayer and colleagues applied the concept of multicellular spheroids to the *in vitro* study of human gastric carcinomas.⁷¹ In this study, a large panel of GC cell lines were cultured as multicellular spheroids, through a liquid overlay technique. From these spheroids, ~70% could recapitulate the growth pattern and differentiation levels of corresponding xenografts in immunodeficient mice. Heterogeneous levels of cell adhesion molecules were found between 2D and 3D conditions, and across spheroids. For instance, E-cadherin, an important player in epithelial differentiation,⁷² which is often deregulated in GC, was expressed in spheroids representing adenosquamous or tubular differentiated tumours but not in those representing poorly differentiated carcinomas, mimicking the observations found in tumour tissue samples.

Few years later, by culturing human GC cell lines under serum-free media and non-adherent conditions, Takaishi and colleagues verified that some of these could produce spheroids.⁷³ Interestingly, only the cell lines that were able to form spheroid colonies were found to be tumorigenic in severe combined immunodeficient mice. These observations suggested the existence of a population of cells with cancer stem cell phenotype among GC cell lines. Such GC-initiating cells could be identified through the expression of CD44 surface marker, which became the first GC stem cell marker to be used in the isolation of cancer stem cells from primary human tissues.⁷⁴ Endowed with self-renewal and tumorigenic capabilities, cancer stem cells play a central role in tumour development, metastasis, and drug resistance.⁷⁵ Thus, after the discovery of GC stem cells, the use of 3D sphere formation assays to study the biology and clinical value of these cells grew exponentially.

Alongside, GC spheroids have been used to test drug delivery, efficacy, and toxicity due to the improved capacity to recapitulate tumour growth, architecture, and differentiation, as compared to traditional monolayered cultures.⁷⁶⁻⁸⁰ Indeed, by culturing GC cells lines in nanofiber scaffolds, Kim and co-workers showed that increased drug resistance, in 3D conditions, was not due to limitations in mass transport, but rather to intrinsic cellular properties.⁸¹

GC cells cultured within collagen-I, Matrigel, or alginate hydrogels, have been shown to lose the expression of standard isoforms of CD44, and gain *de novo* expression of CD44 variant 6.⁸² Notably, the same CD44 splicing pattern observed in 3D spheroids, but not in 2D monolayers, was identified in human premalignant and malignant gastric lesions.⁸³ Of notice, CD44 splicing switch was associated with increased expression of genes involved in metastasis-related processes, like epithelial-to-mesenchymal transition (*Snail*, *ZEB1*), angiogenesis (*ANGPT2*), and aberrant metabolic pathways (*ACLY*, *IGFB7*). Moreover, GC cells cultured as 3D spheroids may undergo extensive modulation of the glycosylation pattern.⁸⁴ Balmaña and colleagues demonstrated that changes in the glycocalyx of GC cells resulted in increased expression of cancer-associated glycan antigens and mucin (MUC)1, reproducing the profile of glycans and glycoproteins observed in tumours. As such, this model provides a new platform to study the therapeutic potential of aberrant or *de novo*

expression of glycan structures arising along the malignant transformation of the gastric mucosa.⁸⁵

This better representation of tumour features by 3D models has been exploited to improve the scarce knowledge on the pathogenesis of GCs. Namely, spheroids and organoids have been used to enlighten the functional relevance of *RHOA* mutations (~14-25%),^{22,86-88} to unveil the molecular mechanisms underlying recurrent mutations of *KMT2C* gene (~41%)⁸⁹, and to validate and modulate the metastatic potential of *TGFBR2* loss⁹⁰ in diffuse-type GCs.

Due to the high resemblance between gastric organoids and the physiological gastric mucosa, these 3D models have also been quite useful to revise the mechanisms underlying GC initiation and development, among which the crosstalk between gastric cells and *H. pylori*.⁹¹⁻⁹⁸

The use of 3D models to study premalignant lesions has shown that IFN- γ promotes parietal cell atrophy during the progression of gastritis to gastric atrophy and metaplasia⁹⁹ and that Trefoil Factor 1 (TFF1) may suppress gastric pathogenesis by blocking the IL6-STAT3 pro-inflammatory signalling axis.¹⁰⁰ Min and colleagues demonstrated that Kras activation controls cellular dynamics and progression to dysplasia, and described cellular heterogeneity in dysplastic cells as the consequence of two simultaneous stem cell populations in dysplasia.¹⁰¹ By further exploring somatic alterations in cancer genes detected in normal and premalignant tissue, Sethi and co-workers showed that the co-deletion of *Cdkn2a* and *Trp53* genes was sufficient to promote GC. Interestingly, *Cdkn2a*^{-/-} *Trp53*^{-/-} mice organoids showed replication stress, which exposed a potential susceptibility to DNA damage response inhibitors.¹⁰² The development of gastric organoids also empowered the study of GC pathogenesis in areas with junction of two types of gastric epithelium.¹⁰³ Although, these regions are known to be cancer-prone, the mechanisms underlying such malignant transformation are not well understood. Fu and colleagues reported that the squamous-columnar junction contained a highly proliferative pool of immature LGR5⁺CD44⁺ cells that were prone to transformation and that originated early dysplastic lesions.

The high fidelity and flexibility rendered by 3D models is also supporting a better integration of molecular and phenotypic heterogeneity observed in human GCs. A comprehensive analysis of organoids developed from 36 GC patients, carrying tumours with distinct molecular features, showed a high prevalence of Wnt and R-spondin signalling independence.¹⁰⁴ The authors showed that Wnt signalling independence was achieved through distinct mechanisms, such as epigenetic regulation and loss of APC protein function. Moreover, simultaneous mutations in *CDH1* and *TP53* genes conferred R-spondin signalling independency. This study showed, for the first time, a successful translation of genotype-based knowledge into important phenotypic features of human GCs.

Other studies have indicated a high predictive value of organoids to determine therapy response and resistance to standard-of-care chemotherapy or targeted therapies, according to the molecular profile of human GC organoids (e.g., trastuzumab for *ERBB2* alterations and palbociclib for *CDKN2A* loss).^{105,106} On the other hand, the analysis of the gene

expression profiles of intestinal and diffuse spheroids led to the identification of candidate genes for targeted therapy.¹⁰⁷ *ERBB3* expression was specifically increased in intestinal spheroids, and its downregulation resulted in suppression of both *in vitro* spheroid formation and *in vivo* tumour growth. Of notice, *ERBB3* expression in intestinal-type GC correlated with decreased patient survival.

Finally, the great flexibility of spheroid cultures facilitates the study of the interactions established between the tumour and its microenvironment. Notably, co-cultures between GC spheroids and fibroblasts demonstrated the important role of fibroblasts in the *in vitro* invasion and *in vivo* peritoneal dissemination of GC cells.^{108,109} Similarly, Zhao and colleagues addressed the molecular mechanisms underlying tumour innervation through the analysis of organotypic cultures of GC cells and neurons.¹¹⁰ These authors found that neuron stimulation promoted tumour growth *via* Wnt and cholinergic signalling pathways. Concordantly, pharmacological inhibition or genetic knockout of the muscarinic acetylcholine M3 receptor suppressed gastric tumorigenesis.

2.3. New approaches to improve gastric cancer preclinical studies

The aforementioned models have greatly contributed to the understanding of GC pathogenesis, and may be of utmost importance for the development of personalized therapies for GC patients.

Following the success of organoid-based drug screenings performed for breast, colorectal and prostate cancers, high-throughput screening platforms using patient-derived organoids may revolutionize treatment strategies in GC.¹¹¹⁻¹¹³ Due to the high heterogeneity of GC, organoid biobanks should include models of the distinct molecular and histological phenotypes encompassed in GC pathogenesis.¹¹⁴ The simultaneous use of these organoids for molecular profiling and drug response assessment, may unveil causal genetic and epigenetic alterations that could improve patient stratification and patient-centred treatment regimens. Additionally, organoids may be particularly relevant for the study of hereditary or less common forms of GC, which are often challenging to faithfully recapitulate using *in vitro* and animal models.

The recognized impact of tumour microenvironment elements on drug response highlights the need for upgraded *in vitro* cultures including vasculature and immune cells.¹¹⁵ As previously observed for mismatch repair-deficient colorectal cancer and non-small cell lung cancer, tumour organoids can be cultured with patient immune cells to enrich for tumour-reactive T cells and to assess the sensitivity of tumour cells to T cell immune response.¹¹⁶ Of notice, T cells cultured with patient-derived organoids are known to maintain the original tumor T cell receptor (TCR) spectrum and elicit anti-tumour cytotoxicity.¹¹⁷ Overall, these studies suggested that advanced co-cultures with patient organoids and autologous immune cells may be important tools for immuno-oncology investigations and may accelerate personalized immunotherapy testing.

Spheroids provided a flexible option to study the complex tumour intercellular communications, as they can be cultured with a wide range of ECM and cell populations. Nevertheless, to potentiate the value of these models, future efforts should prioritize the use of primary normal and GC cells,¹¹⁸ and the integration with microfabrication and microfluidic techniques.^{119,120} The development of tissue/organ inspired models that integrate local cellular interactions with distant communication may provide new molecular targets to control or prevent GC progression and metastasis.

Overall, the complexity of the model should reflect the complexity of the biological question and include the necessary elements that may influence the outcome. As a model, these approaches will never perfectly reproduce human biology, yet a fine and balanced complexity may highly improve their predictive value and pave the way for ‘clinical trials-on-chip’.

3. Networks of Communication in Gastric Cancer

3.1. Intercellular communication

From macro to micro, life is only sustainable due to the orchestration of several layers of communication. Indeed, physiological complexity and functioning are maintained by deep communication networks established between, and within, systems, organs, tissues, and cells, that ensure a dynamic exchange of messages from one cell to another. This mechanism is also crucial for the successful tumour development and progression.

Intercellular communication is usually described according to the ‘length’ of the interactions.¹²¹ Direct cell communication typically regards autocrine and juxtacrine signalling, depending whether the recipient cell, *i.e.*, the cell that receives the information, is the donor cell itself, or a direct neighbour cell. Indirect cell communications can be established locally *via* paracrine and synaptic signalling, or within a long distance through endocrine communication. Most commonly, these communications are accomplished through molecular signals, such as proteins and small peptides, lipids, carbohydrates, and nucleic acids. These bioactive molecules interact with recipient cells and lead to modifications in the intracellular signalling pathways, often producing a specific cellular response. Intercellular communication is supported by highly regulated mechanisms that may include the production of supramolecular structures, such as tunnelling nanotubes,¹²² that physically connect cells, or membrane-derived vesicles that are secreted to the extracellular *milieu*.

EVs is the general term used to describe small organelle-like vesicles that act as signalling shuttles both in physiological and pathological settings.¹²³ Although the first reports of EVs can be dated to 1946,¹²⁴ only 50 years later researchers realized that these vesicles could be more than ‘platelet-dust’ particles and cellular ‘waste bags’. The study of Zitvogel and colleagues suggested, for the first time, a role of EVs in immune intercellular

communication.¹²⁵ It is now acknowledged that EVs display stable configurations that enable them to mediate intercellular communication not only locally, but also systemically. For this reason, EVs are commonly found in bodily fluids, such as blood, urine, and saliva, from which they can be isolated in a minimal invasive manner. Moreover, since their cargo is, to some extent, representative of the donor cell features, many have hypothesized the use of EVs as markers of disease. Thus, these biological materials could provide a minimal invasive and stable platform to study biomarkers in cancer research.

The term 'EVs' refers to a variety of vesicles with distinct biogenesis, cargo, size, and functional effects, that have been traditionally classified as exosomes or microvesicles. Exosomes are intraluminal vesicles, formed by the inward budding of late endosomal membranes, which are secreted upon fusion of the multivesicular endosome with the plasma membrane.^{126,127} On the other hand, microvesicles result directly from the outward budding of the cellular membrane.¹²⁸ These distinct biogenesis mechanisms (Box 5) are thought to determine different cargoes in the two vesicle subtypes, and a generally smaller size for exosomes (30-150 nm).¹²⁹ Independently of the biogenesis mechanism, EVs maintain the same membrane orientation as the donor cells, ensuring that the extracellular domains of transmembrane molecules continue to be exposed, while the cytosolic components are enclosed.¹³⁰

Once secreted, EVs use these extracellular domains to interact with recipient cells; EVs may either bind to receptors at the surface of recipient cells, fuse with their plasma membrane, or be internalized through endocytosis/phagocytosis mechanisms.¹³⁰ This interaction is expected to induce a functional response in recipient cells, through the growth factors and bioactive lipids expressed at EVs' surface, transfer of membrane receptors, or delivery of EV enclosed cargo. Hence, the result of EV-recipient cell interaction is dependent on the status of these cells and on the cargo of EVs.

Currently, the high heterogeneity of EV populations hampers the identification of specific EV markers and the proper classification of EV subtypes.¹³¹⁻¹³³ Following the guidelines of the International Society of Extracellular Vesicles, the present work makes use of the general term 'EVs' to describe the contributions of both exosomes and microvesicles.¹³⁴

3.2. Extracellular vesicles in the tumour microenvironment

The biological functions and therapeutic potential of EVs have been recurrently studied under the scope of cancer progression.^{134,135} A growing body of evidence has strengthened the crucial role of EVs in the crosstalk between tumour and its microenvironment, as well as in the preparation of the pre-metastatic niche. In fact, tumour-derived EVs have been shown to foster and support several events of tumour development, progression, and metastasis.

To accomplish this, functional molecules, like bioactive lipids,¹³⁶ mRNAs and microRNAs,¹³⁷ proteins,¹³⁸ retrotransposon elements,¹³⁹ and DNA¹⁴⁰ are actively sorted to tumour-derived EVs, that will convey the message to malignant and non-malignant cells,

Box 5 | Biogenesis of extracellular vesicles

The wide heterogeneity of EVs is partially explained by its distinct mechanisms of biogenesis, sorting of cargo and secretion.¹²⁸⁻¹³⁰ Intraluminal vesicles (which give rise to exosomes upon exocytosis) may be formed due to the negative curvature of the membrane of late endosomes induced by ceramide, a product of sphingomyelin hydrolysis by neutral sphingomyelinase 2. Alternatively, endosomal sorting complex required for transport (ESCRT)-I/II may trigger the inward budding of the endosomal membrane, which culminates in the formation of intraluminal vesicles due to membrane constriction and fission promoted by ESCRT-III and ATPase vacuolar protein sorting-associated protein 4 (VPS4). Along these processes, cargo can be clustered and sorted to the vesicles by ESCRT dependent (sorting of ubiquitinated proteins by ESCRT-0) or independent processes, namely mediated by syntenin and small GTPase ADP ribosylation factor 6 (ARF6). After maturation, multivesicular endosomes fated to exocytosis are transported to the plasma membrane where they dock to the soluble N-ethylmaleimide-sensitive component attachment protein receptor (SNARE) proteins to fuse with the plasma membrane. This intracellular trafficking is notably regulated by several RAS-related protein GTPases, including RAB7, RAB35 and RAB27. While the mechanisms applied to exosome biogenesis have been well described along the years, the knowledge on microvesicles remains scarce. It is currently known that microvesicles may emerge from rearrangement of the plasma membrane or Ca^{2+} -dependent mechanisms. In the latter scenario, Ca^{2+} dependent enzymes, such as flippases/floppases, scramblases and calpain, promote the curvature of the plasma membrane through asymmetries of the membrane phospholipids. Thereafter, actin and myosin arrangements, and ATP-mediated myosin contraction enable vesicle release. Moreover, production of microvesicles may be triggered by ceramide-mediated budding, which are initiated by the activity of acid sphingomyelinases. Deregulation of EV biogenesis in cancer supports the general observations that tumour release higher amount of EVs, with distinct cargo, compared to non-malignant cells. Thus, understanding the basis of EV formation may unveil important therapeutic targets and biomarkers of disease.

located at the tumour surroundings, or at distant sites. As previously mentioned, the content and surface molecules in EVs may reflect the identity of donor cells. Consequently, the high heterogeneity observed in human cancers is also represented in the cargo and functional activity of the secreted EVs. Moreover, secreted EVs may interact with virtually any cell type, and produce distinct effects in distinct recipient cells. Such combinatorial events lead to a wide panoply of EVs-associated mechanisms in several cancer models. Here, keynote studies demonstrating potential roles of tumour-derived EVs in the regulation of tumour communication will be addressed, particularly emphasizing the contribution of EVs in GC (GC-EVs) (Figure 1).

The role of EVs in cancer can be generally deconstructed into three components: tumour-tumour communication, local tumour-stroma interactions, and preparation of metastatic niches at distant sites. Studies performed in several cancer models, including GC, have shown that tumour-derived EVs induce tumour cell proliferation through distinct mechanisms. This effect was firstly reported by Skog and colleagues, in 2008, who demonstrated that messages delivered by glioblastoma EVs were translated by recipient

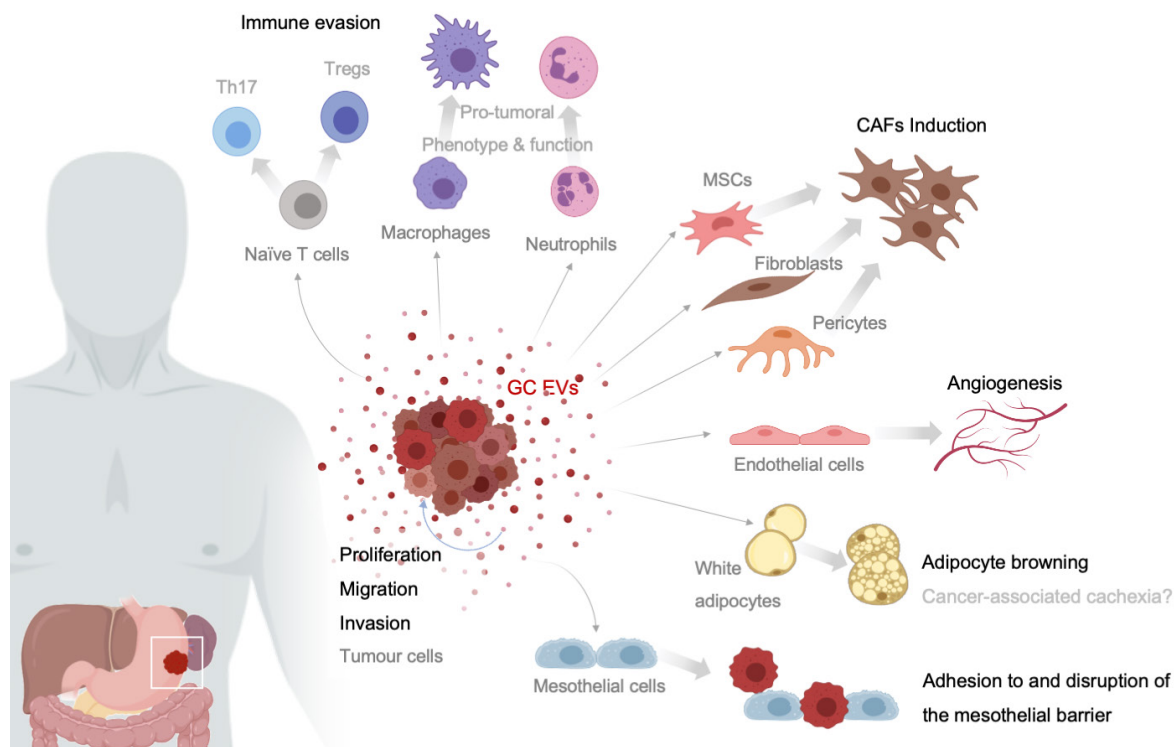


Figure 5. Contribution of EVs to GC development and progression. Schematic representation of the main roles of EVs in the communication between tumour cells and the cell populations located at their microenvironment.

cells, and that these EVs could induce proliferation of a human glioma cell line.¹⁴¹ One year later, Qu and co-workers showed a similar auto-promoting effect in GC cells, in the first report on the role of EVs in GC.¹⁴¹ GC-EVs perceived by GC cells promoted tumour proliferation through the activation of PI3K and MAPK pathways, an effect that was further validated in other *in vitro* and *in vivo* GC studies.^{142,143} Among these, Li *et al.* found that CD97 was involved in the EV-mediated enhancement of tumour cell proliferation and invasion.^{144,145} Additionally, miR-423-5p transported by GC-EVs inhibited the expression of suppressor of fused protein (SUFU), thus enhancing tumour cell proliferation and migration.¹⁴⁶ The horizontal transfer of information among tumour cells is also at the basis of drug resistance. For instance, EVs released by paclitaxel-resistant GC cells, carrying miR-155-5p, could reduce paclitaxel chemosensitivity in sensitive cells.¹⁴⁷ Similar drug resistance mechanisms have been additionally identified in prostate, lung, breast and liver cancers.¹⁴⁸⁻¹⁵¹ Notably, these mechanisms, albeit distinct, seem to be mainly mediated by microRNAs, e.g. miR-100-5p in lung, or miR-222 in breast cancers.^{152,153}

Tumour local and systemic progression entails constant interactions with immune cells, either from innate or adaptative system. Hence, it is not surprising that EVs have been extensively implicated as mediators of anti-tumour immunity and tumour immune evasion.¹⁵⁴ In GC, tumour-derived EVs modulate the phenotype of T cells, macrophages, and neutrophils towards a pro-tumoral environment. Specifically, GC-EVs promote differentiation of naïve T cells into regulatory T cells and Th17 subpopulations *via* TGF- β and miR-451-mediated mTOR activation, respectively.^{155,156} As for macrophages, two distinct mechanisms were

identified. Firstly, GC-EVs were shown to convert macrophages into a pro-inflammatory phenotype due to the stimulation of nuclear factor (NF)- κ B pathway;¹⁵⁷ then, treatment with GC-EVs promoted increased expression of PD-1 in tumour-associated macrophages, which was sufficient to suppress the activity of cytotoxic T cells expressing PD-L1.¹⁵⁸ In both situations, the modulation of macrophage phenotype contributed to tumour growth and progression. A similar scenario was found when neutrophils were treated with GC-EVs.¹⁵⁹ In this case, GC-EVs carrying high mobility group box (HMGB)-1 interacted with toll-like receptor (TLR)4 to activate NF- κ B pathway. Consequently, neutrophils polarized into N2 tumour-associated neutrophils, that display increased autophagic response and promote GC cell migration. In other cancer models, tumour-derived EVs were shown to inhibit the maturation and function of dendritic cells, and induce apoptosis or suppress the activity of cytotoxic T cells and natural killer cells.^{143,160} Notwithstanding, EVs carry tumour antigens that elicit anti-tumour responses mediated by CD8⁺ and CD4⁺ T cells, and by cytokines or heat shock proteins that enhance tumour immunity.^{161,162}

As the tumour crosstalk is bidirectional, EVs released by immune cells may also impact GC progression. As example, macrophage-derived EVs carrying miR-21 stimulated GC cell migration, suppressed apoptosis by inhibition of programmed cell death (PDCD) 4, and conferred cisplatin resistance due to down-regulation of phosphatase and tensin homolog (PTEN) and PI3K activation.^{163,164} Furthermore, macrophages produced apolipoprotein E-EVs that promoted GC cell migration, though PI3K signalling pathway.¹⁶⁵ Again, immune cells may also produce anti-tumour EVs that act on other immune cells to potentiate tumour immunity.

Fibroblasts are a dominant population in most solid tumours. Under specific stimuli, these stromal cells may undergo conversion into CAFs, whose main functions include ECM deposition and remodelling, supporting tumour growth, and interaction with infiltrating immune cells. Accordingly, tumour-derived EVs have been implicated in the mechanisms of CAF induction. GC-EVs induced CAFs from normal fibroblasts, umbilical cord-derived mesenchymal stem cells, or pericytes, *via* exosomal miR-27a, TGF- β and bone morphogenic protein (BMP)2, respectively.¹⁶⁶⁻¹⁶⁸ TGF- β carried by GC-EVs was shown to interact with its receptor in human umbilical cord-derived mesenchymal stem cells, promoting Smad pathway; whereas BMP2 induced PI3K and MAPK signalling pathways in pericytes. CAF induction supported proliferation, motility and metastasis of GC cells, through mechanisms that also include EV-mediation.^{166,169}

Recent studies have highlighted the participation of GC-EVs in other local tumour-associated events. Yang and colleagues showed that the uptake of GC-EVs by vascular endothelial cells promoted angiogenesis by miR130a-mediated inhibition of *c-myc* gene.¹⁷⁰ Moreover, by functioning as a miR-133 sponge, ciRS-133 activated PRDM16 expression, which promoted the conversion of white to brown adipocytes.¹⁷¹ Adipocyte tissue browning is known to underly adipose tissue loss, indicating that ciRS-133 may mediate the development of cancer-associated cachexia.

Lastly, Arita *et al.* showed that EVs isolated from GC cells lines were internalized by other GC cells and human normal mesothelial cells.¹⁷² GC-EVs promoted migration on GC cells, and adhesion between mesothelial and GC cells, due to increased expression of fibronectin-1 and laminin γ -1. Following these observations, GC-EVs were shown to induce peritoneal mesothelial cell infiltration, which accelerated tumour invasion through the stomach wall.¹⁷³ Moreover, GC-EVs promoted apoptosis of peritoneal mesothelial cells and mesothelial-to-mesenchymal transition, resulting in the destruction of the mesothelial barrier and peritoneal fibrosis.^{174,175} MiR-21-5p carried by GC-EVs targeted Smad7 in peritoneal mesothelial cells to promote mesothelial-to-mesenchymal transition, and consequent GC peritoneal dissemination.¹⁷⁵ Altogether, these findings provided strong evidence on the role of GC-EVs in peritoneal dissemination, a particularly relevant aspect of diffuse-type GC progression.

As the tumour develops locally, EVs may disseminate systemically to modulate future metastatic niches. Indeed, the successful colonization by disseminated tumour cells relies on a permissive metastatic environment. The first step to improve such permissiveness is to facilitate the arrival of tumour cells at the metastatic site. Peinado and colleagues unveiled that EVs secreted by melanoma cells fostered metastasis through the transfer the oncoprotein c-Met to bone marrow-derived cells.¹⁷⁶ C-Met induced the mobilization of provasculogenic and hematopoietic bone marrow progenitor cells to ensure a vascularized and immunosuppressive environment at the premetastatic niche. Accumulation of bone marrow-derived macrophages was also observed in liver metastasis.¹⁷⁷ Briefly, pancreatic cancer-derived EVs were shown to contain macrophage migration inhibitory factor (MIF), and induce a pro-inflammatory microenvironment (expression of TGF- β , S100A8 and S100P) upon uptake by Kupffer cells. Under this inflammatory environment, hepatic stellate cells produced and accumulated fibronectin, which promoted recruitment of bone marrow-derived cells at the liver premetastatic niche.

Current understanding on the EV-mediated metastasis indicates that tumour EVs inherit a certain organotropism from their donor cells that could be partially determined by the pool of integrins that EVs express at their surface.¹⁷⁸ Interestingly, the organotropism displayed by EVs was predictive of the metastatic sites. Mice treated with EVs isolated from lung-tropic cancer cells could redirect metastasis of bone-tropic cancer cells towards the lung.¹⁷⁸

To date, although EVs have been implicate in GC metastasis, the underlying mechanisms remain poorly exploited. Following the abovementioned studies, Zhang *et al.* showed that GC-EVs containing epidermal growth factor receptor (EGFR) were uptaken by stromal cells, when delivered into the liver.¹⁷⁹ Thereafter, EGFR-EVs activated hepatocyte growth factor (HGF) by suppressing miR26a/b, which facilitated liver colonization by GC cells expressing c-MET receptor.

3.3. Clinical heterotypic contributions of extracellular vesicles

The uniqueness of EVs' stability and systemic spread, along with their ability to recapitulate tumour heterogeneity and dynamic changes, have pointed EVs as promising sources of GC biomarkers. Moreover, the functional role played by EVs in local and systemic GC communication may be harnessed to develop new therapeutic strategies.

Early studies have shown that cancer patients often display increased concentrations of EVs in plasma, as compared to normal healthy donors.¹⁸⁰ Notably, EVs from patient samples may enclose distinct expression profiles or even genetic alterations found in the original tumour samples.¹⁸¹⁻¹⁸³ In GC patients, few genomic alterations have been found in GC-EVs. In fact, there is only one study showing that EVs isolated from the gastric juice of GC patients, displayed distinct methylation status of *BARHL2* gene, which discriminated GC patients from healthy donors with a 90% sensitivity and 100% specificity.¹⁸⁴

On the other hand, EVs isolated from patient and healthy donors often display distinct transcriptomic profiles, mainly regarding microRNA, long non-coding RNA (lncRNA), and circular RNA (circRNA). The close analysis of these profiles has provided a great number of potential biomarkers which, alone or as a panel, could be used in GC diagnosis and prognosis.¹⁸⁵

EV-proteins that have demonstrated a role in the communication between tumour cells and their microenvironment, may also have clinical value. Both GASTROKINE (GKN)1 and tripartite motif containing (TRIM)3 EV-associated proteins were found to be significantly decreased in the serum of GC patients, as compared with healthy donors.^{186,187} In fact, GKN1 levels in EVs could even discriminate GC patients from patients with non-neoplastic gastric lesions (atrophic gastritis), hepatocellular or colorectal carcinomas.¹⁸⁶ Exosomal TGF- β 1 was correlated with advanced stage GC and lymph node metastasis, suggesting a putative predictive role of GC prognosis.¹⁸⁵ Of notice, these observations await validation in larger and independent cohorts.

As the role of EVs in GC is only now being unveiled, the therapeutic potential of GC-EVs remains fairly unknown. A pioneer study from Zhong and co-workers, demonstrated that EVs isolated from malignant ascites of GC patients induced maturation of dendritic cells and tumour-specific cytotoxic T cell response.¹⁸⁸ Moreover, it was suggested that the release of EVs by GC cells could be hindered using proton-pump inhibitors, which would reduce CAF induction and improve drug response.¹⁸⁹ Although therapeutic strategies targeting the release of EVs by tumour cells have been widely suggested, the effects on EV-mediated physiological communications remain unknown.^{190,191} Further studies on the secretion of EVs in physiological and pathological conditions are required to develop strategies with minimal secondary effects.

Lastly, the ability of EVs to carry and deliver distinct bioactive molecules has been exploited for the development of smart drug delivery systems. In GC, anti-miR-214 loaded in EVs reversed GC cell resistance to cisplatin, while HGF siRNA-loaded EVs suppressed

proliferation and migration of GC cells *in vitro*, and tumour growth and angiogenesis *in vivo*.^{192,193}

Currently, several challenges and caveats are hampering the clinical translation of EV research. Firstly, the nomenclature.¹³³ A growing body of evidence has demonstrated that distinct biogenesis mechanisms originate EVs with specific features. As the cargo and biochemical characteristics change, the role of the different subtypes of EVs in tumour progression may also differ.¹⁹⁴ Hence, a clear classification method is required to enlighten the mechanisms of action for specific EV categories. This first challenge is intrinsically related with the absence of standardize protocols for isolation, quantification, and analysis of EVs. Indeed, isolation methods are known to directly influence the representation of EV subtypes, introducing a bias in the downstream analysis of EVs and their functional effects. Together, these issues are hindering the translation of EV-based therapeutic strategies into clinics. The use of EVs as drug vehicles further requires the definition and validation of appropriate cellular sources of EVs, methods of large-scale production, storage and formulation of EV preparations.^{195,196} As for the targeting of EVs, well-defined mechanisms of action and toxicity effects are yet to be determined.

As discussed in section 2, improved 3D *in vitro* models may better resemble physiological and tumour features. However, the impact of 3D cell organization has been widely disregarded in EV research. Understanding the effect of 3D cell architecture in the biogenesis, release, uptake, and functional translation of EVs' cargo, is of utmost importance and may clarify the aforementioned concerns in EV investigations.

4. The Immune Landscape of Gastric Cancer

4.1. Tumour immunity

The tumour microenvironment is composed by a complex *milieu* of malignant and non-malignant cells, including adjacent epithelial cells, fibroblasts, endothelial cells, and immune cells (Figure 6). The close proximity between tumour cells and these stromal cells intensifies their interactions, which are known to affect tumour progression. The final result, *i.e.*, tumour promotion or tumour damper, relies on the balance between pro- and anti-tumour effects, and on the ability of tumour cells to subvert stromal cells towards pro-tumoral phenotypes. Immune cells play a key role on this balance, as tumours develop under a tight immune surveillance and need to evade anti-tumour immune response to progress. Thus, understanding the immune cell landscape and the mechanisms of immune evasion, is of utmost importance for the development of effective cancer therapies.¹⁹⁷

Innate and adaptative cells involved in the anti-tumour immune response differently infiltrate tumours, leading to specific immunological contexts. Hence, tumours have been classified as 'hot' or 'cold', regarding their infiltration by immune cells: hot tumours, also known as T cell inflamed, present abundant levels of tumour-infiltrating lymphocytes (TILs),

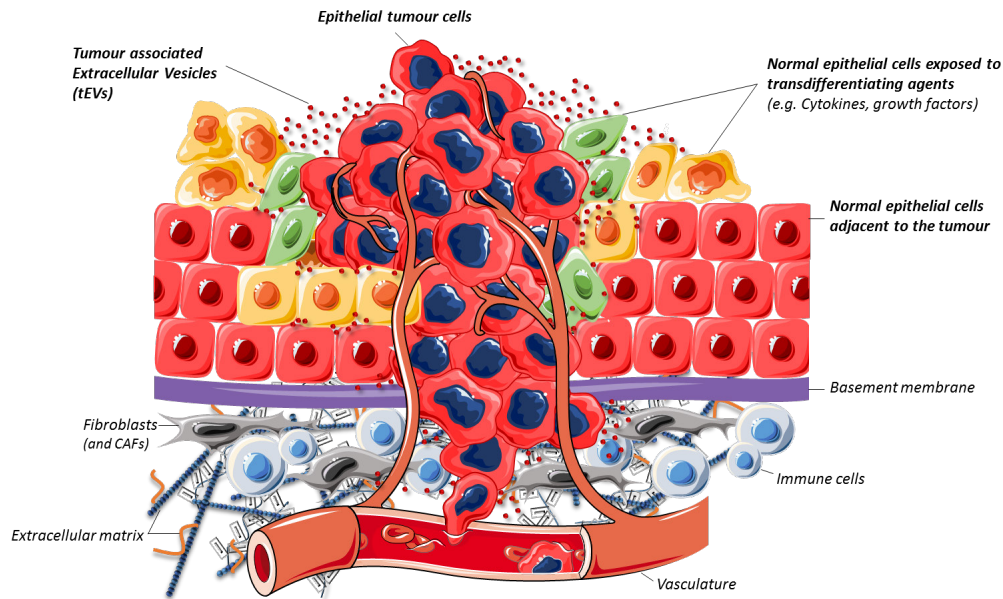


Figure 6. Tumour microenvironment. Invasion of the basement membrane renders tumours a high contact with stromal cell populations, including with immune cells.

whereas cold tumours display absence or very low number of TILs.¹⁹⁸⁻²⁰⁰ The former tumours elicit an initial immune response that is later on inhibited, either by upregulation of immune checkpoints or by an exacerbated immunosuppression activity. Cold tumours may also elicit immune response, however, in this case, immune cells cannot infiltrate the tumour; these tumours display an immune-excluded phenotype. In contrast, cold tumours that do not stimulate immune cells have an ignorant phenotype.²⁰¹ As the anti-tumour immune response differs among tumours, so it will the mechanisms of evasion. Moreover, tumours that have an immune ignorant phenotype will not respond to current immunotherapeutic strategies, such as immune checkpoint inhibitors.

Tumours that elicit an immune response often display aberrant antigen expression, or production of neoantigens, that can passively diffuse through lymphatic vessels to regional lymph nodes, or be actively transported by antigen presenting cells (e.g., dendritic cells and macrophages). At the tumour-draining lymph nodes, antigen presenting cells display tumour antigens to helper (CD4⁺) and cytotoxic (CD8⁺) T cells, inducing their activation. Specific chemokine gradients and adhesion molecules will guide these activated effector T cells to the tumour region, where they recognize and kill tumour cells, by secretory granule exocytosis (e.g., perforins and granzymes) or through the activation of death receptor pathway. Importantly, antigens resulting from tumour cell death may again be transported to draining lymph nodes to potentiate the anti-tumour immune response.²⁰² If this cancer-immunity cycle functions properly, the immune system would be able to fight and eradicate tumours.

However, to successfully thrive some tumours develop mechanisms of immune evasion that impair effector T cell function, activation, proliferation, and survival. These mechanisms include upregulation of inhibitory immune checkpoint molecules, activation and/or attraction of inhibitory immune cells (e.g., myeloid-derived suppressor cells, Tregs, and immature

dendritic cells), or conversion of anti-tumoral macrophages and neutrophils into pro-tumoral phenotypes. Under this scenario, Tregs have a central role on the immunosuppressive activities developed at the tumour microenvironment.

4.2. Immune evasion by regulatory T cells

Tregs are primarily formed during thymic development (natural Tregs) by agonist selection, *i.e.*, the selection of T cells with intermediate self-reactivity.²⁰³ Alternatively, naïve T cells exposed to TGF- β , indole amine 2,3-dioxygenase (IDO), or retinoic acid, may be converted into Tregs at the periphery (peripheral Tregs).^{204,205} Treg development requires TCR signalling, co-stimulation by CD28 and glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR), and high concentrations of exogenous IL(interleukine)-2.²⁰⁶⁻²⁰⁹ Tregs are not known to express any specific marker, and forkhead Box P3 (FoxP3), a transcription factor that orchestrates their immunosuppressive function, has been suggested as the most reliable molecular marker.²¹⁰⁻²¹² In addition to FoxP3, Tregs also express high levels of α -chain IL-2 receptor (IL2R or CD25), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), IL-10, TGF- β , and GITR.

Under physiological conditions, Tregs account for a minor subpopulation of CD4⁺ T cells (4-5%), and they negatively regulate effector T cells to avoid self-reactivity and maintain peripheral tolerance. In cancer, Tregs accumulate at early stages of the disease trajectory and their immunosuppressive activity hinders an appropriate establishment of tumour immunity.²¹³ The comparison of Tregs in blood, normal tissues, and tumours demonstrates that, despite some common features, the three types of Tregs are distinct. Notably, Tregs associated with tumour or normal tissues display increased similarity due to their high activation *status*.²¹⁴ At a first glance, such observation could indicate that the majority of tumour-infiltrating Tregs would arise from tissue-resident Tregs. Nevertheless, complementary studies analysing the TCR repertoire of tumours and normal tissue suggested that tumour associated Tregs are generally recruited from outside tissues, like lymphoid tissue, or blood.²¹⁵⁻²¹⁷

Several factors have already been identified as promoters of Treg accumulation in tumours. Tumour cells and innate immune cells that infiltrate tumours, such as macrophages and dendritic cells, produce cytokines and chemokines that activate local Tregs or induce their recruitment from peripheral regions.²¹⁸ Concomitantly, high concentrations of IL-10, TGF- β , and IDO, at the tumour regions, may induce conversion of helper T cells into Tregs.²¹⁹ The metabolic *status* of tumours is also considered to promote immune evasion. To support their energy and proliferation demands, tumours often undergo metabolic changes that include elevated aerobic glycolysis, *de novo* fatty acid synthesis and glutaminolysis.²²⁰ Overall, these changes result in a low-glucose, high-lactate environment that is not supported by cytotoxic immune cells, but in which Tregs thrive and maintain immunosuppression through a FoxP3-mediated response.²²¹⁻²²³

Most recently, Wang *et al.* demonstrated that CD36 modulated Treg metabolism to

promote the adaptation to a lactic acid-enriched environment, by fine-tuning mitochondrial fitness.²²⁴ Once activated, Tregs may directly compete with effector/naïve T cells for antigen presenting cells signals and cytokines, such as IL-2, or cause their metabolic disruption to hinder naïve/effector T cell proliferation.²²⁵ Interestingly, Akkaya and colleagues have shown that antigen-specific Tregs may form strong interactions with dendritic cells, resulting in selective inhibition of the naïve T cell binding to antigen presenting cells.²²⁶ Such strong binding resulted in the removal of the peptide-major histocompatibility complex (MHC)-II from the surface of dendritic cells, reducing their antigen presentation capacity.

Additionally, Tregs secrete inhibitory cytokines that suppress effector T cell activity, such as IL-10, IL-35, and TGF- β , and granules, like perforins and granzymes, that induce apoptosis of effector T cells. Lastly, Tregs may also undergo oxidative stress-induced death, through which ATP is rapidly converted into adenosine that binds receptors at the surface of T cells, affecting their function.^{227,228}

Despite the obvious role in tumour immune tolerance, Tregs have also been implicated in non-immunological events favouring tumour growth and metastasis. Facciabene *et al.* showed that Tregs are recruited to hypoxic tumours by C-C Motif Chemokine Ligand (CCL)28 chemokine to secrete VEGF-A and contribute to tumour angiogenesis.²²⁹ In a mouse breast cancer model, Tan *et al.* demonstrated that tumour-infiltrating Tregs produced receptor activator of nuclear factor-B ligand (RANKL) that acted on its receptor RANK, expressed in tumour cells, promoting formation of lung metastasis.²³⁰ Together, these studies provided strong evidence for putative non-immunosuppressive roles of Tregs in tumour progression.

In GC patients, elevated numbers of peripheral and tumour-infiltrating Tregs have been described.²³¹⁻²³⁵ Interestingly, the role of Tregs in GC seems to be initiated even before the establishment of neoplastic lesions, as patients with gastritis and peptic or digestive ulcers already display high numbers of Tregs.^{236,237} Several studies have shown that *H. pylori* can remodel the gastric microbiota and stimulate the production of cytokines and chemokines that augment the presence of Tregs at the gastric mucosa.^{238,239} The following increased number of Tregs promotes an immunosuppressive environment that sustains persistent *H. pylori* infection.²⁴⁰ In tumour lesions, Treg recruitment in early steps of GC progression is mediated by C-C chemokine receptor (CCR)4 ligands, namely CCL17 and CCL22 chemokines.²⁴¹ Moreover, GC cells produce high levels of TGF- β and IDO, which favours Treg differentiation.^{242,243}

The absolute number of Tregs in TILs is positively correlated with tumour stage, whereas its relative increase, as compared to effector T cells, has been reported in tumour-draining lymph nodes.^{244,245} Indeed, high expression of FoxP3⁺ Tregs in sentinel lymph nodes was shown to predict metastasis in downstream lymph nodes.²⁴⁶ Notwithstanding, the prognostic value of Tregs in GC remains controversial. Abundant Treg infiltration and higher Treg/effector T cell ratios have been associated with poor prognosis and reduced patient survival.²⁴⁷⁻²⁵¹ Likewise, patients that received neoadjuvant chemotherapy, and which displayed increased number of cytotoxic T cells and decreased Tregs in peripheral

blood, had improved prognosis.²⁵² Nonetheless, other studies have demonstrated that Treg infiltration associates with a favourable prognosis, particularly in patients with tumours in the cardia region or with MSI phenotype.²⁵³⁻²⁵⁵ Further, association of intratumoral Tregs and macrophages also predicts survival of advanced GC patients after radical tumour resection.²⁵⁶

To further elucidate the prognostic value of Tregs in GC, some studies have focused on the spatial distribution of Tregs and on their interaction with other cells at the tumour microenvironment. Of notice, Mizukami and colleagues demonstrated that the localization pattern of Tregs in tumours can better predict patient prognosis than their absolute number.²⁴¹ In this study, Treg spreading was predictive of poor prognosis. The distance between Tregs and cytotoxic T cells also seems to influence patient prognosis. Interestingly, when the predominant distance separating Tregs and cytotoxic T cells is between 30-110 μm , patients display better prognosis.²⁵⁷

This controversial prognostic value of Tregs exposes the lack of knowledge on the role of these cells in GC. Tumour location, histology or molecular characterization, as well as the treatment regimen, may be important factors affecting the role and clinical value of Tregs. Additionally, deeper comprehension and consensus on the subpopulations of Tregs, *i.e.*, on the markers used to identify Tregs, may aid the establishment of specific Treg contributions and, consequently, improve Treg-based therapies.

4.3. Regulatory T cells inspired therapy

The holy grail of Treg-based therapies is the reduction of the amount or activity of Tregs, to enhance tumour immunity without eliciting unrestrained autoimmunity. Indeed, depletion or functional modulation of Tregs results in effective anti-tumour responses, however, in tumours that display self- or quasi-self-antigens, effective tumour immunity is accompanied by autoimmunity.^{258,259}

Moreover, the idea of boosting anti-tumour response through the sole expansion of effector T cells and natural killer cells does not seem to be sufficient to prompt tumour suppression. To test this, Vazquez-Lombardi *et al.* developed a variant of IL-2 that lack CD25 binding, which strongly expanded CD25⁻ cytotoxic T cells but prevented the unwanted CD25⁺ Treg activation.²²⁴ Surprisingly, the authors observed that this variant was less effective in mediating tumour rejection, as compared to the wild-type IL-2 form. In fact, effective immune response was only achieved with Treg depletion.

These findings highlight the need for distinctive markers to discriminate tumour-infiltrating Tregs, from peripheral Tregs that maintain immunological tolerance in normal tissues, and from effector T cells at the tumour microenvironment. Preclinical studies have already pinpointed some promising Treg therapeutic targets, such as CCR4, CCR8, and CD36. Firstly, Sugiyama and colleagues showed that anti-CCR4 antibody could specifically deplete CCR4-expressing cells, preserving the expansion of cytotoxic T cells

in response to tumour antigens.²⁶⁰ In another study, *in vivo* experiments with anti-CCR8 antibody specifically depleted tumour resident Tregs, resulting in inhibited tumour growth.²⁶¹ The recently discovered Treg metabolic modulator, CD36, was found to be selectively upregulated in intratumoral Tregs.²⁶² CD36 ablation resulted in decreased intratumoral Tregs, enhancement of anti-tumour activity, and consequent suppression of tumour growth. Excitingly, this effect elicited additive anti-tumour response with anti-PD-1 therapy. Clinical trials using an anti-CTLA-4 antibody, ipilimumab, demonstrated that melanoma patients that responded to this treatment displayed significant reduction of tumour-infiltrating Tregs.²⁶³ To further improve this therapy, Ravi and colleagues propose the use of antibody-ligand traps that fuse anti-CTLA-4 antibodies with a TGF- β receptor II ectodomain sequence.²⁶⁴ These engineered antibodies could provide a dual and most powerful effect of inhibition of CTLA-4- and TGF- β -mediated differentiation of Tregs.

Through a different perspective, Pilato *et al.* explored TCR-dependent signalling pathways that could be targeted to disable tumour-infiltrating Treg function.²⁶⁵ In this work, the authors demonstrated that after a partial disruption of the CARMA1–BCL10–MALT1 signalosome complex, Tregs lost their suppressive function and produced IFN- γ , *i.e.*, gained effector activity. IFN- γ production was concomitant with activation of macrophages, and upregulation of MHC-I and PD-L1 in tumour cells. When combined with PD-1 blockade, this strategy resulted in tumour rejection, thus avoiding the activation of adaptive immune resistance by PD-L1 upregulation.

The role played by Tregs in GC development and progression highlights these cells as putative therapeutic targets. Notably, the identification of markers that distinguish tumour-infiltrating from peripheral Tregs, may render Treg-based therapies more realistic. Thus, understanding the specific scenarios in which these therapies may succeed is of utmost importance for future patient stratification.

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Rational & Aims

Tumours are carved in a complex microenvironment that can be deconstructed into three essential components: the intrinsic factors that characterize tumour cells; the cellular and acellular components that comprise the tumour microenvironment, and; the network of signals that allow efficient intercellular communication.

In GC, tumour microenvironment is enriched in Tregs, however, whether and how Treg cells promote GC progression is still poorly understood. Lessons from other studies have shown that Tregs may generate an immunosuppressive microenvironment that sustains tumour immune evasion. Yet, some findings have also demonstrated that Tregs may promote tumour growth, angiogenesis and metastasis via non-immune targets. These findings led us to hypothesize that Tregs may have an alternative and non-immunosuppressive role in GC progression.

In order to successfully disseminate, tumours have to establish local and systemic networks of communication, which can be mediated by EVs. EVs released by GC cells promote proliferation, migration, and invasion of neighbour tumour cells and are accountable for a deep remodelling of the normal behaviour of stromal cells located at the tumour microenvironment. Less explored is the role of GC-EVs on the metastatic organotropism and preparation of the pre-metastatic niche. It is still unknown how their cargo, their functional effect on recipient cells and, consequently, the impact on the metastatic cascade, may be influenced by tumour intrinsic factors, such as tumour histology and cellular architecture. In this regard, we hypothesized that EVs secreted by tumours with distinct histology have different cargo and induce different responses in recipient cells. Moreover, we hypothesized that 3D cell architecture recapitulates tumour morphology and polarization, which also affects the cargo of GC-EVs. Thereto, as the cargo of EVs is dynamically modulated by these tumour features, we expect consequent modifications on their ability to drive metastatic dissemination of GC cells.

Finally, the dynamic interactions occurring within the tumour microenvironment may also induce modifications in the cargo of GC-EVs, resulting on the reshaping of tumour communication. Thus, by combining the previous concepts, we further predict that the modulation of GC cell phenotype by Tregs will also impact the cargo and functional consequences of GC-EVs in recipient cells, favouring GC progression and dissemination.

Framed on the aforementioned knowns and unknowns, the present work intends to unveil novel molecular mechanisms of GC progression mediated by important cellular and communication players at the GC microenvironment, respectively Tregs and EVs.

Particularly, in this work, we have addressed the following specific aims:

To investigate whether GC intercellular communication mediated by EVs is dependent on the tumour histology, by analysing the effect of EVs-derived from a panel of GC cell lines on normal recipient cells. Chapter II – Paper 1

To characterize the impact of 3D cell architecture in the cargo of GC-EVs, through a comparative and integrative analysis of the microRNA and proteomic content of EVs produced in 2D and 3D GC *in vitro* cultures. Chapter II – Paper 2

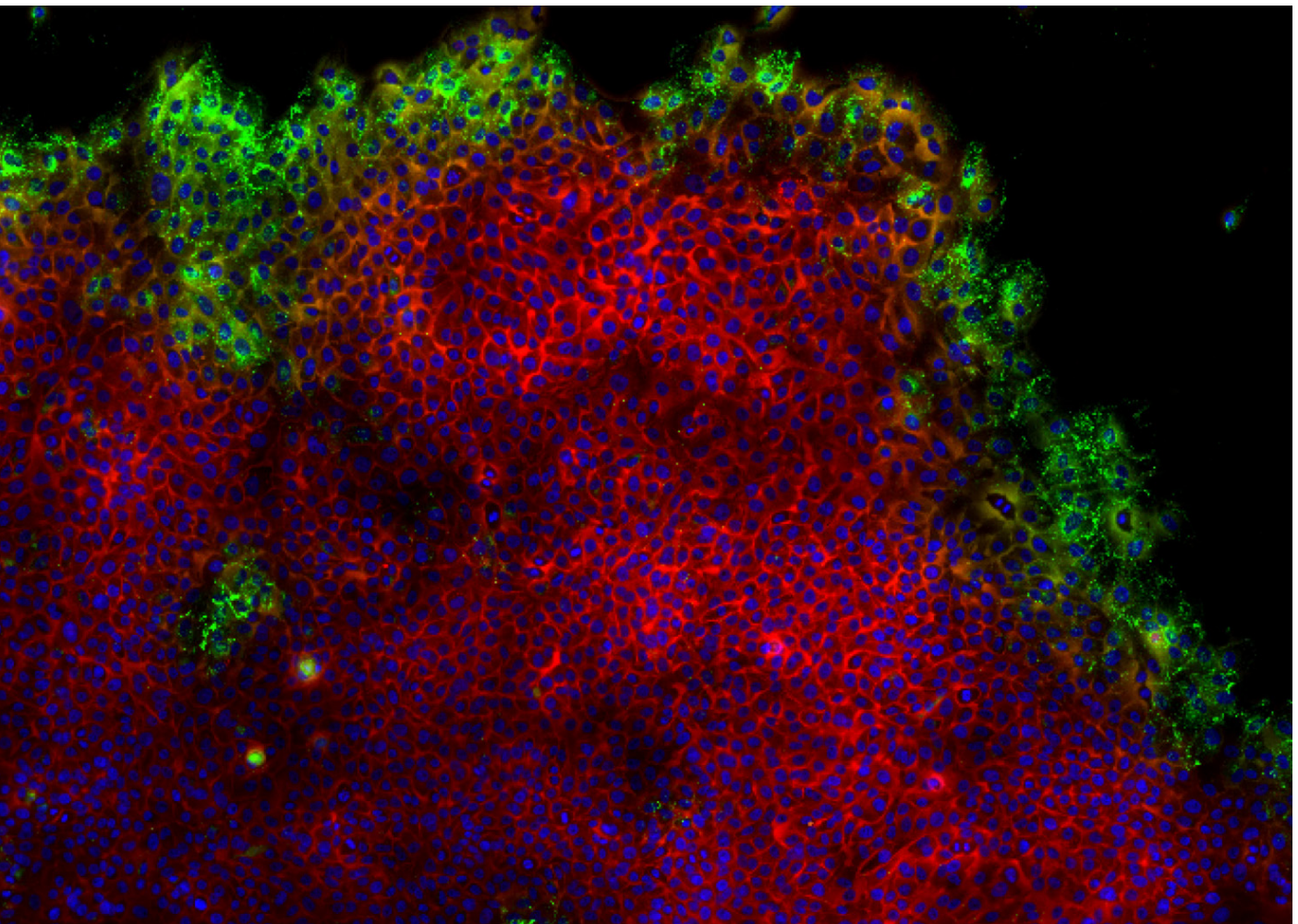
To dissect the role of EVs in GC cells' dissemination, by correlating microRNA and proteomic profiles of EVs and cells with the *in vivo* metastatic burden. Chapter III – Paper 3

To unveil a non-canonical role of Tregs in the modulation of GC cell phenotype, through a comprehensive immunophenotype analysis of GC tumours and *in vitro* direct co-cultures of Tregs and GC cells. Chapter IV – Paper 4

The work developed under the scope of this PhD thesis investigated a poorly understood and immature field of GC research. The resulting findings unravelled new aspects of GC biology and metastasis, involving the contribution of EVs and Tregs. In particular, we identified specific factors that shape the cargo of tumour-derived EVs, and disclosed how they modulate the organotropism of disseminating GC cells. This piece of work may provide candidate biomarkers for GC diagnosis. Furthermore, we unveiled a novel Treg-mediated mechanism of GC development that may hold important clinical implications, and foster innovative therapeutic strategies.

Chapter 1

Intrinsic factors of tumour cells that modulate the cargo and functional effect of extracellular vesicles



Gastric cancer extracellular vesicles tune the migration and invasion of epithelial and mesenchymal cells in a histotype-dependent manner

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International Journal of Molecular Sciences; 20(11). doi: 10.3390/ijms20112608.

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Extracellular vesicles (EVs) secreted by tumor cells modulate recipient cells' behavior, but their effects in normal cells from the tumor microenvironment remain poorly known.

In this study, we dissected the functional impact of gastric cancer cell-derived EVs (GC-EVs), representative of distinct GC histotypes, on the behavior of normal isogenic epithelial and mesenchymal cells.

GC-EVs were isolated by differential centrifugation and characterized by transmission electron microscopy, nanoparticle tracking analysis, and imaging flow-cytometry. Epithelial and mesenchymal cells were challenged with GC-EVs and submitted to proliferation, migration, and invasion assays. Expression of epithelial and mesenchymal markers was followed by immunofluorescence and flow-cytometry.

Our results indicated that GC-EVs secreted by diffuse-type cancer cells decrease the migration of recipient cells. This effect was more prominent and

persistent for mesenchymal recipient cells, which also increased Fibronectin expression in response to EVs. GC-EVs secreted by cancer cells derived from tumors with an intestinal component increased invasion of recipient epithelial cells, without changes in EMT markers.

In summary, this study demonstrated that GC-EVs modulate the migration and invasion of epithelial and mesenchymal cells from the tumor microenvironment, in a histotype-dependent manner, highlighting new features of intestinal and diffuse-type GC cells, which may help explaining differential metastasis patterns and aggressiveness of GC histotypes.

Keywords

Extracellular vesicles
Gastric cancer
Epithelial-to-mesenchymal transition
Invasion
Migration

Introduction

Extracellular vesicles (EVs) are crucial mediators of the 'dialog' between tumor and stromal cells at local and distant microenvironments.¹ These secreted vesicles carry a myriad of pro-tumoral and pro-metastatic factors that qualify them to orchestrate the invasion-metastasis cascade by reprogramming both tumor and stromal cells.²⁻⁴ Several studies have shown that EVs participate in the initial steps of tumor cell invasion by, for instance, inducing a transdifferentiating program known as epithelial to mesenchymal transition (EMT).⁵ Particularly, EVs secreted by tumor cells enclose a pro-EMT cargo that enhances cell migration and invasion via downregulation of epithelial (e.g., E-cadherin and ZO-1) and upregulation of mesenchymal (e.g., Vimentin) markers.⁶⁻⁸ This EMT program can also be triggered by EVs secreted by stromal cells, such as fibroblasts.^{9,10} Indeed, breast cancer-associated fibroblasts secrete EVs that promote breast cancer cell protrusive activity and motility through the Wnt-planar cell polarity signaling.¹¹

Particularly in gastric cancer (GC), several studies have explored the role of EVs in the crosstalk between tumors and several types of stromal cells.¹² GC-derived EVs (GC-EVs) have been shown to sustain tumor growth by triggering the differentiation of human umbilical cord-derived mesenchymal stem cells (MSCs) into carcinoma-associated fibroblasts (CAFs) via Transforming Growth Factor beta (TGF β), or reprogram fibroblasts and pericytes into CAFs by transferring miR-27a and BMP, respectively.^{13,14} GC-EVs may also affect the immunomodulatory functions of MSCs through Nuclear Factor Kappa B (NF- κ B) signaling^{15,16} and induce the expression of pro-inflammatory factors in macrophages or of autophagy in neutrophils,^{17,18} thus promoting tumor cell proliferation and migration. Recently, it was demonstrated that these are bi-directional communications such as CAF-derived EVs induce scirrhous-type GC cell migration and invasion through MMP2 activation,¹⁹ and MSCs-derived EVs induce EMT and stemness of GC cells, further promoting cancer cell growth and migration.²⁰ As reported in other cancer models,^{4,21} GC-EVs have also been implicated in the remodeling of the pre-metastatic microenvironment. Zhang *et al.* showed that EGFR-containing GC-EVs facilitate liver-specific metastasis through the suppression of miR-26a/b expression and activation of HGF on liver stromal cells.²² Further, two independent studies have provided insights into the mechanism for peritoneal metastasis of GC. Whilst Deng *et al.* demonstrated that GC-EVs facilitate peritoneal metastasis through mesothelial-to-mesenchymal transition (MMT) of peritoneal mesothelial cells via upregulation of p-ERK, Li *et al.* found that this role is mediated by miR-21-5p-containing GC-EVs, which promote cancer dissemination by targeting SMAD7.^{23,24}

Notwithstanding, it remains to be elucidated how GC cells modulate not only non-transformed epithelial cells present in the tumor microenvironment but also mesenchymal cells arising in the context of epithelial-to-mesenchymal transition in often-inflamed tumor microenvironments. Further, no evidence exists about the role of EVs in mediating the first steps of tumor cell migration and invasion of intestinal- and diffuse-type GC, the main histological types of this disease. Herein, we sought to dissect the functional impact of

EVs, isolated from GC cell lines, representative of intestinal and diffuse histotypes, on the behavior of normal epithelial and mesenchymal cells.

Results

To explore whether EVs released by GC cells modulate non-transformed epithelial and mesenchymal cells located in the tumor microenvironment, we designed the following study according to the scheme presented in Figure 1. Selected GC cell lines are representative of distinct histotypes of GC. The MKN74 GC cell line represented the intestinal-type GC, Kato III and IPA220 cells presented characteristics resembling the diffuse-type GC, and MKN45 harbored both intestinal and diffuse components (Figure S1A).^{25,26}

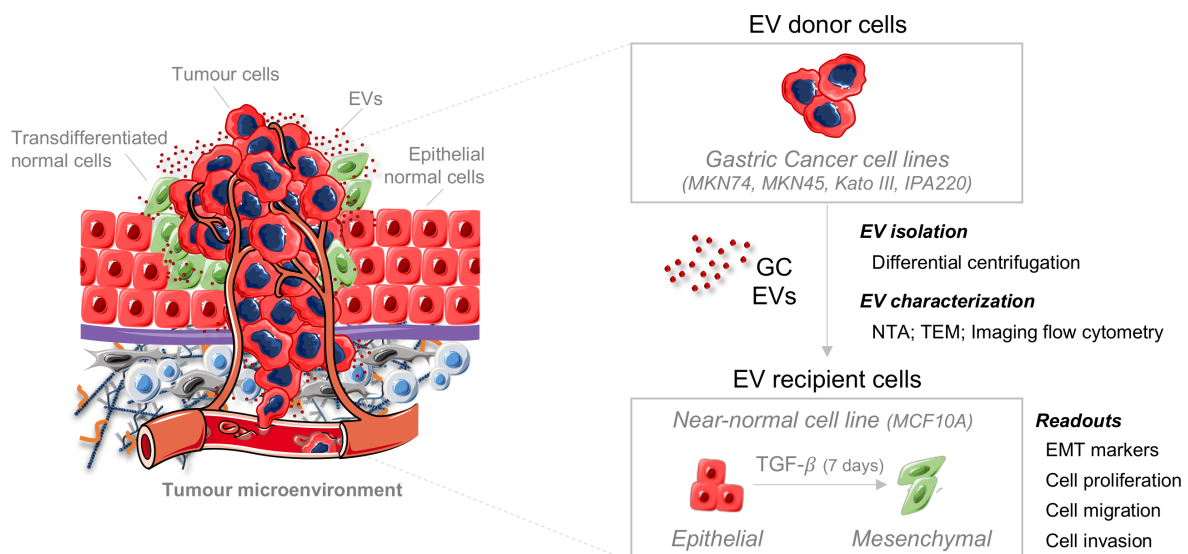


Figure 1. Scheme illustrating the experimental design of the study. Four distinct GC cell lines (MKN74, MKN45, Kato III and IPA220) were used as donor cells of EVs, which were isolated by differential centrifugation and characterized by TEM, NTA and imaging flow cytometry. Epithelial (red) and Mesenchymal (green) cells were used as recipients of GC-EVs and submitted to distinct functional assays.

1. *TGFβ1 induces EMT in a non-tumorigenic epithelial cell line*

First, we established and characterized a human TGFβ-induced EMT cell recipient model. As there is no non-tumorigenic gastric cell line currently available, we induced EMT in MCF10A, a non-tumorigenic breast epithelial cell line.

By adapting a previously described approach²⁷ and using TGFβ1 as a classical inducer of EMT, we derived, from the same genotypic background, two distinct phenotypic cell types: epithelial and mesenchymal. Whilst epithelial cells presented cobblestone morphology, the mesenchymal counterpart acquired an elongated spindle-shape (fibroblastic-like) morphology and lost cell-cell adhesion (Figure 2A). To confirm whether the observed phenotypic alterations were due to EMT, we analyzed the expression of well-described epithelial and mesenchymal markers, both at the RNA and protein levels. Upon EMT, mesenchymal cells displayed a significant decrease of epithelial *CDH1* and *OCLN* and a

substantial increase of mesenchymal *CDH2*, *VIM* and *FN* mRNA expression (Figure 2B). These alterations were also detected at the protein level, where mesenchymal cells lost E-cadherin and gained Fibronectin in comparison to epithelial cells (Figure 2C).

Overall, these results point to the occurrence of EMT, and set the ground for the usage of these two isogenic epithelial and mesenchymal cell lines as recipients of GC-EVs.

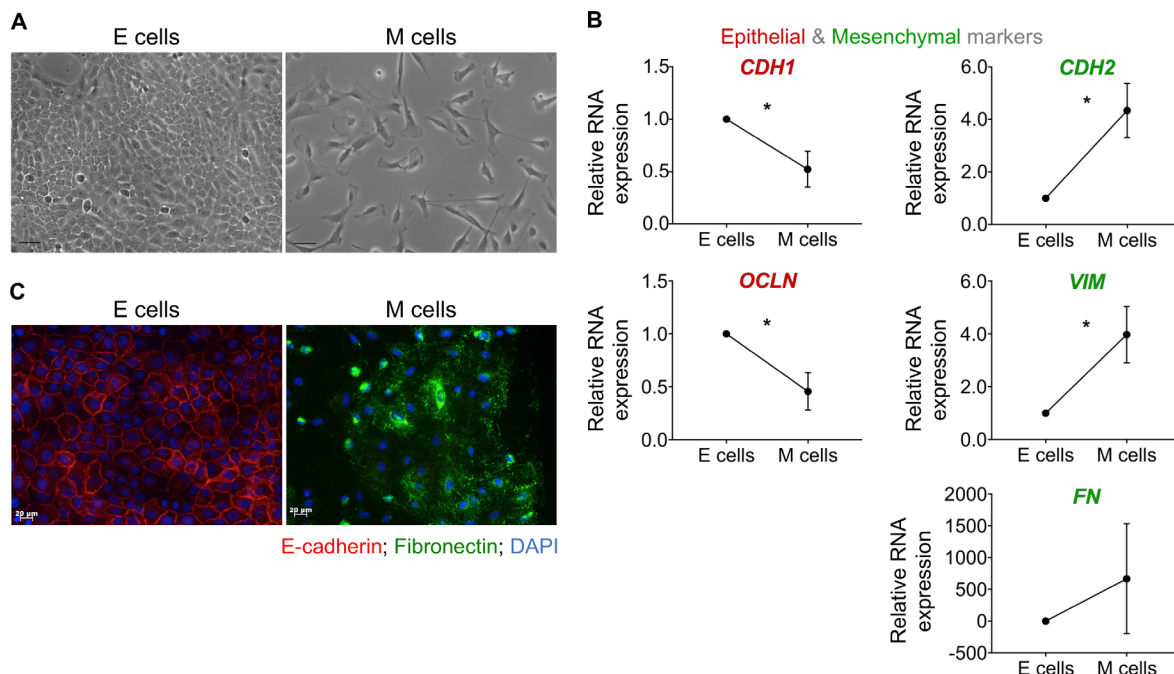


Figure 2. Characterization of a human TGF β -induced EMT cell model. (A) Bright-field microscopic images of epithelial and mesenchymal cells; (B) Quantification of epithelial (*CDH1*; *OCN*) and mesenchymal markers (*CDH2*; *VIM*; *FN*) mRNA expression by qRT-PCR. Data obtained for M cells was normalized for E cells and GAPDH was used as endogenous control. Graphs represent the mean $\pm$ standard deviation of three independent experiments (* $p \leq 0.05$, Unpaired t-test with Welch's correction); (C) Immunofluorescence for E-cadherin (red staining), Fibronectin (green staining) in epithelial and mesenchymal cells. Scale bar: 20 μ m. (E-epithelial cells; M-mesenchymal cells)

2. Distinct GC cell lines secrete EVs with similar physical and biochemical properties

Next, we isolated and characterized EVs secreted by four GC cell lines. We observed that all GC cell lines presented a high percentage of viable cells at the time of conditioned media collection and isolation of EVs (Figure S1B), thus reducing the potential contamination of EV preparations by apoptotic bodies.²⁸ The harvested EVs were analyzed by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and imaging flow cytometry (Figure 3). TEM showed that vesicles recovered at 100 K were of the size generally assigned to exosomes (Figure 3A),²⁹ but in all cell lines except MKN74, a fair concentration of larger EVs (~200 nm) were also present. These results were also confirmed by NTA, which revealed a mean size of 111, 116, 121, 127 nm, for MKN74-, MKN45-, Kato III-, and IPA220-EVs, respectively (Figure 3B). Moreover, imaging flow cytometry detected commonly associated exosomal markers CD9, CD81, and Flotillin-1 in EVs secreted by the four distinct GC cell lines (Figure 3C).³⁰ However, EVs from all four GC cell lines seem to have different contributions of these markers, with IPA220 showing equivalently high

representation of CD9 and CD81, and MKN74 showing the lowest representation of both markers (Figure 3C). Given the differences in these EV-associated markers, it is likely that the cargo of these EVs may also be different.

Despite the later differences, globally our results seem to indicate that EVs secreted by distinct types of GC cell lines present relatively similar physical and biochemical properties.

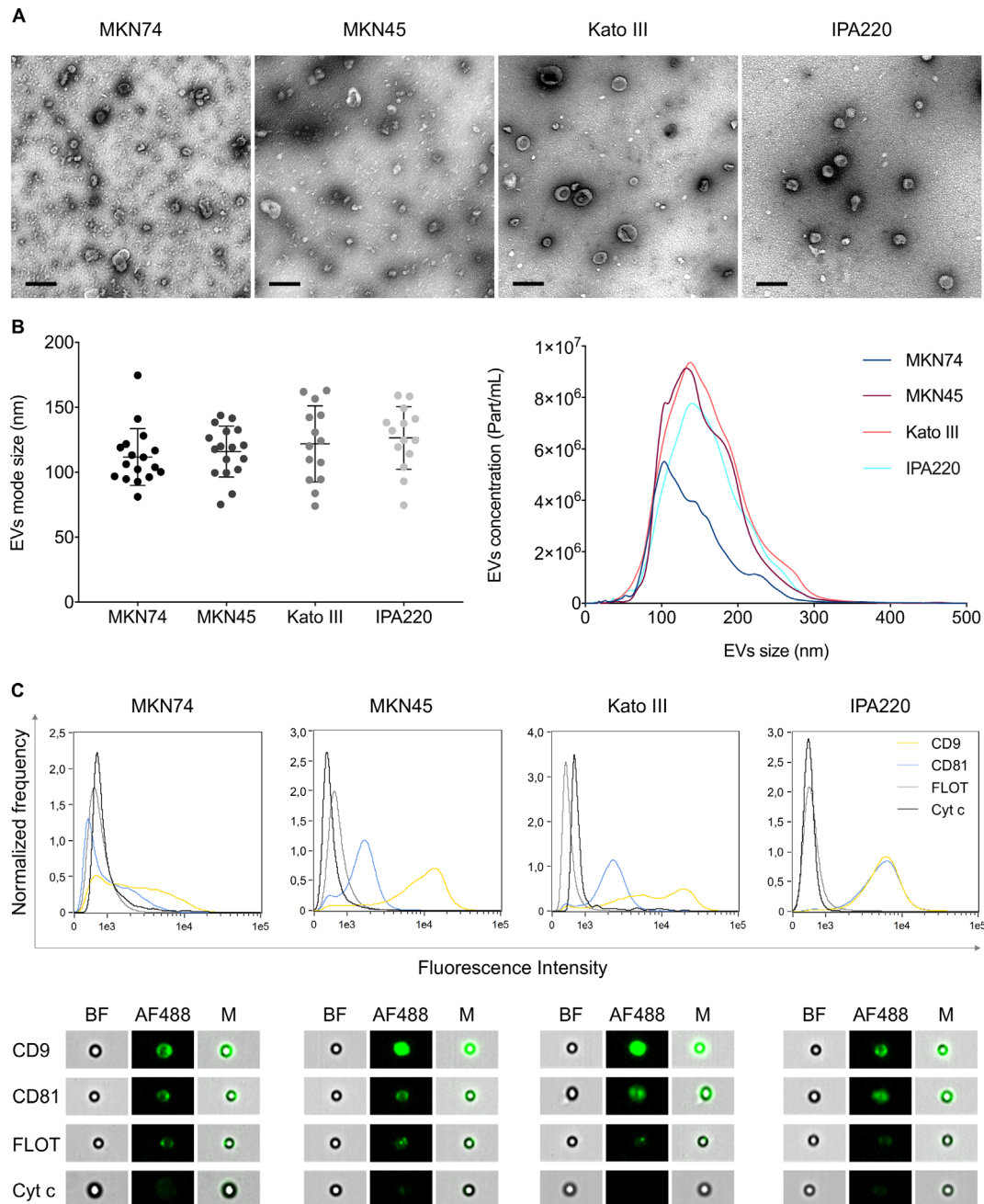


Figure 3. Characterization of EVs secreted by MKN74, MKN45, Kato III and IPA220 GC cell lines. (A) Representative electron microscopy images of EVs isolated from GC cells. Scale bars: 200 nm; (B) NTA of isolated EVs with mode size distribution (left) and particle concentration (right). Graphs represent the mean $\pm$ standard deviation of at least 14 biological replicates; (C) Detection of CD9, CD81, Flotillin-1, and Cytochrome C (negative control) by imaging flow cytometry. Distribution and representative images of the intensity of fluorescence detected for each marker in three biological replicates. Bright-field images (BF) showed beads to which EVs were coupled; fluorescence images (AF488) showed EVs labeled with specific markers; merged images (M) showed labeled EVs coupled to beads.

3. GC-EVs do not modulate E-cadherin expression at the cell membrane

Since E-cadherin is a classical EMT marker, we first checked whether GC-EVs modulate its expression and localization in recipient cells. We treated epithelial and mesenchymal recipient cells with EVs from the four donor GC cell lines and, upon 24 h of treatment, we analyzed the expression and localization of E-cadherin by flow cytometry (Figure 4). As expected, the non-treated (control) mesenchymal cells presented a significantly reduced percentage of membrane E-cadherin-positive cells, in comparison with non-treated (control) epithelial cells ($p \leq 0.0001$, Figure 4A). When epithelial and mesenchymal cells were treated with the four distinct GC-EVs, neither the percentage of E-cadherin-positive cells at the membrane (Figure 4A) nor the intensity of surface E-cadherin expression (Figure 4B) varied between treated and non-treated cells.

These results suggested that GC-EVs do not interfere with the levels of E-cadherin expressed at the membrane of both epithelial and mesenchymal recipient cells.

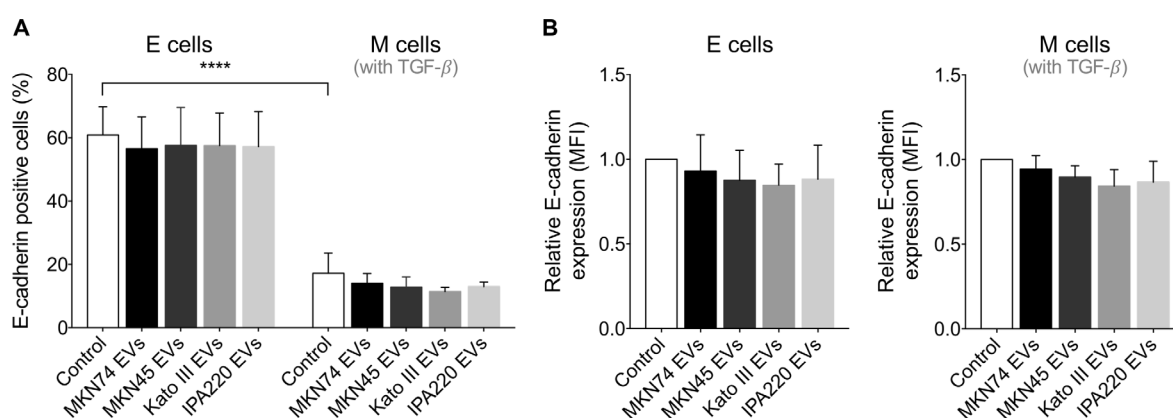


Figure 4. GC-EVs do not modulate membranar E-cadherin expression of epithelial and mesenchymal cells. (A) Percentage of E-cadherin-positive cells in non-treated (control) and treated epithelial and mesenchymal cells were assessed by flow cytometry. Graph represents the mean $\pm$ standard deviation of three independent experiments (**** $p \leq 0.0001$, Two-way ANOVA with Tukey's multiple comparisons test); (B) Intensity of E-cadherin surface expression of non-treated (control) and treated epithelial and mesenchymal cells was assessed by flow cytometry. Graph represents the mean $\pm$ standard deviation of three independent experiments and data was normalized to each respective control cells. (E-epithelial cells; M-mesenchymal cells). TGF β 1 was maintained in M cultures (control and treated M cells).

4. GC-EVs impair migration and invasion of epithelial and mesenchymal cells

Our results suggest that the main hallmark molecule of EMT - E-cadherin - remains unaffected when near-normal epithelial and mesenchymal cells are exposed to GC-EVs. However, growing evidence demonstrate that cell phenotypes, such as migration and invasion, may be modulated even in cancer cells expressing E-cadherin.³¹ For this reason, we assessed whether cell migration, proliferation, and invasion of epithelial and mesenchymal recipient cells could be modulated in the presence of GC-EVs.

We start by examining whether GC-EVs influence the migration rate and pattern of the different recipient cell types, by performing wound-healing assays followed by time-

lapse bright-field microscopy. In the absence of GC-EVs, epithelial and mesenchymal cells (controls) displayed a similar migration rate, although with distinct patterns (Figure 5A, 5B). Whilst epithelial cells migrated in a collective manner, mesenchymal cells displayed a single cell migration behavior (Figure 5A). Then, we analyzed the effects of GC-EVs in recipient cells. We verified that EVs secreted by Kato III and IPA220 GC cells impaired significantly the migration of both epithelial and mesenchymal cells, by decreasing their rate, but without affecting migration patterns (Figure 5A, 5B). To overcome variability among experiments, we normalized the data of treated cells to the respective control cells, considering each time-point (Figure 5C). Specifically, we found that IPA220-EVs led to a reduction in the migration rate of epithelial cells in the first 4 h, which was recovered at later time-points (Figure 5C, left). Regarding the migration rate of mesenchymal cells, both Kato III- and IPA220-EVs led to a more permanent reduction in the first 4 h that was maintained during 12 and 20 h, respectively (Figure 5C, right). Strikingly, mesenchymal cells challenged with IPA220-EVs were not able to close the wound area in 20 h comparing with non-treated mesenchymal cells (Figure 5A, 5B).

To further determine whether distinct levels of proliferation underlined changes in migration rates, we measured EdU incorporation into newly synthesized DNA by flow cytometry. We observed that, in the absence of GC-EVs, mesenchymal cells proliferate less than epithelial cells, although with no statistical significance (18.7% vs. 29.7% of EdU positive cells, Figure S2). When challenged with GC-EVs, both epithelial and mesenchymal cells maintained the proliferation levels of the corresponding non-treated counterparts, meaning that these GC-EVs had no effect on EMT-related proliferation (Figure S2).

Taken together, these results pinpointed that EVs from specific GC cell lines are able to interfere with the migration rates of mesenchymal cells, mainly, without affecting their proliferation levels. Furthermore, in light of the above results on E-cadherin membranous expression, these changes in migration of epithelial and mesenchymal recipient cells may even occur independently of their E-cadherin expression. Importantly the greater impacts in migration were achieved when recipient cells were treated with EVs from cell lines deriving from diffuse-type GC (poorly differentiated).

Next, we evaluated whether EVs from the different types of GC cells differentially modulate the invasion of epithelial and mesenchymal cells (Figure 6). We observed that epithelial cells challenged with MKN74-, MKN45- and IPA220-EVs tend to be more invasive (fold change: 2.37, 3.38, 1.95, respectively) than the non-treated epithelial counterpart; however, this effect was only statistically significant for MKN45-EVs (adjusted *p*-value 0.0143, Figure 6A). In contrast, mesenchymal cells became less invasive upon treatments with MKN45-, Kato III- and IPA220-EVs (fold change: 0.58, 0.36, 0.55, respectively) in comparison with non-treated mesenchymal cells (Figure 6B). Despite the lack of statistical significance observed for all conditions, it was clear that Kato III-EVs had a stronger impact on invasion of mesenchymal cells than MKN45- and IPA220-EVs.

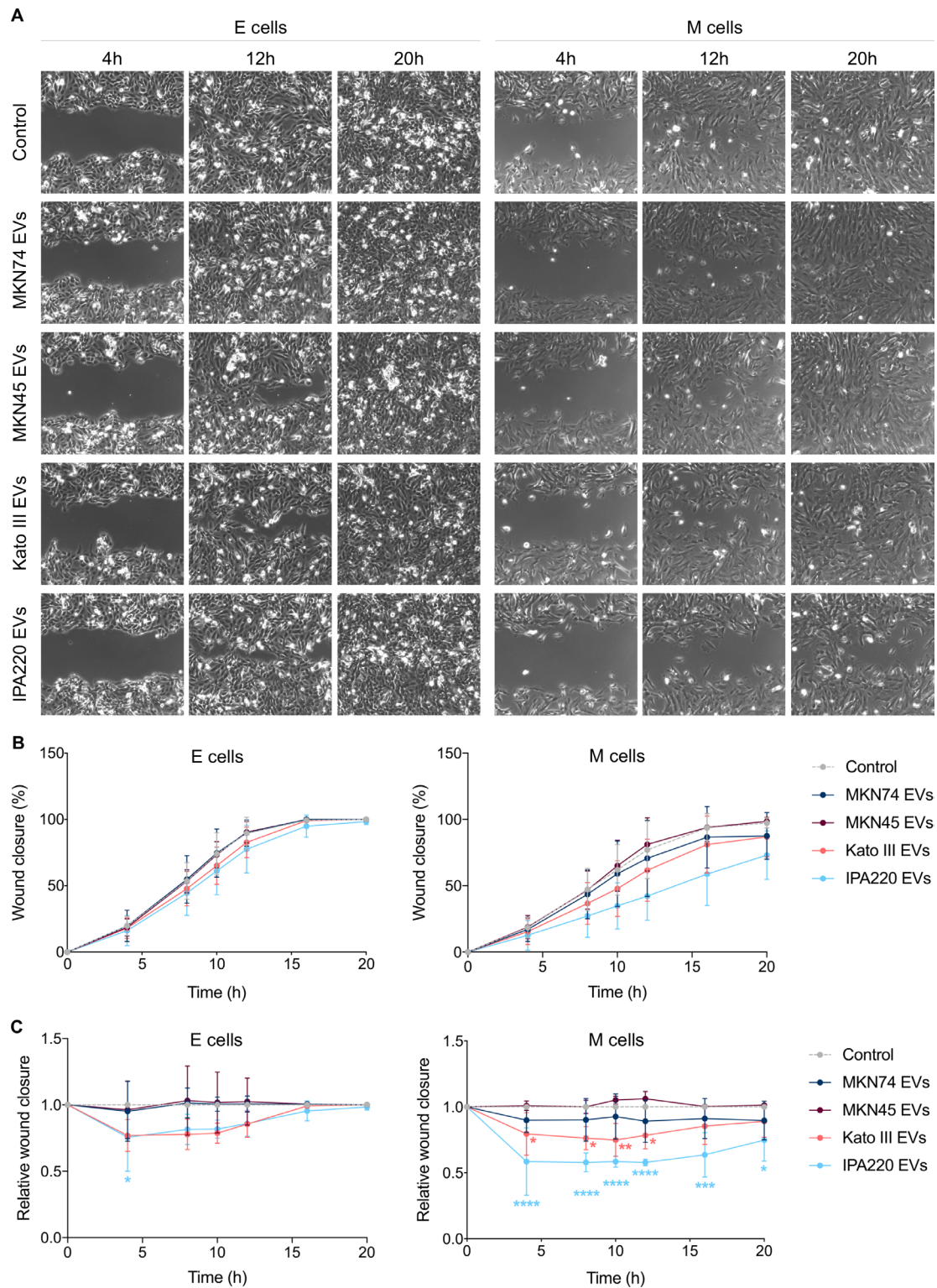


Figure 5. GC-EVs impair the migration of epithelial and mesenchymal cells. (A) Time-lapse microscopic images illustrating the migration of non-treated (control) and treated epithelial and mesenchymal cells, at distinct time-points; (B, C) The migration rate of non-treated (control) and treated epithelial and mesenchymal cells was assessed by time-lapse microscopy; (B) Graphs represent the mean percentage of wound closure $\pm$ standard deviation of three independent experiments; (C) Graphs represent the mean of normalized wound closure $\pm$ standard deviation of three independent experiments; the data was normalized to the control cells at each time-point ($*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$, Two way ANOVA with Dunnett's multiple comparisons test; blue and orange asterisks correspond to E and M cells treated with IPA220-EVs or Kato III-EVs, respectively; (E-epithelial cells; M-mesenchymal cells).

To check whether the impact of GC-EVs on the invasion capacity of epithelial and mesenchymal cells was accompanied by differences in the expression levels of EMT markers, we performed E-cadherin and Fibronectin immunocytochemistry 48 h after EVs treatment, *i.e.*, at the endpoint of the invasion assay. Concerning the epithelial marker E-cadherin, no differences were observed upon challenging of epithelial and mesenchymal cells with GC-EVs comparing with the respective non-treated counterparts (Figure 6C). Interestingly, we observed that the reduced invasion capacity of mesenchymal cells upon treatment with Kato III- and IPA220-EVs was accompanied by an increase of Fibronectin expression (Figure 6C).

Invasion of epithelial and mesenchymal recipient cells seems to be correlated with the histotype from which GC cell lines have been derived. Indeed, EVs from MKN74 and MKN45 cell lines, which display an intestinal component, promote invasion in epithelial recipient cells, but do not affect mesenchymal recipient cells. In contrast, EVs from diffuse-type GC cell lines (Kato III and IPA220) do not influence invasion of epithelial cells but demonstrate a trend towards decreasing the invasion of mesenchymal recipient cells.

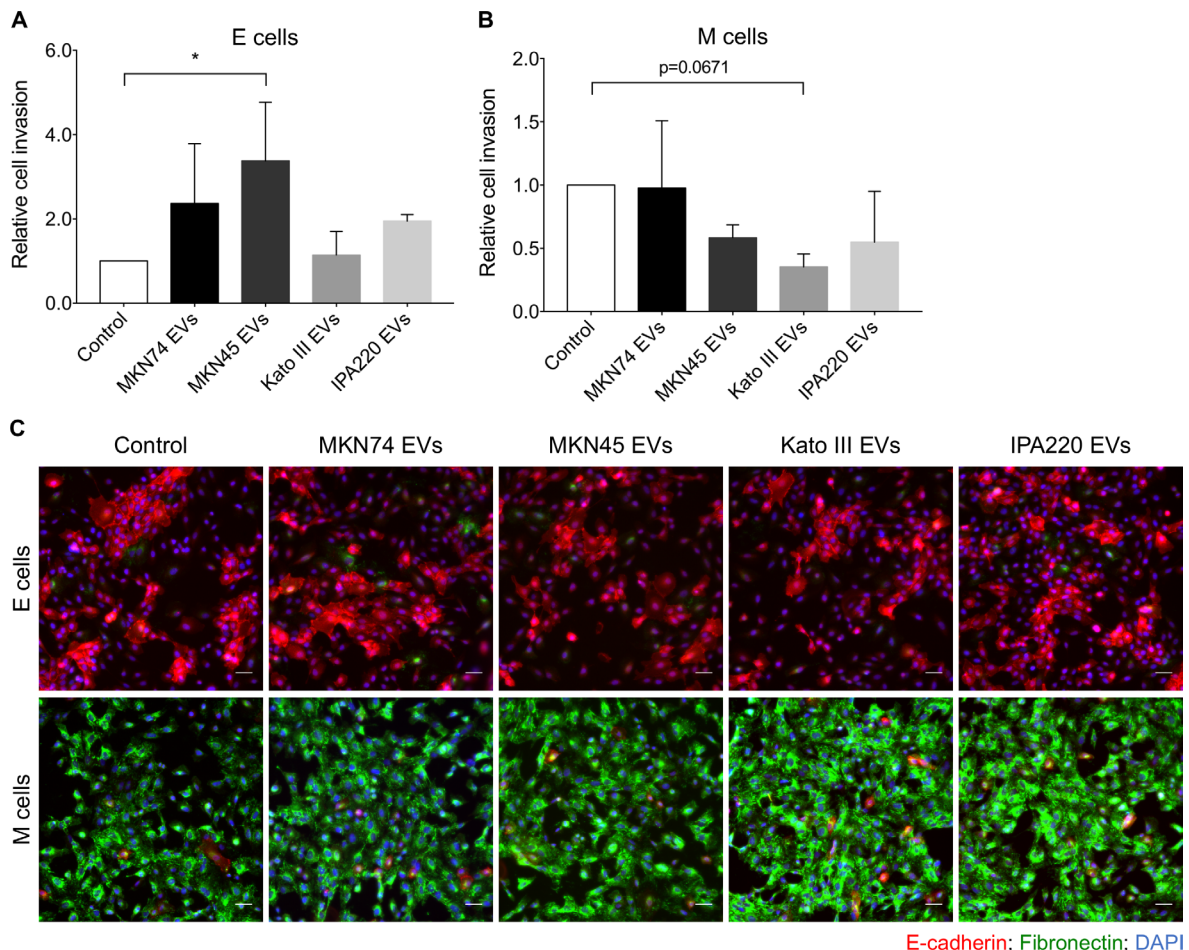


Figure 6. GC-EVs impair the invasion of epithelial and mesenchymal cells. The invasion levels of non-treated (control) and treated epithelial (A) or mesenchymal cells (B) were assessed by matrigel invasion assay; Graph represents the mean $\pm$ standard deviation of three independent experiments and data was normalized to control cells ($*p \leq 0.05$, one-way ANOVA with Kruskal-Wallis test); (C) Immunofluorescence for E-cadherin (red staining) and Fibronectin (green staining) in non-treated (control) and treated epithelial and mesenchymal cells. Scale bars: 50 μ m (E-epithelial cells; M-mesenchymal cells).

Collectively, these findings indicated that GC-EVs modulate the behavior of epithelial and mesenchymal recipient cells by impacting their cell migration, their invasion, and the expression of EMT markers. In particular, our results suggest that epithelial and mesenchymal recipient cells respond differentially to the same GC-EVs. Moreover, the effects of GC-EVs over recipient cells may depend on the histotypes of the EV-donor cells.

Discussion

Communication between tumor cells and the associated stroma plays a key role in driving tumor progression, but how GC cells modify the environment for their own benefit is less clear. In the present study, we hypothesized that EVs secreted by GC cells are able to subjugate normal cells within the tumor stroma to participate in cancer progression. We tested this hypothesis by focusing on two different recipient stromal cell types that often co-exist in the vicinity of cancer cells—normal epithelial and mesenchymal cells. Normal epithelial cells represent the adjacent normal cells surrounding the tumor, while mesenchymal cells represent epithelial cells exposed to transdifferentiating agents, such as inflammatory factors (e.g., TGF β), often arising during EMT.³² To this end, we established and characterized a human TGF β 1-induced-EMT cell model to obtain both epithelial and mesenchymal recipient cells for further treatment with distinct GC-EVs. We chose to use the non-tumorigenic breast epithelial cell line (MCF10A) as a recipient cell type to induce EMT, given that (1) non-tumorigenic gastric cell lines are currently not available; (2) primary epithelial gastric cells hardly proliferate and maintain viability *in vitro*, which makes them incompatible with our experimental design; and (3) breast epithelial cancers, but rarely other cancers from the digestive system, have been identified in the tumour spectrum of GC-associated syndromes, such as hereditary diffuse gastric cancer.³³ This last evidence supports breast epithelia as a tissue mimicking gastric epithelia regarding the tumorigenic process triggered by germline alterations. Altogether, the normal epithelial breast cell line (MCF10A), although not ideal, seems like an acceptable model to be used as a recipient cell line for GC-EVs. This recipient model underwent EMT when treated with TGF β 1, as demonstrated by cell morphological alterations and the differential expression of classical epithelial and mesenchymal markers.³⁴ We then chose four GC cell lines, representative of distinct GC histological types and differentiation patterns, to dissect the functional impact of GC-EVs on epithelial and mesenchymal cells. To the best of our knowledge, this is the first study addressing the impact of GC-EVs from different histological types over otherwise normal epithelial and mesenchymal stromal cells.

GC is histologically classified into two main subtypes—intestinal and diffuse—which are recognized by distinct morphological features, differentiation patterns, and clear-cut differences on the metastatic pattern.^{35,36} Intestinal-type GC is characterized by well-differentiated glandular structures and metastasizes, mainly to the liver, whilst diffuse-type GC consists of individually neoplastic cells that infiltrate the stomach wall and usually forms peritoneal and lymph node metastasis.³⁷ Notwithstanding, the two histotypes share several

sequential and interrelated events, such as local invasion into the surrounding tumor-associated stroma.³⁸

The most striking result of this study shows that as soon as epithelial and mesenchymal recipient cells interact with GC-EVs derived from diffuse-type GC cell lines (Kato III and IPA220), they start migrating less. This effect is equivalent for epithelial and mesenchymal recipient cells at early time-points, but persists longer in mesenchymal recipient cells. Interestingly, Kato III- and IPA220-EVs also induced the highest expression of Fibronectin in mesenchymal cells. These findings together may suggest that diffuse-type GC cells make use of EVs to render the microenvironment permissive for their further colonization and growth at preferred metastatic sites, such as the peritoneum. Actually, our findings may resemble some events occurring in peritoneal metastasis. As such, it has been demonstrated that GC-EVs facilitate peritoneal metastasis by disrupting the mesothelial barrier through induction of mesothelial to mesenchymal transition.²³ Additionally, it has been demonstrated that GC-EVs can stimulate the expression of adhesion-related molecules, such as fibronectin 1 and laminin gamma 1, in mesothelial cells.³⁹ This alteration fosters the adhesion of GC cells to mesothelial cells, thus creating a favorable scenario for dissemination of GC cells and consequent metastasis. From this perspective, it is sensible to hypothesize that GC-EVs derived from diffuse-type GC cell lines act particularly over mesenchymal cells in the tumor vicinity, and eventually adhere to them through fibronectin (yet to be seen) towards peritoneal colonization.

We also found that EVs derived from GC cells with an intestinal-type component were able to promote invasion of normal epithelial cells, with the most pronounced effect observed for EVs secreted by a GC cell line displaying both diffuse and intestinal morphologies (MKN45). Interestingly, this invasive phenotype was not accompanied by an increased migration. As such, one may hypothesize that the cargo of MKN45-EVs is more important for the remodeling of the basement membrane than for the direct movement of epithelial cells. Additionally, E-cadherin and Fibronectin expression levels remained unchanged in recipient cells, suggesting that other epithelial/mesenchymal markers may be responsible for promoting the observed invasive phenotype.

Our results indicate that GC-EVs secreted by cancer cells, derived from diffuse-type tumors, increase migration of recipient mesenchymal cells, while GC-EVs secreted by cancer cells derived from tumors with an intestinal component increase invasion of recipient epithelial cells in the tumor microenvironment. These results suggest that GC-EVs representative of different GC histotypes may have distinct cargo and membrane compositions, which may influence their uptake by recipient cells. On the other hand, epithelial (E) and mesenchymal (M) cells may also uptake and perceive GC-EVs differently. In fact, potential distinctive uptakes of GC-EVs by recipient cells may represent how effectively these EVs may convey tumor messages to their microenvironment. Several studies on different cancer models have demonstrated that EVs may indeed deliver signals able to induce EMT, thus initiating the invasion-metastasis cascade. For instance, Franzen *et al.* proved that bladder cancer-derived EVs increased the expression of several mesenchymal

markers (including α -smooth muscle actin, S100A4, and snail), as well as the motility and invasiveness of normal recipient uroepithelial cells.⁶ These phenotypes were also detected when lung-cancer EVs derived from highly metastatic cells were applied to normal epithelial cells.⁸ Specifically, these lung-cancer EVs have been found to increase the expression of Vimentin and N-cadherin and decrease E-cadherin and ZO-1. Additionally, breast and colon cancer-EVs containing amphiregulin boosted invasiveness of both epithelial and breast cancer cells.² These and others studies highlighted that EVs isolated from different cancer types induce similar phenotypes on normal epithelial cells, although using distinct players and molecular pathways.^{40,41}

In summary, the present study demonstrated that GC-EVs modulate the migration and invasion of epithelial and mesenchymal cells in a histotype-dependent manner, highlighting new features of intestinal and diffuse-type GC cells, which may help explaining the metastasis pattern, aggressive phenotypes, and prognosis of GC histological types.

Materials and Methods

1. Establishment and Characterization of EMT recipient model

Treatment of MCF10A epithelial cell line with TGF β 1

A human TGF β 1-induced-EMT cell model was established as previously described.²⁷ The non-tumorigenic epithelial cell line MCF10A (E-cells) was cultured in D-MEM/F12-Glutamax™ (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with horse serum (5%, Thermo Fisher Scientific), penicillin-streptomycin (PS, 1%, Thermo Fisher Scientific), recombinant human insulin (5 μ g/mL, Sigma-Aldrich, St. Louis, MO, USA), recombinant human epidermal growth factor (20 ng/mL, Sigma-Aldrich), cholera toxin (20 ng/mL, Sigma-Aldrich), and hydrocortisone (500 ng/mL, Sigma-Aldrich). Mesenchymal cells (M cells) were obtained by treating 6×10^4 epithelial cells (E cells) during seven days with the abovementioned culture medium supplemented with transforming growth factor- β 1 (TGF β 1, 8 ng/mL, Sigma- Aldrich).

Co-immunofluorescence of E-cadherin and Fibronectin

MCF10A cells (E and M) were co-immunostained for E-cadherin (1:100, 24E10 Cell Signaling, Danvers, MA, USA) and Fibronectin (1:100, clone 2755-8, Santa Cruz, Dallas, Texas, TX, USA). Briefly, cells were fixed in 4% PFA (20 min), followed by treatment with NH₄Cl (50 $\times$ 10⁻³ M, 10 min, Merck, Darmstadt, Germany) and Triton X-100 (0.2%, Sigma-Aldrich, 5 min) and blocking with BSA (Bovine Serum Albumin, 5%, 30 min, NZYTech, Lisbon, Portugal). Upon overnight (ON) incubation at 4°C with a mixture of rabbit anti-E-cadherin and mouse anti-Fibronectin primary antibodies, cells were co-incubated with secondary antibodies anti-rabbit Alexa 594 and anti-mouse Alexa 488 (1:500, 1 h, Thermo Fisher Scientific) at room temperature (RT). Images were taken with a Zeiss Imager.Z1, AxioCam MRm (Zeiss, Oberkochen, Germany).

RNA expression quantification of epithelial and mesenchymal markers

RNA was extracted from three biological replicas of E and M cells following instructions of the mirVana miRNA Isolation Kit (Thermo Fisher Scientific), and reversed transcribed (1000 ng) to cDNA with Superscript II Reverse Transcriptase and random hexamer primers (Thermo Fisher Scientific). Quantitative real time-PCR (qRT-PCR) was carried out in triplicates for the epithelial (*CDH1* and *OCLN*) and mesenchymal (*CDH2*, *VIM* and *FN*) target genes and for the endogenous control *GAPDH* using PrimeTime-qPCR assays (IDT, Hs.PT.53.2388193; Hs.PT.49.14927371; Hs.PT.58.45367437; Hs.PT.47.14705389; Hs.PT.58.40986315; and Hs.PT.51.1940505) on a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific). Data was analyzed by the comparative $2^{-\Delta\Delta CT}$ method.⁴² For all data comparisons, the Unpaired t test with Welch's correction was used.

2. Characterization of GC-EVs

GC cell culture and viability

Human GC cell lines MKN74, MKN45, Kato III (ATCC), and IPA220 (established at Ipatimup²⁶) were cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, Biowest, Nuaille, France) and 1% PS (Penicillin-Streptomycin, Thermo Fisher Scientific) at 37°C in 5% CO₂ humidified atmosphere. GC cells were seeded in T175 flasks and maintained with the abovementioned medium until a confluence of 60–70% was reached. GC cells were further cultured in EV-depleted medium during 48 h. This medium was obtained as previously described.⁴³ Briefly, RPMI medium supplemented with 20% FBS and 1% PS was centrifuged ON at 100,000×g, filtered, and diluted in the same amount of RPMI medium supplemented with 1% PS and without FBS. EV-depleted medium was further submitted to TEM to ascertain that the depletion was correctly performed.

GC cell viability was measured using annexin V-FITC and PI double staining followed by flow cytometry, as described.⁴³

EV isolation by Differential Centrifugation

GC-EVs were isolated from the conditioned media of GC cell cultures, growing in EV-depleted medium, by differential centrifugation, as previously described.⁴³ Briefly, the conditioned media of each GC cell line was submitted to two sequential centrifugation steps (at 300×g for 10 min and 2000×g for 20 min) followed by a filtration step. The filtered supernatant was centrifuged at 100,000×g, 4°C, in a SW32 rotor (Beckman Coulter, Fullerton, CA, USA) for 4 h to pellet EVs, and was further washed in PBS without Ca²⁺ and Mg²⁺ (Thermo Fisher Scientific) and centrifuged at 100,000×g, 4°C, for 2 h. Each pellet containing GC-EVs was resuspended in an appropriate volume of 0.9% NaCl, in accordance with downstream applications.

EV morphology by Transmission Electron Microscopy (TEM)

GC-EVs were negatively stained and visualized by Transmission Electron Microscopy. Briefly, EV preparations were added to Formvar-carbon-coated grids and incubated for 1

min at RT in the dark, to allow adsorption to the grid. EVs were further stained with 5% uranyl acetate during 1 min and examined with a JEM 1400 electron microscope (JEOL, Tokyo, Japan). Images were recorded using a SC1000 Orius CCD camera (Gatan, Pleasanton, CA, USA).

EV characterization by Nanoparticle Tracking Analysis (NTA)

The size and concentration of GC-EVs were measured by NTA, using the NanoSight NS300 (Malvern, Worcestershire, UK) as described.⁴³ Mode and mean size were calculated from the three videos per biological replicate. Results represent the mean $\pm$ standard deviation of at least 14 biological replicates of each cell line.

EV characterization by Imaging Flow Cytometry

GC-EVs were analyzed for the expression of CD9, CD81, and Flotillin-1 using imaging flow cytometry (ImageStreamXAmnis Corporation, Seattle, WA, USA) as described.⁴³ Briefly, EVs were coupled to previously sonicated aldehyde/sulfate latex beads (1×10^9 particles determined by NTA per 3 μ L of beads) for 1 h at RT with agitation followed by ON incubation at 4°C and blocking with glycine solution with agitation (100×10^{-3} M 30 min; Merck). Next, EVs–beads were immunostained with mouse monoclonal anti-CD9 (sc-59140, Santa Cruz), anti-CD81 (sc-23962, Santa Cruz), and rabbit polyclonal anti-Flotillin-1 (sc-25506, Santa Cruz) for 1 h, followed by incubation with goat anti-rabbit/anti-mouse Alexa Fluor 488 secondary antibodies (Invitrogen, 30 min) and analysis by imaging flow cytometry. For each sample, 100 000 events were acquired at a 40 $\times$ magnification. Fluorescence of the stained EVs–beads was excited with a 488 argon laser and collected on channel 2 (505–560 nm). A 745 nm laser was activated for side scatter and collected on channel 6, and bright-field images with adjusted intensity were collected on channel 1. Data analysis was performed using the IDEAS software (Amnis Corporation).

3. Functional assays with recipient cells treated with GC-EVs

Treatment of Epithelial and Mesenchymal cells with GC-EVs

After establishing M cells by treating E-cells (60 000 cells) for seven days with TGF β 1 (as abovementioned), both E and M cells were seeded in 6-well plates (100 000 cells/well) and maintained during 24 h in normal culture medium. Then, the normal culture medium was replaced by EV-depleted medium and cells were treated with 1×10^9 of GC-EVs (TGF β 1 was maintained in M cultures). After 24 h of treatment, E and M cells were collected and submitted to different assays.

Flow cytometry analysis of E-cadherin

Flow cytometry was performed as described.⁴⁴ Briefly, E and M cells (treated and non-treated with GC-EVs) were detached with Versene (Thermo Fisher Scientific) and resuspended in ice-cold PBS with 0.05 mg/mL CaCl₂. For all conditions, cells were centrifuged at 1500 rpm for 5 min at 4°C and washed in PBS with 0.05 mg/mL CaCl₂ and 3% BSA. Cells were then incubated for 1 h with mouse anti-E-cadherin primary antibody HECD-1 (1:50 dilution, Thermo Fisher Scientific), washed twice, and incubated with secondary antibody

anti-mouse Alexa 488 (1:250 dilution, Thermo Fisher Scientific) in the dark for 1 h. At least 2×10^4 single cells were acquired using FACS Canto II cytometer, and further analyzed using FlowJo software (Ashland, Oregon, OR, USA). Results represent the mean $\pm$ standard deviation of three biological replicates. Data was analyzed by Two-way ANOVA with Tukey's multiple comparisons test.

Wound-healing migration assay

Wound-healing assays of E and M cells (treated and non-treated with GC-EVs) were performed using μ -Plate 24 well with culture-inserts (Ibidi) and time-lapse microscopy. Briefly, 4×10^4 cells were seeded in each of the two wells of the culture insert and grown to confluence for, approximately, 24 h. After this period, the culture insert was removed, cells were washed with PBS, and fresh EV-depleted medium supplemented with GC-EVs (5×10^8) was added. Bright field images of the wounds were photographed immediately after removing the insert (0 h) and again at 4, 8, 10, 12, 16, and 20 h using Leica DMI6000 (Wetzlar, Germany). Five images were automatically captured per well per time point to completely image each wound. Cells were maintained at 37°C in 5% CO₂ humidified atmosphere during the time-lapse imaging process. The total area of the wound was measured at each time point using ImageJ software (MRI_Wound_Healing_Tool). Results represent the mean $\pm$ standard deviation of three biological replicates. Data was analyzed with a two-way ANOVA with Dunnett's multiple comparison test.

Proliferation assay

Proliferation assays of E and M cells (treated and non-treated with GC-EVs) were performed following the instructions of the Click-iTTM Plus EdU Pacific Blue Flow Cytometry Assay Kit (Thermo Fisher Scientific). At least 2×10^4 single cells were acquired using FACS Canto II cytometer, and further analyzed using FlowJo software. Results represent the mean $\pm$ standard deviation of three biological replicates.

Matrigel invasion assay

Invasion assays of E and M cells (treated and non-treated with GC-EVs) were performed using Corning BioCoat Matrigel Invasion Chambers (Thermo Fisher Scientific). Briefly, after 24 h of treatment with GC-EVs as abovementioned, 5×10^4 of E and M cells were seeded in the upper compartment and incubated for 24 h at 37°C in 5% CO₂. Filters were then washed in PBS and fixed in ice-cold methanol for 10 min. After removal of non-invasive cells adherent to matrigel with a prewet cotton swab, each filter was washed and mounted in glass coverslips with Vectashield/DAPI. A mosaic of the entire filter was obtained with Axiovert 200 M (Zeiss), and the total number of invasive nuclei was counted using ImageJ software. Results represent the mean $\pm$ standard deviation of three biological replicates. Data was analyzed with a one-way ANOVA with Kruskal-Wallis test.

Co-immunofluorescence of E-cadherin and Fibronectin

E and M cells (treated and non-treated with GC-EVs) were seeded in coverslips and grown during 24 h. The choice of this time-point was based on the one adopted for the invasion assay. After this, co-immunostaining for E-cadherin and Fibronectin was performed

according to the protocol described above. Images were acquired with Zeiss Imager.Z1, AxioCam MRm, or Leica DMI6000.

4. Statistical Analysis

The Unpaired *t* test with Welch's correction was used for comparisons of RNA expression levels of epithelial and mesenchymal markers. Flow-cytometry and wound-healing data were analyzed by a Two-way ANOVA with Tukey's and Dunnett's multiple comparisons tests, respectively. Matrigel invasion data was analyzed with a one-way ANOVA with a Kruskal–Wallis test.

Author Contributions

Conceptualization, S.R., J.C., and C.O.; Formal analysis, S.R., S.P.T., P.O., J.C., and C.O.; Funding acquisition, C.O.; Methodology, S.R., S.P.T., M.A., and J.C.; Supervision, J.C. and C.O.; Writing—Original Draft, S.R. and J.C.; Writing—Review and Editing, S.R., S.P.T., P.O., J.C., and C.O.

Funding

Ipatimup integrate the i3S Research Unit, which is partially supported by FCT, the Portuguese Foundation for Science and Technology. This work was supported by (1) FEDER—Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020—Operacional Programme for Competitiveness and Internationalisation (POCI), Portugal 2020, and by Portuguese funds through Portuguese Science and Technology Foundation (FCT)/Ministério da Ciência, Tecnologia e Inovação in the framework of the project “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274); (2) the project NORTE-01-0145-FEDER-000029, supported by Norte Portugal Regional Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); (3) Portuguese FCT with the Grant Reference PTDC/BBB-ECT/2518/2014; (4) FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), specifically by the BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences); (5) Ipatimup Board of Directors: Project “The role of gastric cancer cell-derived extracellular vesicles”; and (6) FCT fellowships PD/BD/128406/2017 to SR, SFRH/BPD/86543/2012 to JC, and SFRH/BPD/89764/2012 to PO.

Acknowledgements

The authors thank Joana Paredes for having generously shared the MCF10A cell line, and Cecília Durães for technical support with nanoparticle tracking analysis. The authors acknowledge the support of the HEMS, ALM, and Bioimaging i3S Scientific Platforms as well as the assistance of Rui Fernandes, Maria Azevedo, and Maria Lazaro.

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Supplementary Information

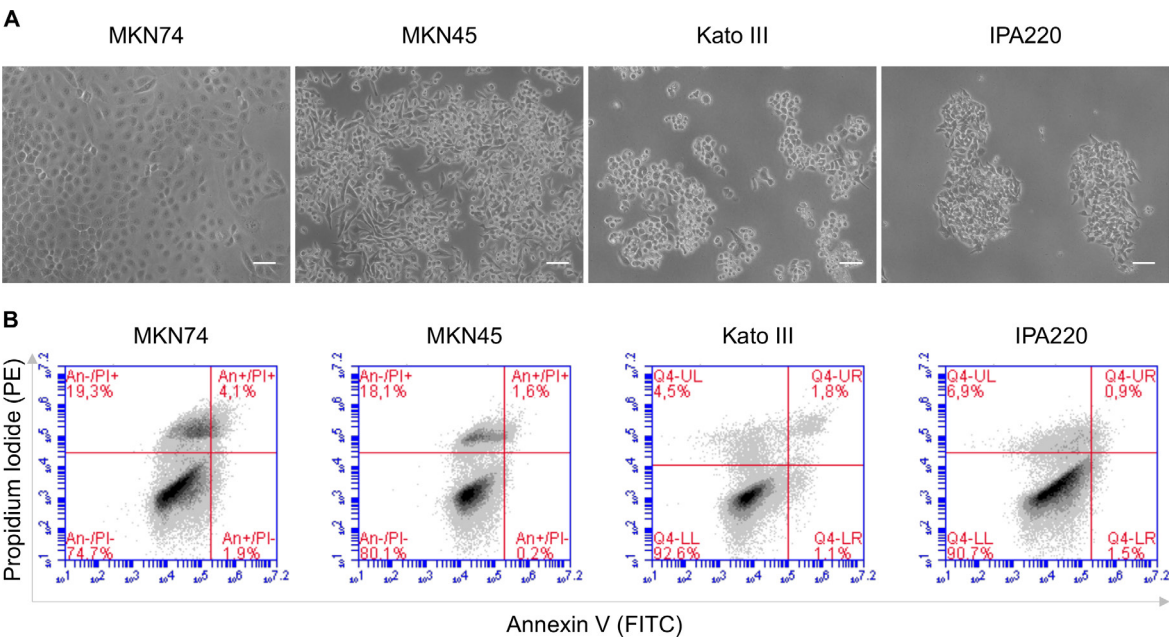


Figure S1. Characterization of GC cells before EVs isolation. (A) Representative bright-field microscopic images of MKN74, MKN45, Kato III and IPA220 GC cell lines. MKN74 and MKN45 displayed a more differentiated morphology, resembling the intestinal-type GC. Kato III and IPA220 presented a poorly differentiated phenotype, representative of diffuse-type GC. (B) Viability of GC cells tested by flow cytometry of annexin V (FITC) and propidium iodide (PE).

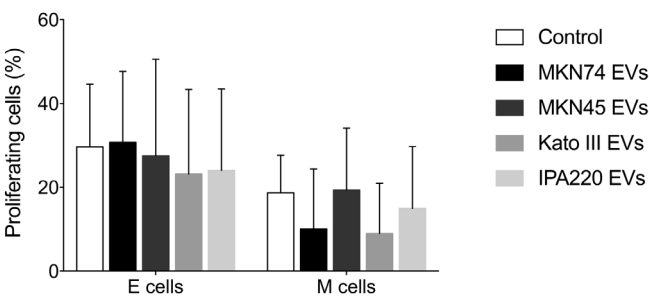


Figure S2. GC-EVs do not affect proliferation of epithelial and mesenchymal recipient cells. Graphs represents the mean percentage of proliferating cells $\pm$ standard deviation of three independent experiments.

3D cellular architecture affects microRNA and protein cargo of extracellular vesicles

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Advanced Science (Weinh); 6(4):1800948. doi: 10.1002/adv.201800948.

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The success of malignant tumors is conditioned by the intercellular communication between tumor cells and their microenvironment, with extracellular vesicles (EVs) acting as main mediators. While the value of 3D conditions to study tumor cells is well established, the impact of cellular architecture on EV content and function is not investigated yet. Here, a recently developed 3D cell culture microwell array is adapted for EV production and a comprehensive comparative analysis of biochemical features, RNA and proteomic profiles of EVs secreted by 2D vs 3D cultures of gastric cancer cells, is performed. 3D cultures are significantly more efficient in producing EVs than 2D cultures. Global upregulation

of microRNAs and downregulation of proteins in 3D are observed, indicating their dynamic coregulation in response to cellular architecture, with the ADP-ribosylation factor 6 signaling pathway significantly downregulated in 3D EVs. The data strengthen the biological relevance of cellular architecture for production and cargo of EVs.

Keywords

3D cell culture
Cancer
Extracellular vesicles
Integrative network analysis
Microwell arrays

Introduction

Epithelial tumors account for 90% of the tumors in all organs¹ and the success of tumor initiation and progression ultimately depends on the dynamic interactions taking place between the tumor cells and their microenvironment.^{2,3} Recent findings have showed that extracellular vesicles (EVs) act as key mediators of this crosstalk.⁴⁻⁶ Depending on their sub-cellular origin, various types of EVs, different in their content and function, can be distinguished, such as: exosomes (vesicles of endosomal origin in the size range from 50-150 nm), microvesicles (vesicles budding from the cell surface in the size range from 100-500 nm) and oncosomes (large vesicles of 1-10 μ m in diameter produced by some tumors).^{7,8} EVs serve as a universal mechanism of intercellular communication, transporting genetic information and bioactive molecules, such as DNA,⁹⁻¹¹ coding and non-coding RNA,¹²⁻¹⁴ proteins^{15,16} and lipids^{17,18} from donor to recipient cells.¹⁹ The information conveyed by tumor-derived EVs may functionally influence recipient cells and foster their reprogramming,²⁰ thus contributing to the regulation of different stages of tumor progression.²¹⁻²⁵ Therefore, once released into the circulation, EVs and EV-associated molecules can be used as biomarkers in diagnosis,²⁶⁻²⁸ prognosis^{29,30} and therapeutic follow-up of cancer patients.^{31,32} The role of EVs in tumor progression has been addressed using mostly *in vivo* and conventional two-dimensional (2D) *in vitro* models.^{22,24} In these studies, EVs have been isolated either from biological fluids of cancer patients or, more often, from 2D cell cultures of cancer cells.^{33,34} However, it is widely accepted that under 2D conditions, cells lack the *in vivo* spatial polarization and architecture, intrinsically related to their function, as well as cell-cell and cell-matrix interactions.^{35,36} Moreover, 2D sources of EVs are poorly efficient as a large input volume is needed and the yield of isolated EVs is normally low. Altogether, these facts may hamper the scale-up of 2D sources of EVs and their clinical implementation. In the last years, three-dimensional (3D) *in vitro* models have been developed and tailored to better recapitulate the *in vivo* features of tumors.³⁷⁻⁴¹ In fact, it has been demonstrated that tumor cells growing in 3D conditions acquire morphology,^{42,43} differentiation,⁴⁴ growth pattern^{40,45} and gene expression signatures similar to those observed in human tumors.⁴⁶ Importantly, it has already been shown that some of these cellular features, such as differentiation⁴⁷ and phenotype,^{48,49} affect the content and function of EVs. However, the impact of 3D cellular architecture on the production, content and function of EVs remains to be elucidated.⁵⁰⁻⁵² Additionally, it is unclear whether 3D models provide an efficient and cost-effective production of EVs as compared to 2D models.

In this work, we addressed these questions by studying the differences and commonalities between EVs released by tumor cells growing in 2D and 3D cultures. For this purpose, we used a customized 3D *in vitro* model⁵³ to isolate EVs released by cell aggregates from two gastric cancer (GC) cell lines. Thereafter, we performed a comprehensive comparative analysis of the biochemical features, small-RNA transcriptome and proteome of EVs released by cells growing in 2D and 3D conditions. We found that GC cells cultured in 3D produce higher amounts of smaller EVs, as compared to the same cells grown in 2D. The small RNA profiles of EVs produced in 2D and 3D conditions were similar; however, there were specific

microRNA signatures distinguishing EVs released in these two conditions. Moreover, EVs obtained from 2D and 3D cultures exhibited different protein expression profiles, showing significant downregulation of proteins assigned to the ARF6 signaling pathways in 3D. We also observed increased association between cells and EVs and invasion capacity of recipient cells upon treatment with EVs derived from 3D cultures of a specific tumor cell line, without affecting proliferation and viability. Analysis of microRNAs and proteins differentially expressed under 2D and 3D conditions showed a global upregulation of microRNAs and downregulation of proteins in 3D. Integrative network analysis of these data revealed a dynamic co-regulation of microRNAs and proteins in cells and EVs associated with the change of cellular architecture from 2D to 3D.

Altogether, these results indicate that the 3D cellular architecture modifies production levels and cargo of EVs, which may affect efficiency of association and consequently uptake and induce different functional behavior on recipient cells, rendering 3D cultures a more physiological high-throughput alternative to conventional 2D *in vitro* systems.

Results and Discussion

Establishment and characterization of 3D microwell array culture of GC cells

To dissect a potential influence of 3D cellular architecture on the release and cargo of EVs, we designed and carried out a comprehensive study according to the scheme presented in Figure 1.

First, we established a new method for EVs production using a recently developed and intensively characterized agarose microwell array,⁵³ which allowed long-term culture of cell spheroids and aggregates (Figure 2a).

In this system, MKN45 and MKN74 GC cells grew in aggregates with a maximum size of 200 μm when seeded at a density of 1×10^5 cells/mL and cultured for six days in EVs-depleted medium, without replenishment (Figure S1a, b). While MKN45, a poorly differentiated cell line derived from GC liver metastases,⁵⁴ formed aggregates of cells loosely attached to each other (Figure 2b, upper panel), MKN74, a moderately differentiated tubular adenocarcinoma cell line,⁵⁴ formed compact cell aggregates with a glandular-like organization (Figure 2b, bottom panel). Next, we examined the expression of E-cadherin and Mucin-1, proteins natively located at the basolateral and apical surface of normal gastric epithelial cells, respectively.⁵⁵ MKN45 and MKN74 cells displayed expression of E-cadherin at the basolateral membrane in both 2D and 3D cultures, resembling its physiological localization (Figure 2b). Mucin-1 is frequently upregulated in invasive GC⁵⁶ and its expression has been correlated with worse prognosis and tumor differentiation.⁵⁷ In agreement with these observations, Mucin-1 was only expressed at the apical localization in MKN74 cells cultured in 3D conditions (Figure 2b), suggesting that in 3D this differentiated cell line displays a phenotype that is similar to the one observed in tumors. Since MKN45 cell line was derived from a poorly

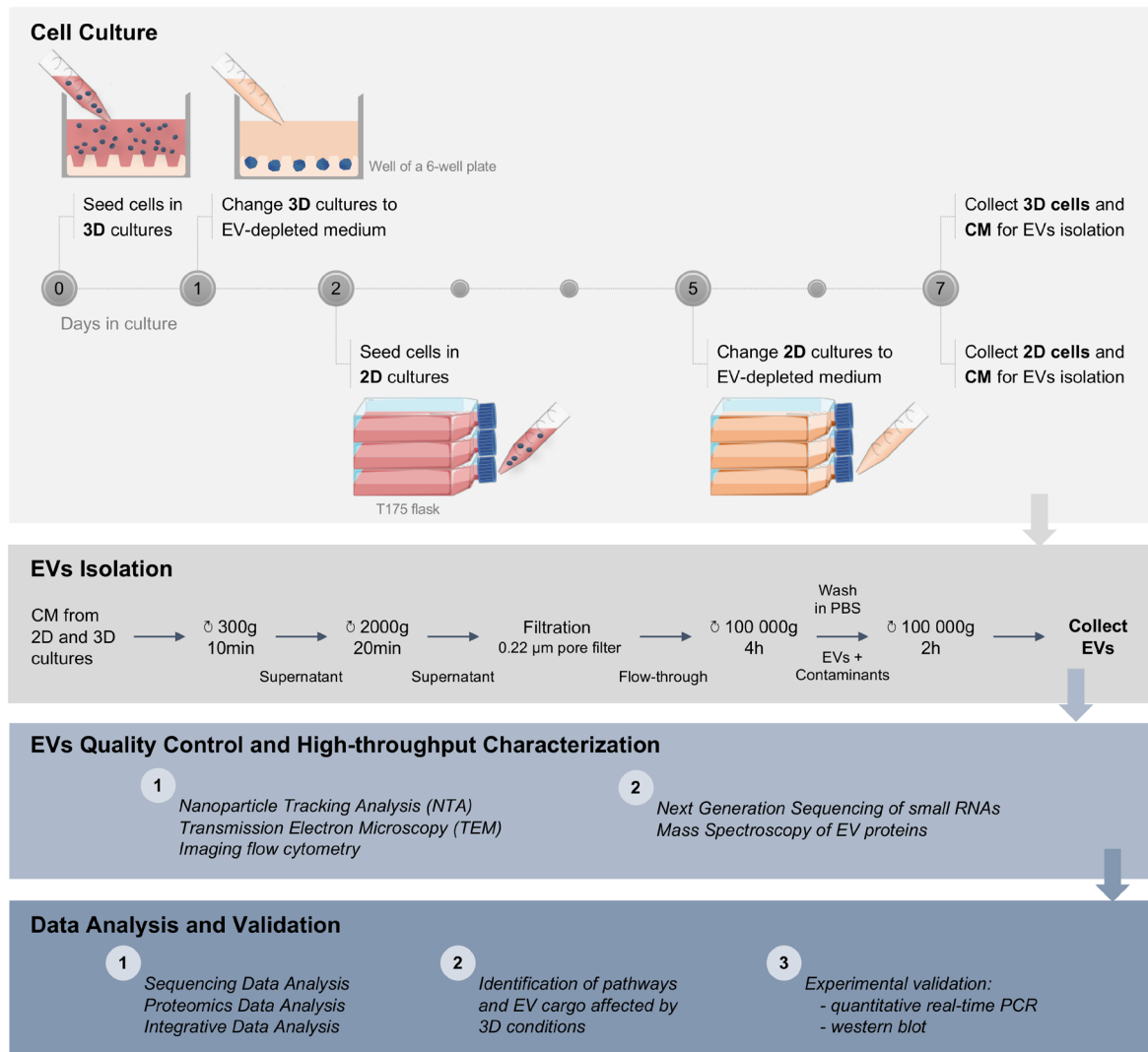


Figure 1. Scheme of the experimental flow for EVs production, isolation, characterization and data analysis. Conditioned medium (CM) isolated after six days of 3D cell culture and two days of 2D cell culture was used for EVs isolation by differential centrifugation. Quality control techniques (NTA; TEM; Imaging flow cytometry) were performed on the isolated EVs prior Next Generation Sequencing of small RNAs and Mass Spectrometry of proteins. Resulting data were analyzed separately and further integrated for the identification of altered pathways and EVs cargo in 3D cell culture conditions. Results were validated using independent samples and technical approaches (quantitative real-time PCR and Western Blotting).

differentiated tumor metastasis, apical Mucin-1 expression was expected neither in 2D nor in 3D conditions. Furthermore, hematoxylin-eosin staining showed phenotypical similarities between the structure of cell aggregates grown in microwell arrays and the structure of *in vivo* tumors, grown as xenografts in mice (Figure 2b, right panel). All these findings corroborated previous results using an *in vitro* multicellular gastric cancer spheroid model based on liquid overlay technique.⁵⁸ To estimate proliferation, we performed Ki-67 staining and detected proliferating cells in both MKN45 and MKN74 cell aggregates (Figure 2c). Cellular viability, measured after spheroid dissociation into single cell suspension, was determined by propidium iodide/annexin V staining. We observed that the percentage of viable cells was higher than 90% (Figure 2d), thus reducing the potential contamination of EVs preparations by apoptotic bodies.⁵⁹ Of notice, the percentage of dead cells growing in

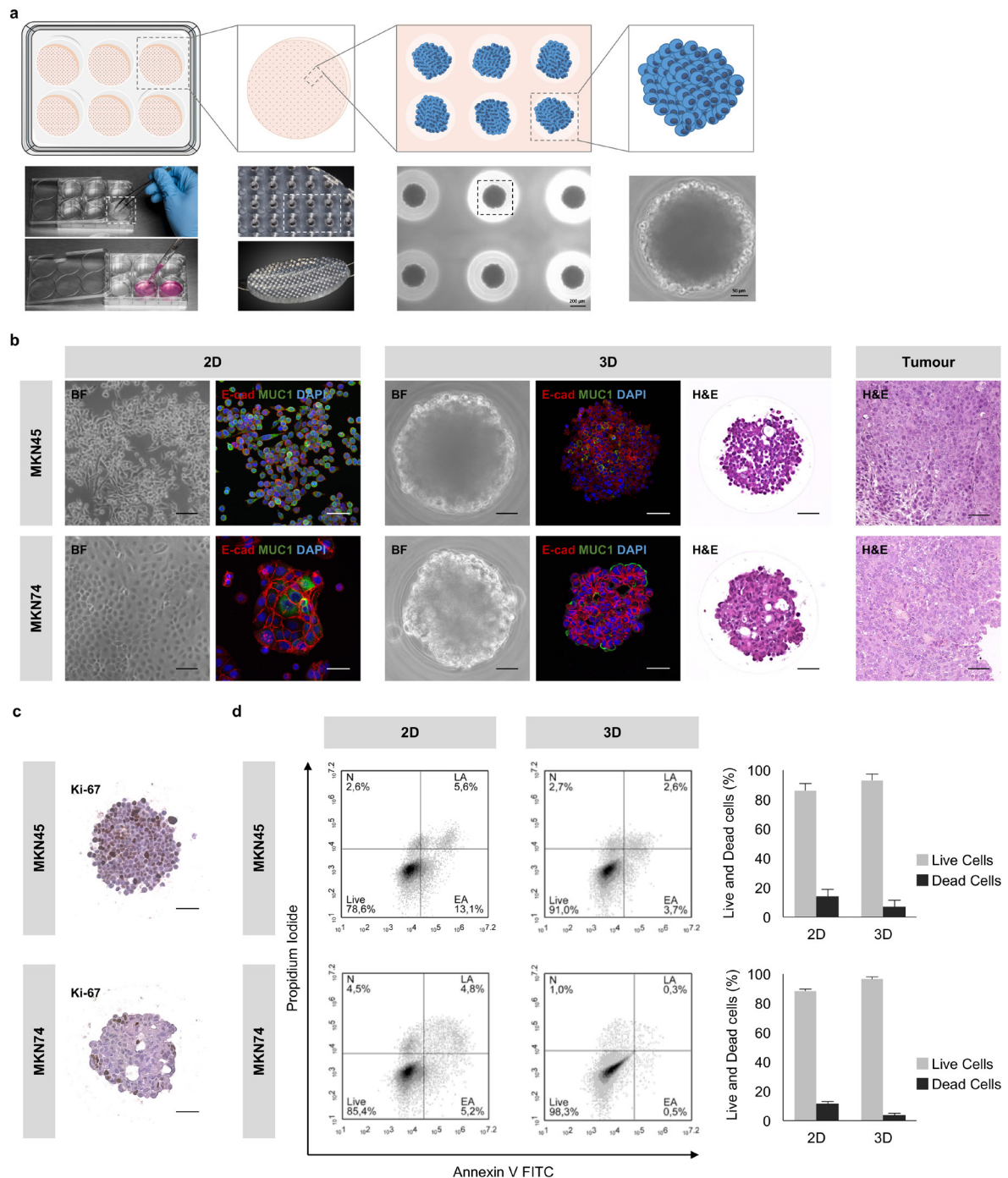


Figure 2. 3D culture of GC cells resemble the native GC histological subtype. **a)** Illustration of the 3D culture method. Agarose microwell arrays with 1000 conical microwells were placed in 6-well plates and 1×10^6 GC cells were seeded on the top of each matrix. GC aggregates, formed by liquid overlay, were allowed to grow for six days under EV-depleted medium. **b)** Representative images of the morphology (BF), expression of epithelial markers (E-cad/MUC1) of 2D and 3D cultures, and histology (H&E) of 3D cultures and xenograft tumors of GC cells. Scale bars, 50 μ m. BF: Brightfield; Ecad/MUC1: E-cadherin/Mucin1; H&E: Hematoxylin and eosin staining. Xenograft tumors were obtained from subcutaneous injection of 1×10^6 MKN45 cells and 5×10^6 MKN74 in athymic nude mice. **c)** Ki-67 staining showing representative proliferative patterns in 3D cultures. Scale bars, 50 μ m. **d)** Viability of cells harvested from 2D and 3D cultures (dissociated spheroids) tested by flow cytometry of Annexin V and Propidium Iodide. Data representative of five independent experiments were shown. N: Necrotic cells; LA: Late apoptotic cells; EA: Early apoptotic cells; Live: Live cells.

2D conditions was already above 10% (Figure 2d). This result combined with the fact that cells grow fast and become over-confluent latest after day five, prevents longer periods of 2D cultures.⁵⁹

The high cell viability of 3D cultures suggested the absence of a hypoxic or necrotic core, which was expected since the maximal aggregate diameter observed was near 200 μm .⁶⁰ It is noteworthy that larger spheroids could be obtained, by increasing cell density at seeding, to mimic the hypoxic/necrotic core that characterizes tumors.

Altogether, these results indicate that the 3D microwell array is a suitable system for the culture of GC cells. Knowing that differentiation and polarization play a role in the regulation of EVs release and content,⁶¹⁻⁶³ and that GC aggregates reproduced better the properties of *in vivo* tumors, one may speculate that EVs produced in this 3D system may be closer to those produced by patient tumors, than standard 2D culture systems. Therefore, this system may constitute a useful model for EV production and functional studies under a more physiological environment.

3D cellular architecture favors the release of smaller EVs expressing exosomal markers

To understand the impact of the 3D cellular architecture on the physical and biochemical properties of EVs, we performed transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and imaging flow cytometry on EVs isolated by ultracentrifugation from 2D and 3D cultures. TEM revealed vesicular structures of a size typically assigned to exosomes and microvesicles (Figure 3a).⁸ Additionally, EVs derived from 3D cultures tended to be smaller than EVs isolated from 2D cultures, which was further confirmed by NTA (Figure 3b, c). While EVs derived from 3D cultures had mean sizes between 85-135 nm, EVs isolated from 2D cultures had mean sizes of 100-180 nm (Figure 3b, c). By normalizing the total number of EVs isolated in each experiment to the total number of cells producing these EVs (cells detached from 2D monolayers or dissociated from 3D aggregates), we observed that the average number of EVs per cell was significantly higher in 3D in comparison to 2D (Figure 3d; Table S1). This result may reflect two key parameters of the culture method: culture time and cell number. Whereas in 2D, the maximum period for conditioned media collection was 48 h, in 3D this period could be extended for another 4 d without compromising cell viability. Moreover, in 3D cultures the number of cells used for producing EVs is considerably lower than in 2D (Table S1): three fold at the seeding and six- to ninefold at the end point, depending on the cell line. The volume of media required for 3D cultures was only 30% comparing with 2D conditions. Altogether, these observations strengthened the efficiency of the 3D system.

These findings were in agreement with previous studies showing that exosomes released by tissue-engineered tumors were smaller than exosomes released by the same cells cultured as monolayers, and had similar size distribution and concentration as exosomes isolated from human patient plasma.⁵² As EV preparations were enriched in small-sized

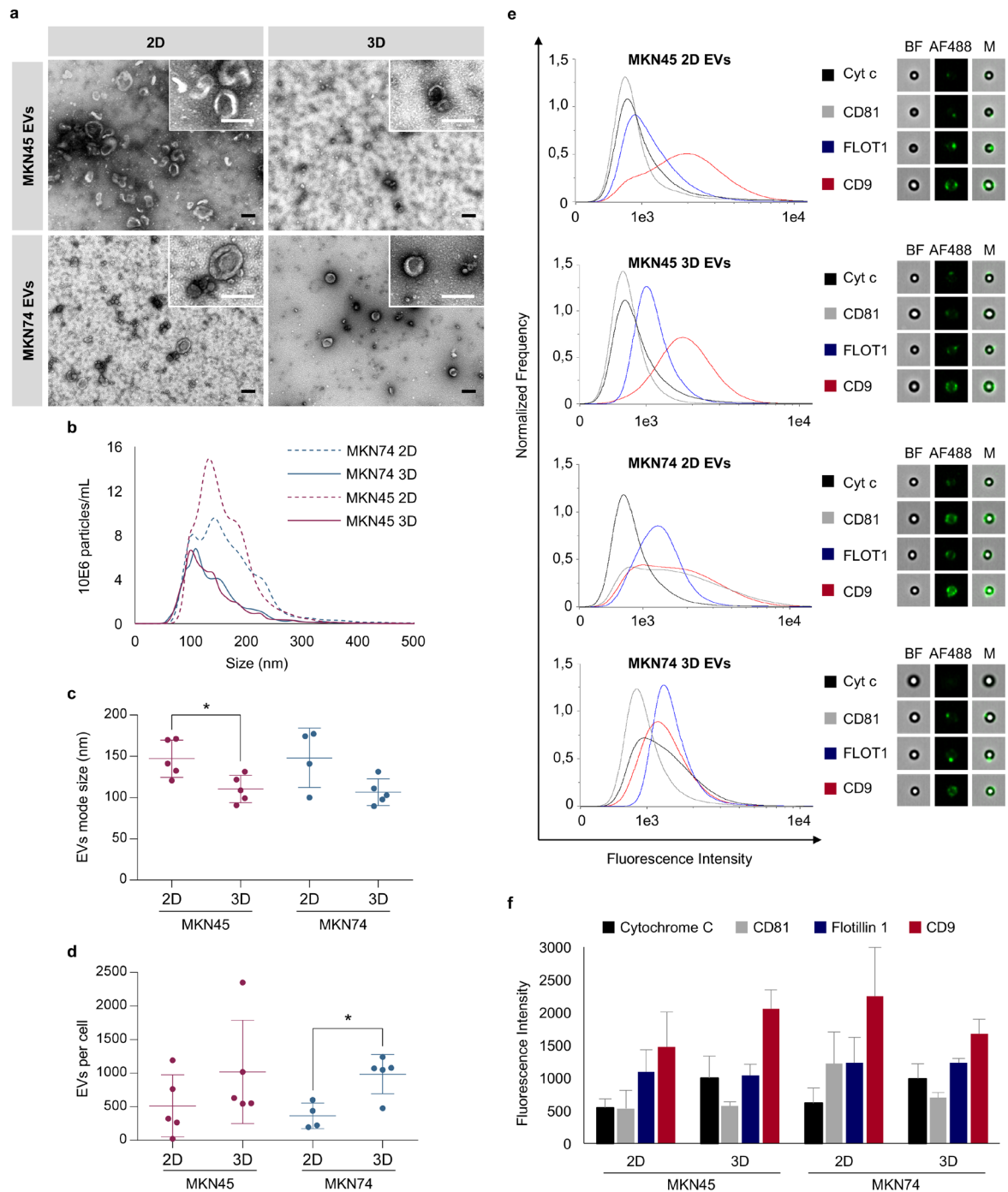


Figure 3. Increased EVs production by GC cells growing in 3D cultures a) Representative electron microscopy images of EVs isolated from 2D and 3D GC cultures. Scale bars, 100 nm. b-d) Nanoparticle Tracking Analysis (NTA) of isolated EVs. Distribution of b) size, c) mode size, and d) number of EVs per cell; graphs represent the mean $\pm$ standard deviation of at least four biological replicates. The number of EVs per cell represents the ratio between the total number of isolated EVs and the total number of cells retrieved from the respective cultures. * $p < 0.05$, Mann-Whitney Test. e,f) Detection of CD9, CD81 and Flotillin-1 by imaging flow cytometry. e) Distribution and representative images of the intensity of fluorescence detected for each marker. Bright field images (BF) showed beads to which EVs were coupled; Fluorescence images (AF488) showed EVs labelled with specific markers; Merged images (M) showed labelled EVs coupled to beads. f) Quantitative analysis of the intensity of fluorescence of each marker. Results represent the mean $\pm$ standard deviation of at least three biological replicates.

vesicles corresponding to the size of exosomes,⁶⁴ we tested the expression of exosome markers CD9, Flotillin-1 and CD81 (Cytochrome C – as a negative control) by imaging flow cytometry (Figure 3e, f; Figure S2). We observed that EVs from 2D and 3D cultures harbored comparable amounts of CD81 and Flotillin-1, while the portion of CD9-positive EVs tends to be increased in MKN45 3D cultures (Figure 3f). Taken together, these results showed that GC cell lines cultured in 3D conditions are highly efficient in producing EVs that maintain the expression of exosomal markers.

EVs isolated from 2D and 3D cultures exhibit similar small RNA profiles

To evaluate the impact of 3D cellular architecture on the small RNA profile of EVs, we generated and sequenced small RNA libraries from EVs and their donor cells. Since characterization of the small RNA content of EVs is still accompanied by a number of technical challenges, we followed the recommendations of the International Society of Extracellular Vesicles (ISEV) for the processing of EVs for RNA isolation, quality control and sequencing analysis.⁶⁵ Of notice, the sequencing of one of the biological replicates of MKN45 2D cells was less efficient, exhibiting a lower number of reads. To overcome this, findings from one biological replicate were considered to be sufficient for all described comparisons. Comprehensive sequencing analysis of cellular and EVs small RNAs revealed that more than 89% of the reads detected in cellular RNA, and 39%-94% of the reads in EVs RNA mapped to the reference genome (Figure 4a).

Notably, the vast majority of mapped reads from EVs libraries corresponded to reads that mapped to multiple genomic locations, whereas cellular libraries also encompassed sequences mapped to single genomic regions (Figure 4a). To dissect the small RNA profile of each sample, we assigned the mapped reads to the different small RNA classes using the biotypes defined by Ensembl and MirBase. On average, we detected the expression of 303 small RNAs in EVs and 1675 in cellular samples (Table S2). Of the small RNAs detected (FPKM>0), microRNAs were the most abundant, both in EVs and cellular samples, comprising on average 54% and 41%, respectively (Figure 4b).

In fact, other studies have reported that the majority of small RNAs detected in EVs were assigned to microRNAs.⁶⁶ Interestingly, our study used a NGS platform distinct from the ones used in such studies, which goes against the notion that the NGS platform may introduce bias in the RNA biotypes identified.⁶⁷ We also observed that while the second most abundant class of small RNAs in EVs were miscellaneous RNAs (misc-RNA), in cells, the second most abundant class were small nuclear RNAs (snRNA), comprising on average 26% and 29%, respectively (Figure 4b). Moreover, the high proportion of other types of small RNAs enclosed by EVs may reflect the different origins of EVs as described elsewhere.⁶⁷⁻⁶⁹ The sorting of all these small RNAs into EVs remains unclear, hence future investigations should focus on understanding how different types of small RNAs are recruited to EVs. By hierarchical clustering, we observed that the expression profiles of small RNAs were not affected by the culture condition, neither among cells nor among EVs (Figure 4c). Principle

component analysis revealed striking differences between small RNAs detected in EVs and in donor cells. While the small RNA profiles of all EVs samples exhibited high similarity, strong differences were observed between small RNA profiles of cells (Figure 4d).

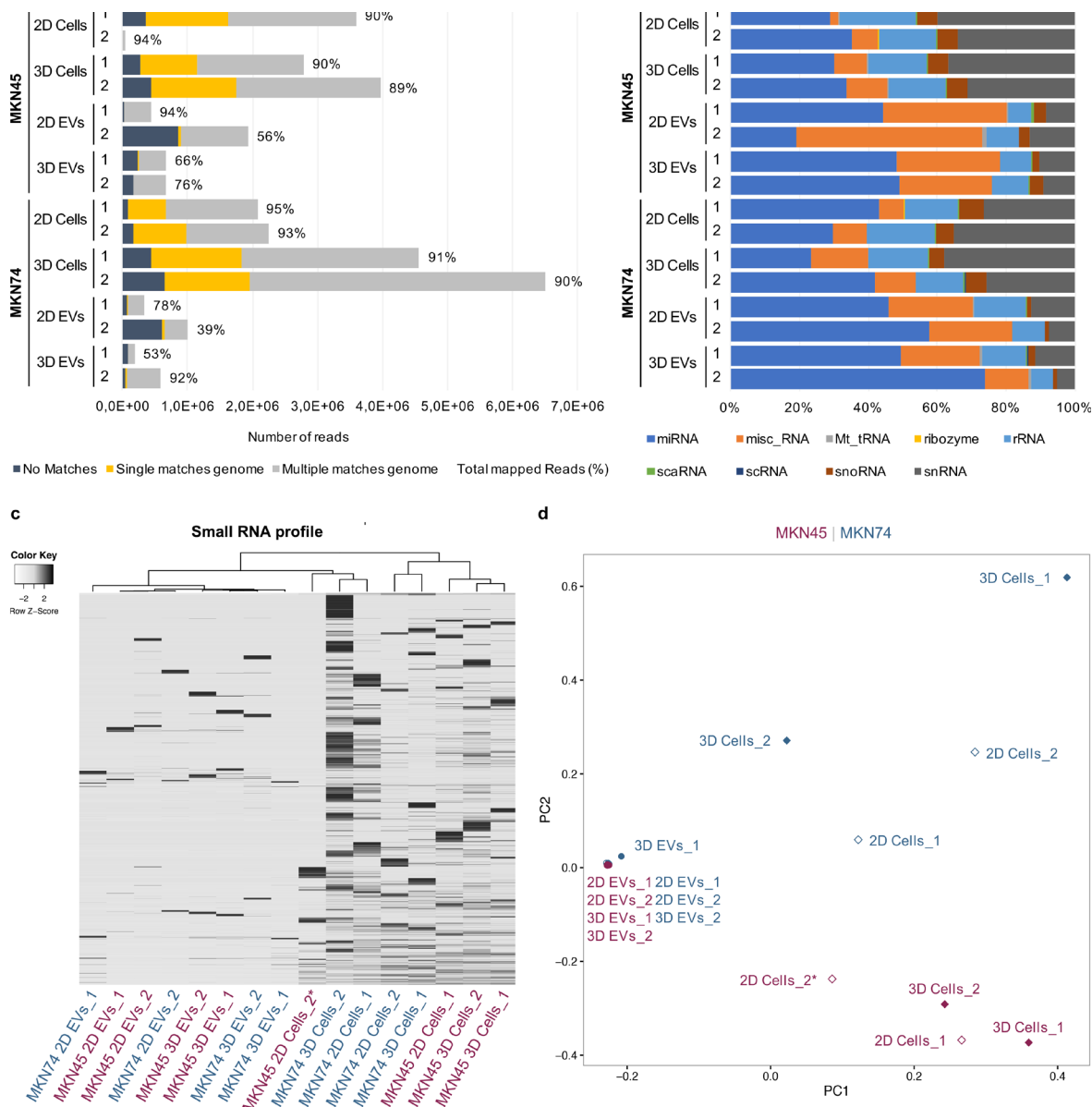


Figure 4. EVs released by GC cells under 2D and 3D conditions exhibit similar small RNA profiles. a) Total number of reads and percentage of mapped reads detected by small RNA sequencing. Reads that could not be mapped in the genome are shown in black; unique mappable reads are shown in dark grey; and reads that were mapped to multiple regions are shown in light grey. b) Distribution of mapped reads by small RNA classes. c) Heatmap and dendrogram of small RNA profiles of MKN45 and MKN74 EVs and cells in 2D and 3D cultures (Z-score normalized expression values). d) Principal Component Analysis (PCA) of small RNA profiles of MKN45 and MKN74 EVs and cells in 2D and 3D cultures. 1 and 2 represent two independent biological replicates. *Sample with reduced number of reads.

EVs and donor cells from 3D and 2D cultures exhibit specific microRNA signatures

Since microRNA was the most prevalent biotype identified both in EVs and cells, we further explored the microRNA expression profiles of our samples (Figure 5; Figure S3a-f). Using unsupervised hierarchical clustering, we observed no major differences between cells or EVs derived from 2D and 3D cultures, meaning that clusters were independent of culture conditions (Figure 5a). Next, we investigated if a different cellular architecture affected specific microRNA signatures, by searching for microRNAs exclusively present either in cells or in EVs, derived from 2D and 3D cultures. We identified 58 microRNAs exclusively expressed in cells from 2D cultures and 137 microRNAs exclusively expressed in cells from 3D cultures (Figure 5b). Whereas most microRNAs detected in EVs were also present in cells, we identified four microRNAs specific for EVs isolated from 2D cultures and 10 microRNAs specific for EVs isolated from 3D cultures. A single microRNA was shared by EVs from 2D and 3D cultures and not by cells (Figure 5b). Interestingly, these specific microRNAs, which discriminate spatial condition (2D vs 3D) and entity (cells vs EVs), corresponded to a small percentage of the total number of detected microRNAs (Figure 5c). The observation that EVs harbored a specific set of microRNAs, distinct from donor cells, was also seen in a breast-cancer model, suggesting that it might be a general phenomenon in tumor-derived EVs.⁶⁷ Moreover, we observed that a set of 64 microRNAs was shared by cells from 2D and 3D cultures and present only in EVs derived from 3D cultures (Figure 5c). Of notice, the number of specific microRNAs present in cells or in EVs from 3D cultures was higher than the number of microRNAs detected exclusively in 2D (Figure 5c). Although, experimental data explaining this phenomenon are scarce, sorting of certain microRNAs to EVs may require specific RNA-binding proteins and recognition of specific sequence motifs.⁷⁰

To explore the biochemical categories and pathways significantly enriched among the set of microRNAs present in EVs from 3D cultures, we performed an over-representation analysis (ORA) of two groups of microRNAs using the miEAA tool.⁷¹ Firstly, from the 10 microRNAs exclusively present in EVs from 3D cultures (Figure 5b), hsa-miR-155-5p, hsa-miR-143-3p, and hsa-miR-127-3p were significantly enriched in 48 categories, mostly associated with disease-state *e.g.*, lymphoma, KRAS pathway, hypermethylation and inflammatory response (Table S3).

Next, from the group of 64 microRNAs shared by cells growing in 2D and 3D conditions and present only in EVs derived from 3D cultures (Figure 5b), six significant categories were obtained (Table S4). The lowest *p*-value 0.01 was determined for 5/64 miRNAs, targeting the PIP5K1A signaling pathway, involved in the regulation of phosphoinositides metabolism, which plays a role in cell differentiation⁷² and invasion⁷³ (Table S4).

To identify pathways enriched among the 164 microRNAs detected in EVs from 3D cultures, regardless of their presence in EVs from other conditions, we performed a gene set enrichment analysis (GSEA), adapted for microRNAs (Table S5).⁷¹ Among the top 20 hits, main cancer-associated signaling pathways p53, MAPK, TGF beta and Ras were

identified (Figure S4).

Finally, to validate the NGS data, we quantified six randomly selected microRNAs, detected in both cells and EVs, using quantitative Real-Time PCR (qRT-PCR) (Figure S5a-c). We observed a strong correlation between the qRT-PCR results and the small RNA sequencing data in cellular RNA, showing higher levels of the selected microRNAs in 3D samples (Figure S5d). For EVs, the qRT-PCR data correlated better in MKN74 than in MKN45 cell line, which in general exhibits a high level of plasticity and phenotypic heterogeneity (Figure S5d).

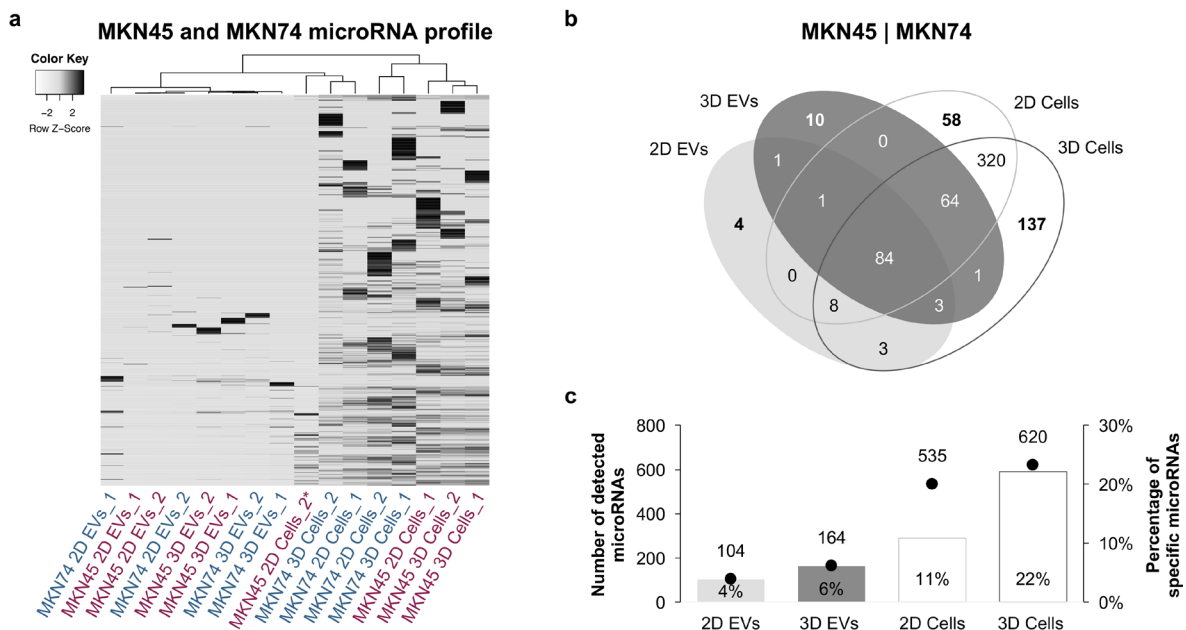


Figure 5. EVs and cells cultured under 2D and 3D conditions display distinct microRNA repertoires. a) Heatmap and dendrogram of microRNA profiles of GC EVs and cells in 2D and 3D cultures (Z-score normalized expression values). b) Venn diagrams showing the distribution of detected microRNAs in both cell lines. c) Plots showing the number of total microRNAs and percentage of specific microRNAs detected in each condition (2 biological replicates for each cell line).

ARF6 signaling pathway is downregulated in EVs isolated from 3D cultures

Next, we performed a comprehensive proteomics analysis of EVs released under 2D and 3D conditions by mass spectrometry. In total, 430 proteins were identified in EVs released by MKN45 and MKN74 cells in 2D and 3D. Following normalization, unsupervised hierarchical clustering was tested to estimate the impact of EVs origin (MKN45 vs MKN74) and spatial architecture (2D vs 3D). The protein expression profiles showed two separate clusters corresponding to MKN45 and MKN74 EVs, indicating that the cellular origin modulated the protein cargo of EVs (Figure 6a). Moreover, within these clusters, a clear separation was detected corresponding to 2D and 3D conditions (Figure 6a; Figure S6).

To highlight the differences between EVs released under 2D and 3D conditions, supervised clustering was applied to the 20 proteins with highest significance (Figure 6b). Remarkably, 17 out of 20 proteins had decreased expression in 3D comparing to 2D, demonstrating an overall downregulation of proteins in EVs from 3D cultures (Table S6). To

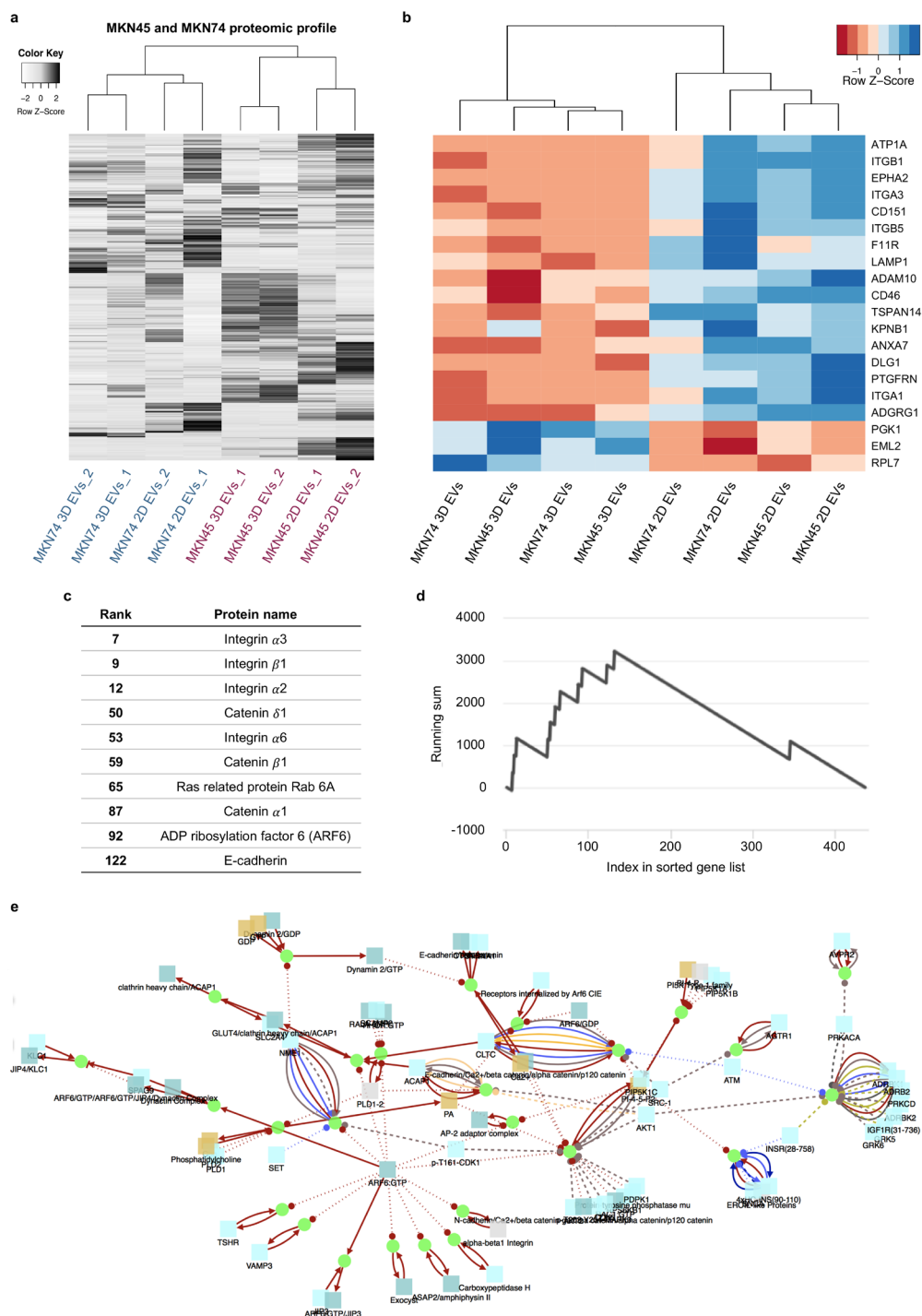


Figure 6. Protein set enrichment and network analysis show ARF6 signaling pathway significantly altered in EVs secreted by 3D cell cultures. a) Heatmap and dendrogram of protein profiles of GC EVs in 2D and 3D cultures (Z-score normalized expression values). b) Supervised clustering was performed to highlight the differences between EVs secreted by 2D and 3D cultures. Heatmap and dendrogram of the 20 most significantly different proteins between EVs isolated from 2D and 3D cultures (Z-score normalized expression values). c) Protein set enrichment analysis revealed ARF6 signaling pathway as the only pathway significantly altered between 2D and 3D conditions in both cell lines. The corresponding proteins were ranked according to the degree of deregulation between 2D and 3D. d) Running sum statistics highlighting the significant enrichment of the proteins listed. e) Representation of the ARF6 signaling network. f-g) Western blot validation and proteomics data of significantly altered proteins in 3D EVs. f) Representative Western blot images. g) Protein levels of significantly altered proteins quantified by densitometry analysis of Western Blot and validation of proteomics data. Tubulin was used as endogenous control for cells and GAPDH for EVs. Each biological replicate was independently represented.

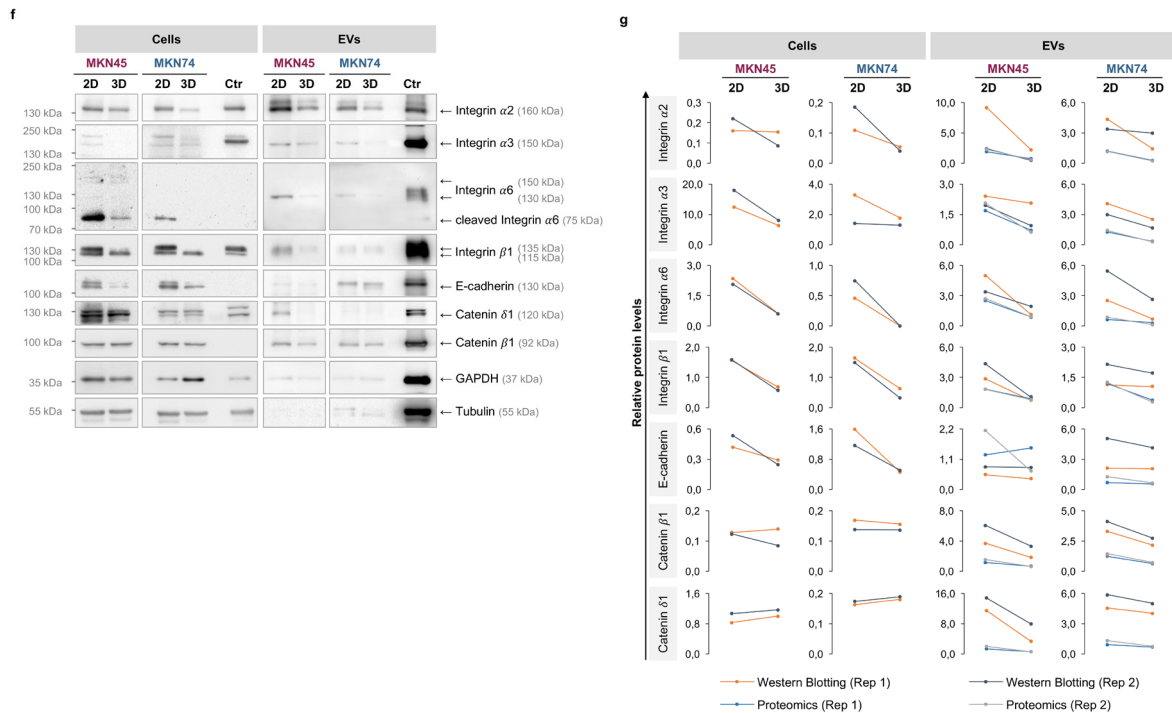


Figure 6. Continued.

unveil the proteins most affected by the spatial conditions, we applied protein set enrichment analysis (analogous to gene set enrichment analysis), listing proteins with respect to the degree of deregulation. We observed that the most downregulated proteins in 3D cultures are at the top of the sorted list (Figure 6c).

Next, we addressed whether proteins belonging to a certain biological category or pathway accumulate at the top or bottom of the sorted list by computing a running sum (Figure 6d). Using an algorithm relying on dynamic programming,⁷⁴ we defined ARF6 (ADP-ribosylation factor 6) signaling pathways to be significantly downregulated in EVs derived from 3D cultures (Figure 6e). ARF6 is a ubiquitously expressed protein involved in the regulation of membrane trafficking under physiological conditions and, together with syntenin, serves as one of the main regulators of exosome biogenesis.^{75,76} In addition, it has been shown that ARF6 activation leads to an increased shedding of microvesicles from tumor cells, carrying proteases that may degrade the surrounding extracellular matrix.⁷⁷ In fact, ARF6 upregulation/activation has been associated with an increase of cell motility and invasiveness in tumors.⁷⁸ Taking into account that the proteomic profile of EVs from cells grown in the 3D system suggests downregulation of ARF6-associated signaling pathways and, knowing that these cells released higher numbers of smaller EVs, we may hypothesize that the cellular spatial architecture influences the biogenesis of EVs in an ARF6-associated manner.

We validated seven proteins significantly downregulated in EVs from 3D cultures, by western blotting, and a good correlation with proteomics data was observed (Figure 6f, g). Additionally, by evaluating these proteins also in the donor cells, we observed that all proteins except catenin $\beta 1$ and catenin $\delta 1$ were downregulated in 3D (Figure 6f, g). Specifically for ARF6 protein, we observed no significant alterations at the protein levels

in the donor cells (Figure 6f, g). Analysis of ARF6 in EVs by western blotting confirmed results of proteomics data, showing downregulation of ARF6 in EVs from the MKN45 3D cultures (proteomics fold change: 0.41), and no differential expression of ARF6 in MKN74 EVs (proteomics fold change: 1.04) (Figure 6f, g). These data suggested that the regulation of ARF6 expression may be cell line specific. However, taking into account the observed regulation of proteins involved in the ARF6 signaling pathway (Figure 6f, g) we cannot exclude other ARF6 regulatory mechanisms, such as its activation *via* phosphorylation,⁷⁹ which were not addressed in our study. Therefore, the role of ARF6 and ARF6-associated signaling pathways in the biogenesis and regulation of EVs content, mediated by changes in cellular architecture, remains to be elucidated.

Dynamic co-regulation of microRNAs and proteins in response to changes in spatial cellular architecture

Given that in 3D cultures, we observed an upregulation of microRNAs in cells and EVs (Figure 5), together with a downregulation of proteins in EVs (Figure 6), we hypothesized that changes in cell architecture induced a microRNA-based regulation that targets cellular proteins, thus affecting EV cargo and their potential impact on recipient cells. Therefore, we analyzed the efficiency of association between EVs derived from 2D and 3D cultures with by different recipient cells: normal human dermal fibroblasts (NHDF), immortalized normal epithelial cells (MCF10A), and the EV-donor cancer cells MKN45 and MKN74 (Figure S7). Furthermore, proliferation, viability and invasion capacities of MCF10A treated with EVs isolated from MKN45 and MKN74 cancer cells growing in 2D and 3D conditions were also assessed (Figure S8). We observed increased association during the first 15 min of the EVs derived from the 3D culture of the MKN45 cells when MCF10A and MKN45 were used as recipient cells. No difference between 3D and 2D conditions was obtained when testing MKN74 EVs (Figure S7). The treatment with EVs from both cell lines did not change the proliferation and viability of recipient cells. Nevertheless, a significant increase in the invasion of recipient cells was induced by treatment with 3D EVs but not 2D EVs from the MKN45 cell line, indicating that despite similarity of cargo between EVs isolated from MKN45 and MKN74 3D cultures, their functional effect also relies on the inherited cell-specific context.

Next, we performed an integrative network analysis of NGS and LC-MS/MS data to pinpoint clusters of microRNAs and proteins reflecting the 3D cellular architecture. For this, two main criteria were considered: 1) miRTarBase indicated strong or weak evidence that a microRNA targeted a specific protein, and 2) the expression patterns of microRNAs and their target proteins were negatively correlated. With this analysis, several clusters were identified, three of which depicted in Figure S9. Of notice, such clusters may be constituted by more than one microRNA targeting the same protein.⁸⁰ By combining the full set of significant clusters, we identified a network specific for 3D conditions (Figure 7a, b).

Next, we selected four upregulated microRNAs (hsa-miR-26b-5p, hsa-miR-30d-5p, hsa-

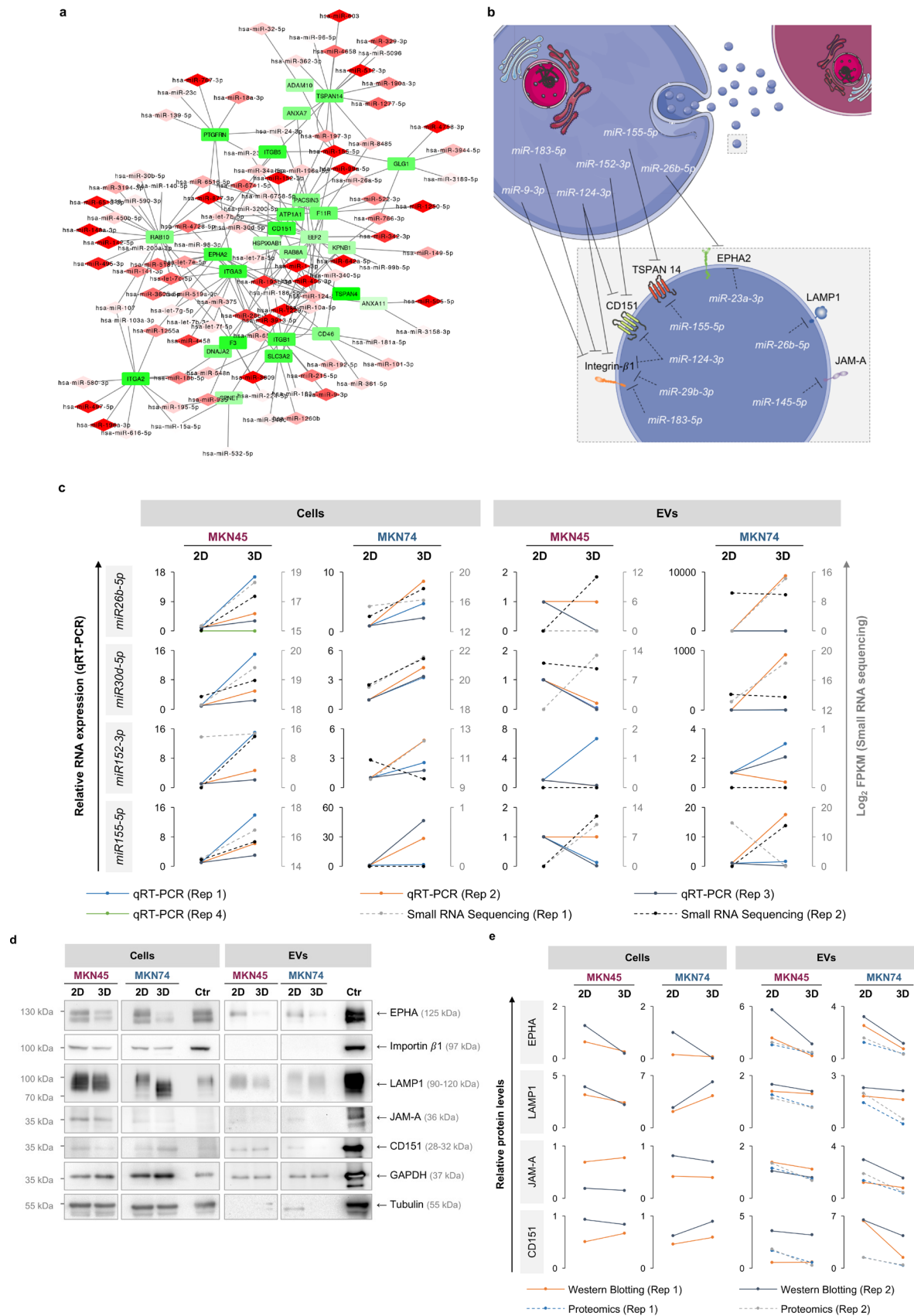


Figure 7. Integrative network analysis reveals overall up-regulation of microRNAs and down-regulation of proteins in EVs in 3D. **a)** Network of microRNAs (red rhombs) and specific target proteins (green rectangles) de-regulated in opposite direction. Upregulation and downregulation in 3D conditions were represented in different scales of red and green, respectively. **b)** Schematic representation of dynamic co-regulation of microRNAs in

miR-152-3p and hsa-miR-155-5p) and five downregulated proteins (Ephrin receptor A2, CD151, Junctional adhesion molecule A, Importin β 1 and Lysosome-associated membrane glycoprotein 1) for validation by qRT-PCR (Figure 7c) and Western Blot (Figure 7d, e). In cells, the expression levels of the four microRNAs correlated well with the data obtained by NGS (Figure 7c). In EVs, this correlation was maintained for MKN74 samples, however the same was not observed for MKN45 samples (Figure 7c). Validation of protein data in EVs supported the postulated hypothesis, showing consistent downregulation of all selected proteins, with the exception of importin β 1, which was not detected (Figure 7d, e).

Concluding Remarks

Our study focused on two main objectives: firstly, to establish a robust experimental system allowing efficient EVs production under controlled conditions and closer to the physiological environment, in comparison with conventional 2D cultures; secondly, to understand the influence of cellular architecture on the release and content of EVs. Here, we described the application of a newly developed agarose microwell array for isolation of EVs released by long-term culture of GC cell aggregates. Altogether, the high viability, the polarization and morphology of cells grown in 3D and the increased number of obtained EVs, empower the usage of this 3D system for scale-up of EV production, allowing further high-throughput experiments.

Using this system, we provided comprehensive evidence of the cellular architecture impact on EVs, including a comparative analysis of small RNAs and proteins present on EVs released by cells growing under 2D and 3D conditions, and functional assays.

Integrative network analysis of these data allowed us to raise the hypothesis of a dynamic co-regulation of microRNAs and proteins, in cells and EVs, in response to changes on cellular spatial architecture. In summary, we observed that the 3D cellular architecture favored an upregulation of certain microRNAs, leading to the downregulation of the target proteins, thereby affecting the cargo of EVs released from cells.

Although the impact of spatial architecture in cell properties is widely accepted, this study is the first, to our knowledge, addressing the impact of 3D culture conditions on EVs. Our findings suggest that spatial architecture influences the biogenesis and cargo of EVs and that our 3D system represents an improvement for a more physiological, highly efficient

cells and EVs (white) and proteins in EVs (black) highlighting specific sets of miRNAs and their common target proteins in 3D conditions. c) Quantitative RT-PCR validation and small RNA sequencing data of microRNAs of the integrative network in MKN45 and MKN74 cells and EVs. d,e) Western blot validation and proteomics data of proteins of the integrative network in MKN45 and MKN74 cells and EVs. d) Representative Western Blot images for proteins of the integrative network in MKN45 and MKN74 cells and EVs. e) Relative levels of proteins of the integrative network quantified by densitometry analysis of Western blot data and proteomics. Tubulin was used as endogenous control for cells and GAPDH for EVs. c-e) Each biological replicate was independently represented.

and cost-effective production of EVs in comparison to conventional 2D *in vitro* systems. Understanding the relationship between cellular spatial organization and its consequences on the content and function of EVs will improve our knowledge on EVs-mediated intercellular communication and its implications in health and disease.

Materials and Methods

Cell culture and characterization

Human GC cell lines MKN74 and MKN45 (ATCC, Manassas, VA) were cultured in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 10% foetal bovine serum (FBS; Biowest, Nuaille, France) and 1% penicillin-streptomycin (PS; ThermoFisher Scientific, Waltham, MA, USA). MCF10A (ATCC, Manassas, VA) were culture in DMEM/F12 supplemented with 5 $\mu\text{g mL}^{-1}$ insulin, 1 $\mu\text{g mL}^{-1}$ hydrocortisone, 5% horse serum, 1 ng mL^{-1} EGF, NHDF were cultured in DMEM (low glucose), supplemented with 10% foetal bovine serum (FBS; Biowest, Nuaille, France). All cells were maintained at 37°C in 5% CO_2 humidified atmosphere.

EVs-depleted medium was obtained by overnight ultracentrifugation at 100 000 $\times$ g in a 70Ti rotor (Beckman Coulter, Fullerton, CA, USA) of RPMI medium supplemented with 20% FBS and 1% PS. This EVs-depleted medium was filtered through a 0.22 μm filter and further diluted in the same amount of RPMI medium supplemented with 1% PS and without FBS, to reach a final 10% FBS concentration.

For 2D cultures, GC cells were seeded in T175 flasks, and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% PS until a confluence of 60%-70% was reached. Next, GC cells were washed with phosphate buffer solution (PBS; ThermoFisher Scientific, Waltham, MA, USA) and cultured in EV-depleted medium (10% FBS final concentration) during 48 h. The conditioned medium was used for EVs isolation and cells for downstream analysis. Cells growing in monolayer were detached using TrypLE™ Express Enzyme (ThermoFisher Scientific, Waltham, MA, USA; 5 min, 37°C), resuspended in normal medium and counted.

For 3D cultures, GC cells were seeded in customized microwell arrays developed at the Medical Center University of Freiburg,⁵³ which are in the meantime commercially available as 3D CoSeedis (abc biopply, Switzerland). Cell seeding was performed at a density of 1×10^5 cells mL^{-1} , corresponding to $\approx 1 \times 10^6$ cells per array and 1000 cells per microwell. GC 3D cultures were grown in RPMI medium supplemented with 10% FBS and 1% PS during the first day, and then cultured for 6 d in EVs-depleted medium. Optical microscopy was used to follow cell growth and spheroid formation. The conditioned medium was used for EVs isolation and cells for downstream analysis. After spheroid dissociation using 0.5% Trypsin-EDTA (ThermoFisher Scientific, Waltham, MA, USA; 5 min, 37°C), cells were resuspended in normal medium and counted.

Histological analysis and Immunofluorescence

GC cells growing in 3D cultures were fixed overnight, at room temperature (RT), using 2% paraformaldehyde (PFA; Merck, Darmstadt, Germany) followed by Gill's hematoxylin (Bio-Optica, Milan, Italy) staining for 10 min. Then, 2.4% low melting point agarose (50°C; Lonza, Basel, Switzerland) was added on top of 3D cultures followed by gelling (10 min at RT and 20 min on ice). Paraffin sections of 3 µm were used for hematoxylin and eosin (H&E), Ki-67 and immunofluorescence staining.

For H&E staining, slides were incubated with hematoxylin for 4 minutes, rinsed in ethanol and stained with eosin for 2 min.

For Ki-67 staining, antigen retrieval was performed using citrate buffer (1:100 dilution, pH 6.0, at 98°C, 40 min; Abcam, Cambridge, UK). Endogenous peroxidase activity was blocked using 3% hydrogen peroxidase solution (1:10 dilution, 20 min; Sigma-Aldrich, St. Louis, MO, USA) followed by incubation with anti-Ki-67 antibody (SP6, 1:200 dilution, 90 min; ThermoFisher Scientific, Waltham, MA, USA). After washing, slides were incubated with REAL™ EnVision™ Detection System (Dako, Glostrup, Denmark) substrate buffer (30 min) and with DAB Chromogen (10 min).

For immunofluorescence, samples from 2D and 3D cultures were co-stained for E-cadherin and Mucin-1. 2D cultures were fixed in 4% PFA (20 min), followed by treatment with NH₄Cl 50mM (10 min; Merck, Darmstadt, Germany), TritonX-100 0.2% (5 min; Sigma-Aldrich, St. Louis, MO, USA) and blocked with 5% of bovine serum albumin (BSA, 30 min; NZYTech, Lisbon, Portugal). For 3D cultures, antigen retrieval was performed as described for Ki-67 staining, followed by incubation in blocking serum (20 min; Ultra V Block; Lab Vision Corporation, Fremont, CA, USA). 2D and 3D samples were co-incubated (at 4°C, overnight) with rabbit anti-E-cadherin and mouse anti-Mucin-1 primary antibodies, followed by 1 h incubation with secondary antibodies anti-rabbit Alexa 594 and anti-mouse Alexa 488. Images were taken with a TCS-SP5 AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany) and slides were scanned using Axio Scan.Z1 slide scanner system (Zeiss, Oberkochen, Germany). See antibody details on Table S7.

Annexin V-FITC/Propidium Iodide staining

Cell viability was measured using Annexin V-FITC and Propidium Iodide (PI) double staining followed by flow cytometry. Cells from 2D cultures were harvested using TrypLE™ Express Enzyme. Aggregates from 3D cultures were retrieved from the agarose matrix by centrifugation (3 min, at 300×g) and dissociated using 0.5% Trypsin-EDTA. Cells recovered from the centrifuged conditioned media (300×g pellet – EVs isolation protocol described below) were pooled with cells dissociated from 2D or 3D cultures prior to Annexin V-FITC/PI staining. Dissociated 2D and 3D cells were washed with Annexin V binding buffer (AVBB; 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂) and stained with Annexin V-FITC (1:20, 15 min; ImmunoTools, Friesoythe, Germany). Cells were stained with PI (2 µg mL⁻¹, 15 min; ThermoFisher Scientific, Waltham, MA, USA), washed twice with AVBB, filtered through

a 0.22 μm filter and analyzed in a BD Accuri C6 flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Dead cells represent the sum of early apoptotic (EA; Annexin V-FITC+/PI-), late apoptotic (LA; Annexin V-FITC+/PI+) and necrotic (N; Annexin V-FITC-/PI+) cell populations. Live cells were considered to be Annexin V-FITC-/PI-. Results represent the mean $\pm$ standard deviation of three biological replicates of each cell line and culture condition.

EVs isolation by differential ultracentrifugation

EVs were isolated from the conditioned media of 2D and 3D cultures, growing in EVs-depleted medium, by differential centrifugation adapting previously described protocols.⁵⁹ Briefly, the collected conditioned media was centrifuged at 300 $\times$ g, 4°C, for 10 min to pellet cells. The remaining supernatant was transferred to new tubes, centrifuged at 2000 $\times$ g, 4°C for 20 min and filtered through a 0.22 μm filter. The filtered supernatant was further centrifuged in a SW32 rotor (Beckman Coulter, Fullerton, CA, USA) at 100 000 $\times$ g, 4°C for 4 h to pellet EVs. The EVs pellet was washed in PBS without Ca^{2+} and Mg^{2+} (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and centrifuged at 100 000 $\times$ g, 4°C for 2 h before being re-suspended in an appropriate volume of 0.9% NaCl, in accordance to downstream applications. Characterization of EVs is annotated in the EV-TRACK Database as EV-TRACK Nr. EV180053.

Negative staining and visualization of EVs by Transmission Electron Microscopy

The morphology of EVs was analyzed by TEM at the Histology and Electron Microscopy facility of i3S. In brief, an aliquot (10 μL) of fresh isolated EVs preparations were mixed with an equal volume of 4% PFA, to make a final concentration of 2% PFA. Fixed EVs (5 μL) were then added to Formvar-carbon coated grids and incubated for 1 min, in the dark at RT, to allow adsorption to the grid. Thereafter, EVs were stained with 5% uranyl acetate during 1 min and examined at a JEM 1400 electron microscope (JEOL, Tokyo, Japan). Images were recorded using a SC1000 OriusTM CCD camera (Gatan, Pleasanton, CA, USA).

Nanoparticle Tracking Analysis of EVs

The size and concentration of isolated EVs was measured by NTA, using the NanoSight NS300 instrument (Malvern, Worcestershire, UK) with scientific CMOS sensor. Samples were diluted in 0.9% NaCl to achieve a particle count between 1×10^8 - 1×10^9 particles mL^{-1} . For each biological replicate, three technical measurements (videos) were recorded under controlled fluid flow with a pump speed set to 40. Camera focus was adjusted to give a clear sharp image of the particles (camera level between 10 and 16). The three videos of 30 sec, with more than 1000 detected tracks per video, were analyzed using the automatic functions of NTA 3.1 Build 3.1.54 software and a detection threshold fixed at 5. Mode and mean size were calculated from the three videos per biological replicate. Results represent the mean $\pm$ standard deviation of at least four biological replicates of each cell line and were analyzed using the Mann-Whitney test (GraphPad Prism). Statistical significance was considered when $p < 0.05$.

EVs characterization by Imaging Flow Cytometry

EVs isolated from 2D and 3D cultures were analyzed for the expression of CD9, CD81, Flotillin-1 and Cytochrome c using imaging flow cytometry. Firstly, aldehyde/sulphate latex beads (4 μm , 4% w/v; ThermoFisher Scientific, Waltham, MA, USA) were washed in PBS, spun down at 14 000 rpm for 3 min, 4°C, and sonicated for 5 min on ice. EVs were then coupled to sonicated beads (1×10^9 particles determined by NTA per 3 μL of beads) for 1 h at RT with agitation, re-suspended in 1 mL PBS and incubated overnight, at 4°C. EVs coupled to beads (EVs-beads) were blocked in 100 mM glycine solution (30 min; Merck, Darmstadt, Germany) prior to immunostaining. EVs-beads were labelled during 1 h with mouse monoclonal anti-CD9, anti-CD81, anti-Cytochrome c and rabbit polyclonal anti-Flotillin-1. After washing (PBS-2% BSA), labelled EVs-beads were incubated with goat anti-rabbit/anti-mouse Alexa Fluor 488 secondary antibodies (30 min) and analyzed by imaging flow cytometry. EVs-beads incubated only with secondary antibodies were used as negative controls.

Acquisition was performed using ImageStreamX Imaging Flow Cytometer (Amnis Corporation, Seattle, WA, USA) equipped with INSPIRE software. This technology was demonstrated to be able to prevent the acquisition of confounding signal derived from dye aggregates, which may have a size comparable to the used latex beads, allowing to improve accuracy of EV measurements.⁸¹ For each sample, 100 000 events were acquired at a 40x magnification. Fluorescence of the stained EVs-beads was excited with a 488 argon laser and collected on channel two (505-560 nm). A 745 nm laser was activated for side-scatter and collected on channel 6, and bright field images with adjusted intensity were collected on channel 1. Data analysis was performed using the IDEAS software (Amnis Corporation, Seattle, WA, USA) using the pipeline described in Figure S2. Data shown as mean $\pm$ standard deviation. See antibody details on Table S7.

RNA extraction

Small RNA was isolated from 2D and 3D cells using mirVana miRNA isolation kit (ThermoFisher Scientific, Waltham, MA, USA) according to manufacturer's instructions and including the final procedure for small RNA enrichment. For the isolation of small RNA from 2D and 3D EVs preparations, mirCURY RNA isolation kit - Biofluids (Exiqon, Vedbaek, Denmark) was used, according to manufacturer's instructions. Prior to the small RNA isolation, EVs were treated with RNase A (final concentration 0.4 mg/mL, at 37°C, 10 min; NZYTech, Lisbon, Portugal). RNase A was inhibited with RNasin ribonuclease inhibitor (final concentration 1 U/ μL ; Promega, Madison, WI, USA). Samples were treated with proteinase K (final concentration 0.05 mg/mL, at 37°C, 10 min; Qiagen, Hilden, Germany), and inactivated at 75°C for 10 min. Concentration and quality of small RNAs, including microRNAs, were measured using the Agilent 2100 Bioanalyzer with the small RNA kit (Agilent, Santa Clara, CA, USA).

Small RNA library preparation and sequencing

Two independent small RNA samples isolated from cells and EVs collected from 2D and 3D cultures of MKN45 and MKN74 cells were used for small RNA library preparation using the Ion Total RNA-Seq Kit v2 (ThermoFisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. Briefly, 3' and 5' adaptors were attached directionally, and simultaneously, to 3 µL of small RNA input sample (≈10 ng of miRNA). Then, hybridized and ligated RNA was reversed transcribed using Ion RT primer v2 and SuperScript III Enzyme mix followed by purification and size-selection with a magnetic bead-based method. Each cDNA sample was amplified and barcoded using Platinum PCR SuperMix High Fidelity, Ion Xpress RNA 3' Barcode Primer and a unique Ion Xpress RNA-Seq Barcode BC Primer, which allowed sample identification and tracking. Amplified DNA was submitted to purification and size-selection with a magnetic bead cleanup module and the yield and size distribution was assessed with Agilent 2200 TapeStation (S/N 3-PM-1173NA) – HS D1000 Screen Tape (P/N 5067- 5584). The small RNA barcoded libraries were diluted to the same molar concentration and an equal volume of each diluted library was used to prepare the final pool. Pooled libraries were processed on Ion Chef™ System (S/N CHEF00657) using the – Ion 540™ Kit-Chef (P/N A27759) and the resulting 540™ chip (P/N A27766) was sequenced on Ion S5™ XL System (S/N 245717100156). FASTQ files were generated using the Torrent Suit plugin FileExporter v5.0.

Small RNA sequencing data analysis

High quality reads from the resulting sixteen FASTQ files were aligned to Homo sapiens reference genome (Assembly: GCA_000001405.15) using Bowtie2.⁸² Mapped reads were annotated using the corresponding GenBank information and mirBase (v.21).⁸³ Expression data for each sample was calculated using Cufflinks⁸⁴ and obtained in FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped). Cuffdiff⁸⁴ was used to pinpoint differentially expressed genes for each described sample comparison. Private Perl scripts and Ensembl database⁸⁵ information was used for expression data selection of genes with one of the following biotypes: miRNA, misc_RNA, mt_tRNA, ribozyme, rRNA, scaRNA, scRNA, snoRNA, snRNA. Expression data from selected genes was used for: 1) heatmap construction using FPKM values scaled to z-score with the R package 'gplots';⁸⁶ 2) supervised and unsupervised hierarchical clustering for dendrogram construction using calculated Euclidean distances, using R package 'gplots';⁸⁶ 3) Venn diagrams plotting using jvenn.⁸⁷ Of notice, microRNAs with FPKM>0 were considered as expressed and used for Venn diagrams plotting. Moreover, we considered a microRNA as expressed when detected in at least one of the biological replicates. RNA sequencing data will be available upon request.

ORA was performed using the miEAA tool.⁷¹ Ten microRNAs specifically present in 3D EVs and 64 microRNAs present in 3D EVs and in 2D and 3D cells were subjected to the analysis. As a reference list we defined a total number of 164 microRNAs that were detected in all EVs from 3D culture. We considered all categories with nominal p-values below 0.05

as significant but only considered categories containing at least two miRNAs. For a GSEA, the 164 miRNAs detected in EVs from 3D culture were ranked according to their average FPKM value and analyzed using the default settings in miEAA tool, such as significance value 0.05 and threshold level 2. The categories pathways, Gene Ontology, diseases and targets were selected for the analysis.

Quantitative Real-Time PCR

TaqMan™ Advanced microRNA assays (ThermoFisher Scientific, Waltham, MA, USA) were used to quantify specific microRNAs on cellular and EVs RNA samples, on a 7500 Fast Real-Time PCR system (ThermoFisher Scientific, Waltham, MA, USA). Analysis was performed using the cell and EVs RNA samples, previously submitted to small RNA sequencing plus one independent biological replicate for MKN74 samples and two independent biological replicates for MKN45 samples. Briefly, 10 ng of RNA were reversed transcribed using universal RT primers and the TaqMan™ Advanced microRNA cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA), according to manufacturer's instructions. Then, real-time qPCR was performed for each sample using 7 µL of diluted cDNA, TaqMan™ Advanced microRNA assays and TaqMan™ Fast Advanced Master Mix (ThermoFisher Scientific, Waltham, MA, USA) under fast cycling conditions. Reactions were done in triplicates and data was analyzed by the comparative $2^{-\Delta CT}$ method, where $\Delta CT = C(t)_{\text{miRNA 3D cells}} - C(t)_{\text{miRNA 2D cells}}$ or $\Delta CT = C(t)_{\text{miRNA 3D EVs}} - C(t)_{\text{miRNA 2D EVs}}$. Results were represented as mean $\pm$ standard deviation.

Using the Small RNA sequencing data, we searched for a small RNA that could be used as a housekeeping gene. Such small RNA must be expressed in all samples (FPKM>0), with a good sample detection (FPKM>10 000) and with no differential expression between samples ($0.5 \leq \text{Fold change} \leq 1.5$). As we could not find a single candidate for normalization of qRT-PCR data, we focused the analysis on the presence or absence of a certain microRNA, rather than its differential expression. Thus, individual replicates data was shown rather than the mean $\pm$ SD. See microRNA assay details on Table S7.

Protein sample preparation for tandem mass tag labelling

EVs pellets were suspended in RapiGest 0.1% (in TEAB 0.1 M, pH 8). Volumes were adjusted to 100 µL with RapiGest 0.1% (in TEAB 0.1 M, pH 8). Reduction of 10 µg of protein per sample was done with TCEP (tris(2-carboxyethyl)phosphine) at final concentration of 10 mM; the samples reacted for 30 min at 60°C. Alkylation was done with iodoacetamide, added to a final concentration of 40 mM to each sample following incubation for 60 min, in the dark, at RT. Trypsin was added (ratio of 1:25, w/w), and the digestion was performed overnight at 37°C. Then, a 10-plex Tandem mass Tags (TMT) (ThermoFisher Scientific) labeling was performed according to manufacturer's instructions. TMT reagent tags were dissolved in ACN, and each sample was incubated for 60 min at RT with a specific tag. For TMT quenching, 8 µL of hydroxylamin 5% (V/V) were added, and incubated with the samples for 15 min. TMT-labelled samples were pooled. For RapiGest cleavage, samples were incubated with TFA for 45 min at 37°C (pH<2). After centrifugation at 14 000 rpm for

10 min, supernatants were recovered and dried under vacuum. Samples were dissolved in 5% ACN/0.1% FA and desalted with C18 micro-spin columns (ThermoFisher Scientific). Peptides were separated by off-gel electrophoresis, desalted and solubilized in an appropriate amount of 5% ACN / 0.1% formic acid for mass spectrometry (MS) analysis.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The peptides were dissolved in 5% ACN/0.1% FA to a concentration of 0.25 µg µL⁻¹. Mass spectrometry experiments were performed on a Q Exactive Plus equipped with an Easy-nanoLC (ThermoFisher Scientific). Peptides were trapped on 2 cm x 75 µm ID, 3 µm pre-column and separated on an Easy-spray column, 50 cm x 75 µm ID, PepMap C18, 2 µm (ThermoFisher Scientific). The analytical separation was run for 60 min using a gradient of H₂O/FA 99.9%/0.1% (solvent A) and CH₃CN/FA 99.9%/0.1% (solvent B) at a flow rate of 300 nL min⁻¹. For MS survey scans, the OT resolution was set to 140 000 and the ion population was set to 3 × 10⁶ with an *m/z* window from 350 to 2000. Twenty precursor ions were selected for higher-energy collisional dissociation (HCD) with a resolution of 35 000, an ion population set to 1 × 10⁵ (isolation window of 0.5 *m/z*) and normalized collision energy set to 30%.

Protein identification and quantification

Raw data were loaded on Proteome Discoverer 2.1 software for identification and/or quantification of peptides and proteins. Identification was performed in the UniProt/SwissProt human database (2015_03, 20'203 entries) using Mascot (Version 2.5.1, Matrix Sciences, London, UK). Carbamidomethylation of cysteines, TMT-sixplex amino terminus, TMT-sixplex lysine (for TMT labeled samples) were set as fixed modifications and methionine oxidation as variable modifications. Trypsin was selected as the enzyme, with two potential miss-cleavages. Peptide and fragment ion tolerances were respectively 10 ppm and 0.02 Da. Threshold of the average reporter signal-to-noise ratio (S/N) was set to 1 and false discovery ratio (FDR) was set to 1% at peptide-spectrum match (PSM), peptide and protein levels. Only high-confident master proteins with at least two distinct peptide sequences were subjected to identification.

Western blotting

Cells and EVs pellets were lysed in HEPES lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, 1% TritonX-100, 2 mM PMSF, protease inhibitor Tabs, EDTA-free, Promega) for 45 min at 4°C. After lysis, samples were centrifuged at 15 000 × g, 4°C for 20 min. For analysis, 30 µg of cell lysates and 20 µg of EVs lysates were subjected to SDS-PAGE followed by immunoblotting. SuperSignal West DURA Extended Duration Substrate (Pierce) was used for signal detection. See antibody details on Table S7.

Uptake assay

To estimate efficiency of EV uptake, the association between EVs and recipient cells was measured using flow cytometry. EVs released from the 2D and 3D MKN45 and MKN74

cultures were tested in this assay. As recipients MCF10A, NHDF (normal human dermal fibroblasts) and the EV donors, MKN45 and MKN74 cells, were tested. Recipient cells were seeded in 24-well plates, next day by 80% density, association was analyzed using EVs labeled with PKH26 (Sigma) according to the recommendations of the supplier. Briefly, 1.25×10^9 EVs were brought to the final volume of 250 μ L with the diluent C and mixed; 1 μ L of PKH26 was mixed with 249 μ L diluent C; both solutions were mixed and incubated for 5 min in the dark. Staining was stopped with 500 μ L 1% BSA. To remove unbound dye, the EVs were loaded in centrifugal filter units (100 kDa cut-off) and centrifuged 5 min at $2500 \times g$ RT. Flow-through was discarded and EVs were brought to the final volume of 300 μ L using the serum-free medium. As a control, PBS without EVs was stained using the same protocol.

Directly prior EV treatment, recipient cells were washed with PBS, 1.25×10^9 labeled EVs were added to each well and incubated for 5, 15 or 30 min at 37°C . After that EVs were removed and the cells were washed, trypsinized, and measured by flow cytometry (BD Accuri C6) using thresholds FSC-H 80.000, SSC-H 10, flow rate $35 \mu\text{L min}^{-1}$, core size 16 μm and 10 000 counts, for NHDFs 6000 count limit was used.

Functional assays

Cell invasion, proliferation, and viability of normal epithelial cells (MCF10A) were measured upon treatment with EVs derived from 2D and 3D cultures of MKN45 and MKN74 cells.

Firstly, MCF10A cells were washed with PBS and harvested using Versene (ThermoFisher Scientific) followed by staining with CellTrace Cell proliferation kit, accordingly to manufacturer's instructions (ThermoFisher Scientific). Then, 5×10^5 labeled cells were seeded and treated with 5×10^9 EVs in the next day. After 24 h of treatment, cells were collected and submitted to different functional assays. For the invasion assay, Corning™ BioCoat™ Matrigel™ Invasion Chambers (ThermoFisher Scientific) were hydrated with DMEM/F12 medium (ThermoFisher Scientific) during 1 h at 37°C . Then, 5×10^4 cells were seeded in the upper compartment and incubated for 24 h at 37°C in 5% CO_2 . Filters were washed in PBS and fixed in ice-cold methanol for 10 min. Non-invasive cells and Matrigel were removed with a pre-wet cotton swab, and filters were washed and mounted in glass coverslips with Vectashield/DAPI. A mosaic of the entire filters was obtained with Axiovert 200M (Zeiss, Oberkochen, Germany) and the total number of invasive nuclei was counted using ImageJ software. Cell proliferation was determined by analyzing CellTrace dilution using a BD FACSCanto™ II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Cell viability was performed as abovementioned. Results represent the mean $\pm$ standard deviation of at three biological replicates. Data were analyzed with a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.

For proteomic data, protein set enrichment analysis was performed using the multi-omics analysis toolkit GeneTrail2,⁸⁸ freely available at <https://genetrail2.bioinf.uni-sb.de>. Proteins were ordered with respect to the degree of deregulation and significance values were calculated by an exact algorithm relying on dynamic programming.⁷⁴ Next, an integrative analysis of miRNAs and proteins was performed using the criteria described in the Results section. Two criteria were considered: 1) miRTarBase indicated strong or weak evidence that a microRNA targeted a specific protein and; 2) the expression patterns of microRNAs and their target proteins were negatively correlated. Of notice, miRTarBase strong evidence targets were those found by reporter assays, while weak evidence targets derived from high-throughput techniques such as microarrays.

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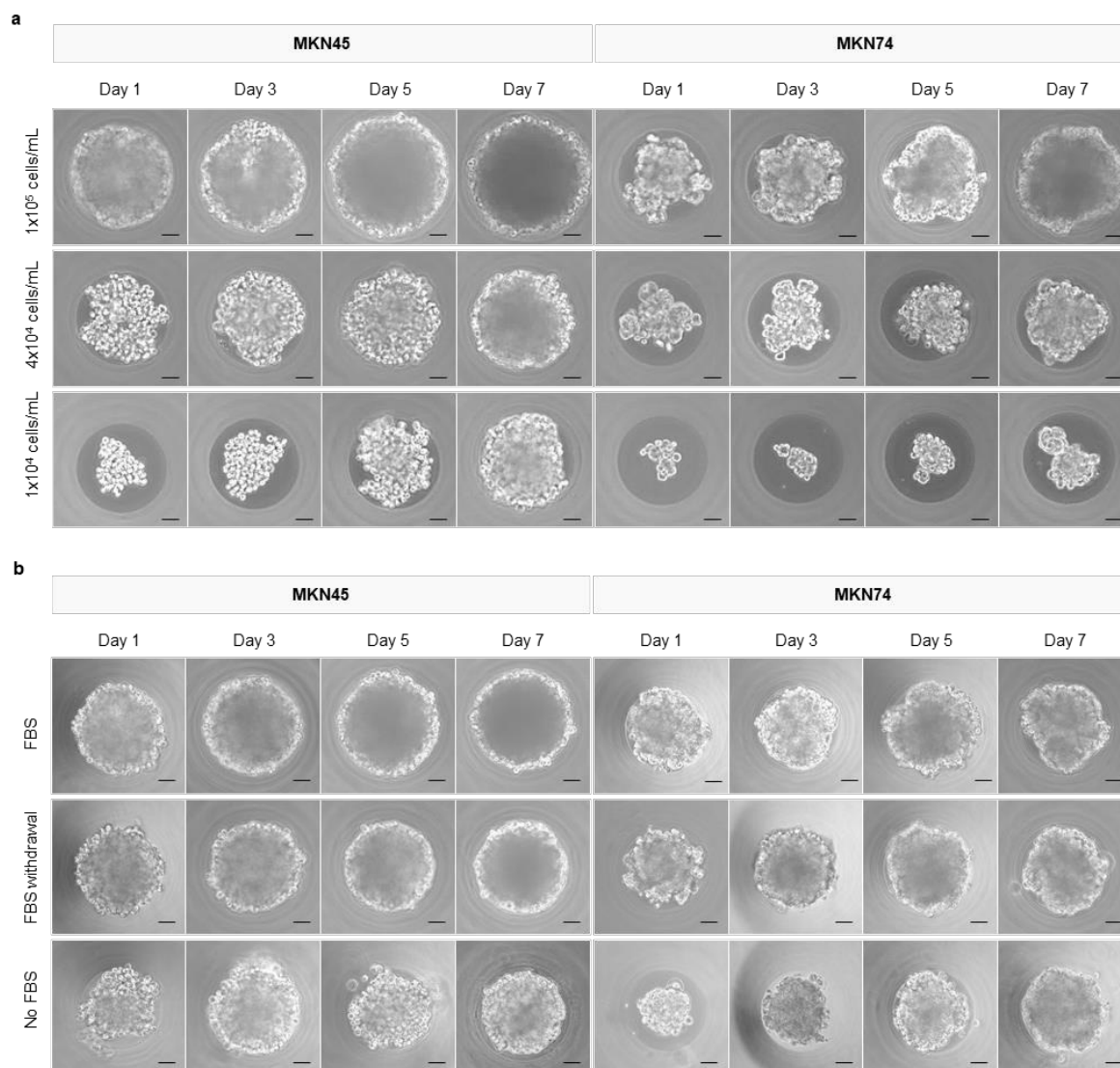
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Acknowledgements

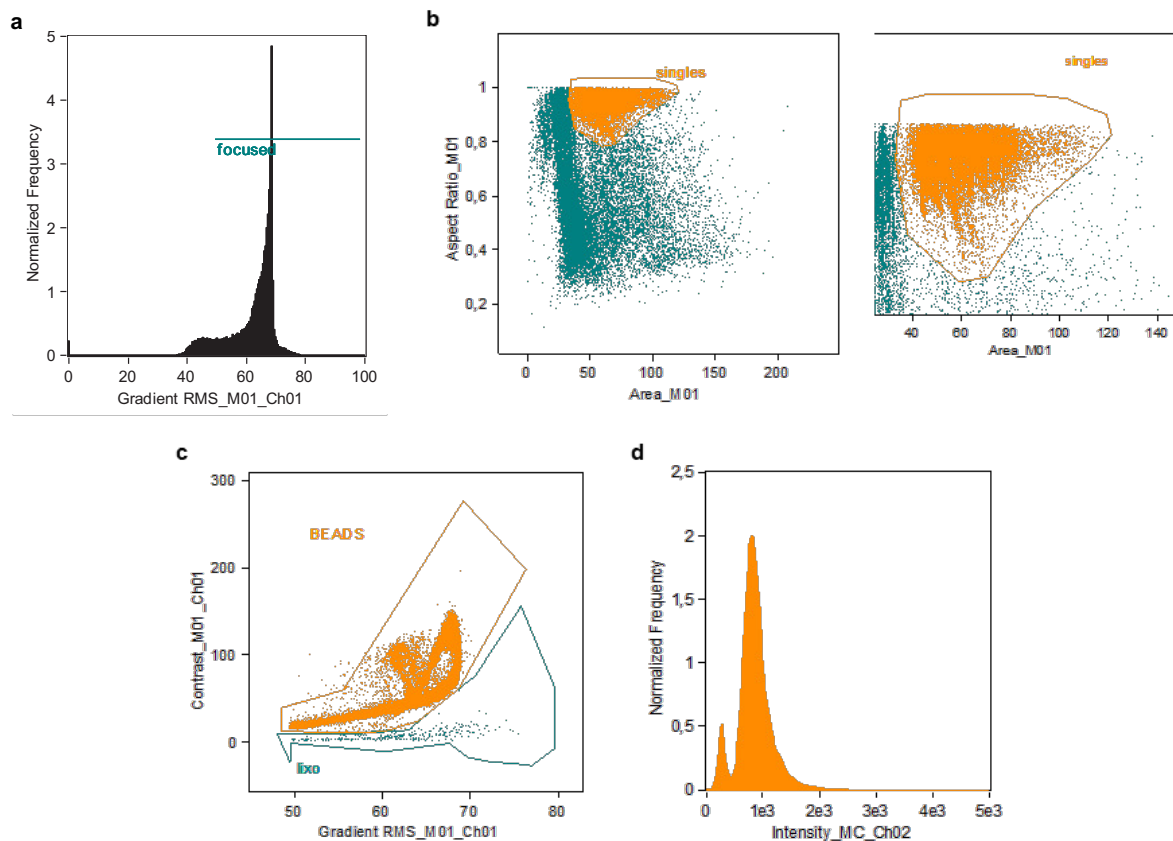
S.R. and J.C. contributed equally to this work as co-first authors. C.O. and I.N. contributed equally to this work as co-senior authors. The authors thank Celso Reis and Joy Burchell for having generously shared the anti-Mucin-1 antibody, Gabriela Almeida and Mafalda Santos for sharing the H&E images from MKN45 and MKN74 tumors in mice, Maria Lazaro for the technical support with imaging flow cytometry data, Tanja Gainey-Schleicher and Elena Grueso Navarro for the support with EV–cell association assay, and Deborah Lawrie-Blum for proofreading the manuscript. S.R., R.K., J.C., C.O., A.T., A.K., and I.N. were involved in the conception and design of the study; S.R., J.C., and P.O. were involved in the establishment and characterization of 3D microwell array culture of GC cells, characterization of EVs, small RNA sequencing experiments, data analysis and validation, and functional assays; D.S., M.V., N.W., and R.K. were involved in all proteomics studies including data analysis and validation, and the analysis of cell–EV association; S.R., J.C., P.O., C.O., D.S., A.K., J.-C.S., A.T., and I.N. analyzed the data; S.R., J.C., P.O., C.O., A.K., and I.N. wrote the manuscript; S.R., J.C., P.O., C.O., A.K., and I.N. critically reviewed the manuscript; and all authors gave final approval of the manuscript.

Supplementary Information

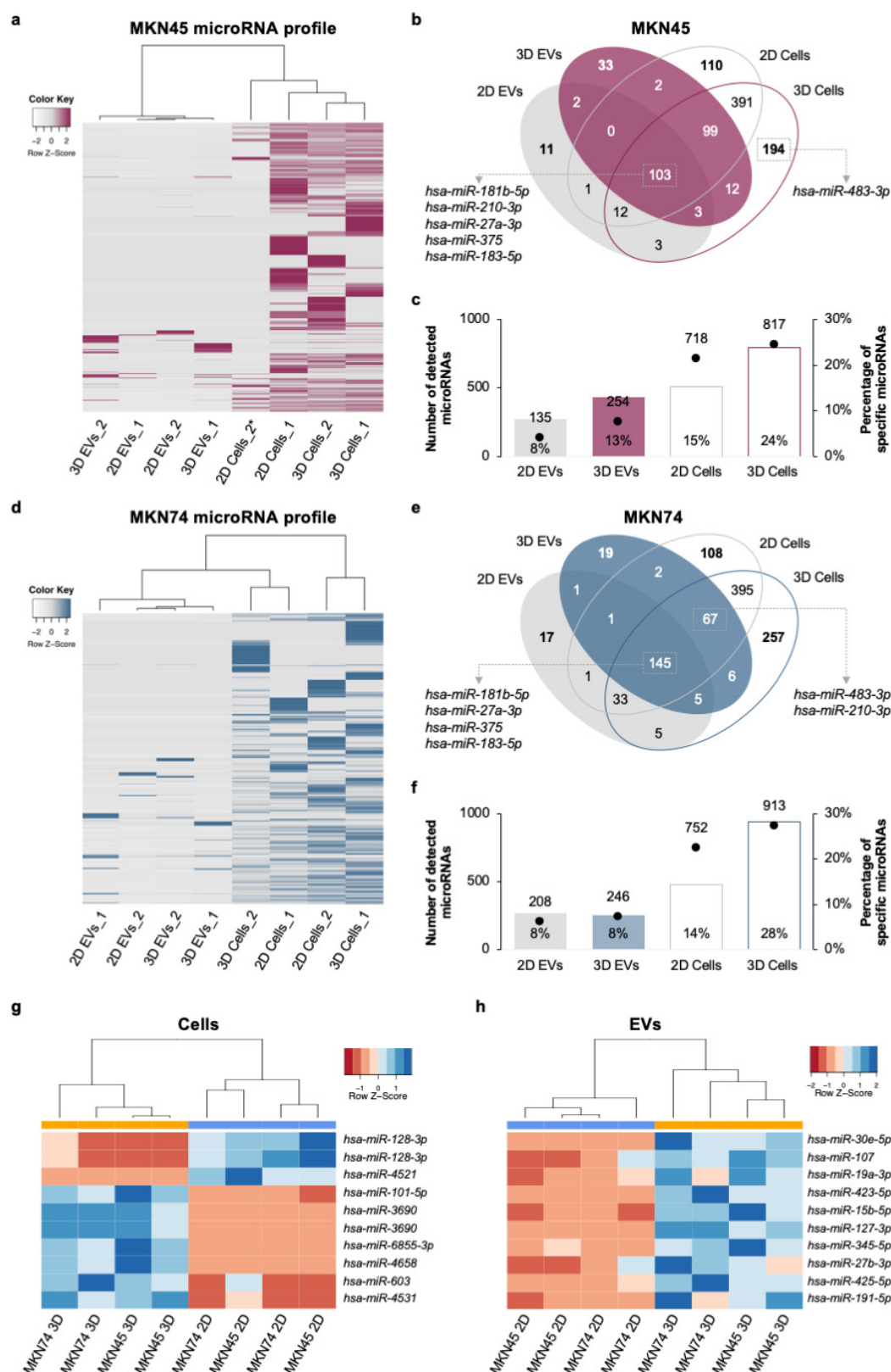
Supplementary Figures



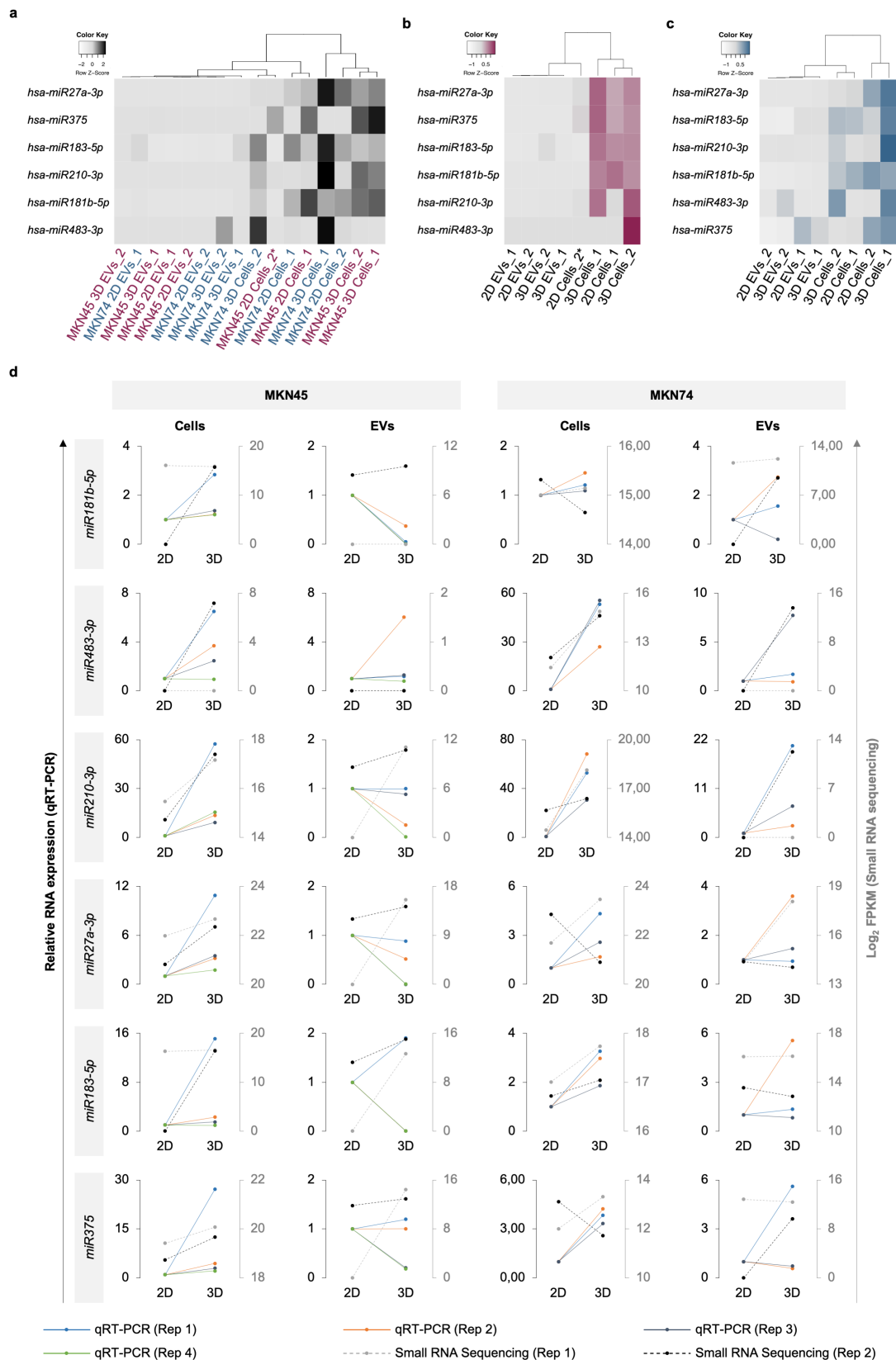
Supplementary Figure 1. Optimization of the 3D culture model. a) Representative microscope images of aggregates obtained using different cell seeding densities. Cell seeding at a density of 1x10⁵ cells/mL (1x10⁶ cells per agarose array) enables the establishment of larger aggregates (~200 μ m). (b) Representative microscope images of aggregates cultured with distinct FBS supplementations. FBS seems to accelerate aggregates growth, particularly during the first hours in culture. Scale bars, 50 μ m.



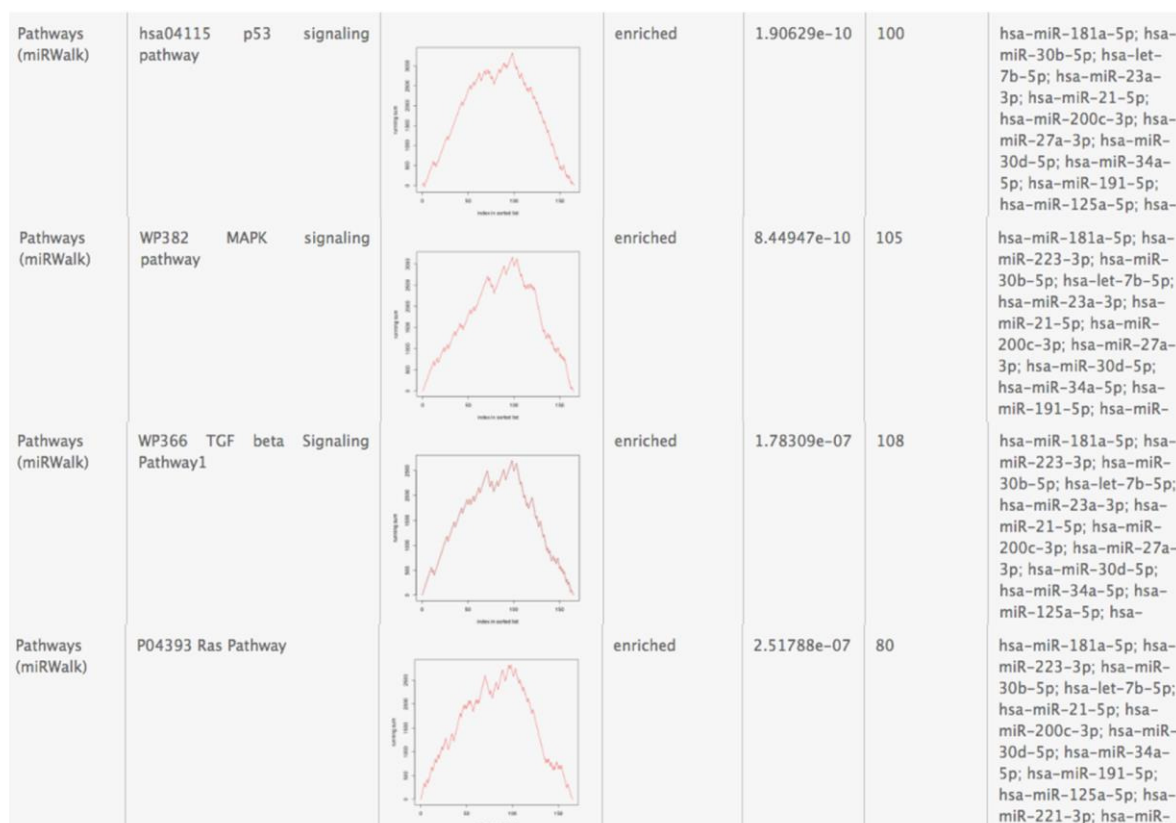
Supplementary Figure 2. Step-wise imaging flow cytometry analysis of EVs-coupled to beads. a) Focused beads were gated using a histogram of gradient RMS, which represents the Root Mean Square for image sharpness. b) An aspect ratio/area scatter plot was used to select single beads based on their roundness (aspect ratio close to 1) and characteristic area (40-120 pixels). c) A contrast/gradient RMS plot was used to distinguish beads from debris, which showed small changes of pixel values (contrast near 0). d) The intensity of the fluorescence staining (Alexa Fluor 488) was plotted in a histogram of channel 2 intensity.



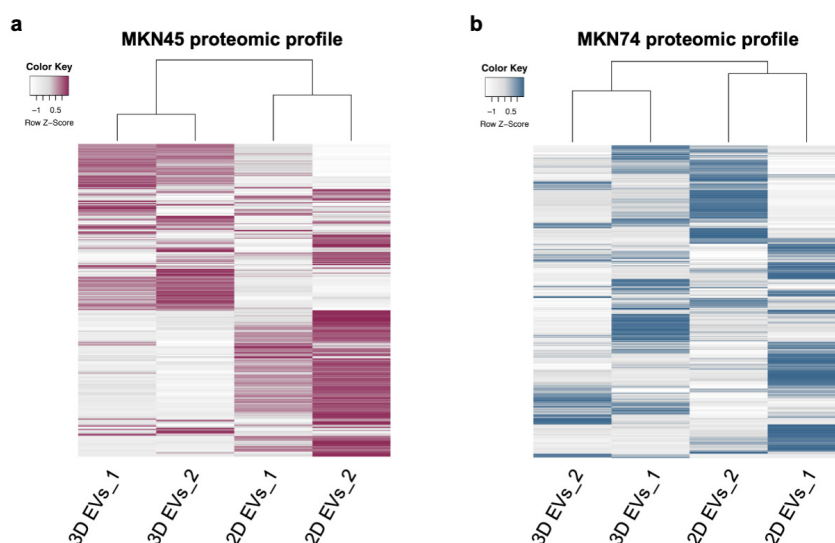
Supplementary Figure 3. Individual analysis of microRNA repertoires of MKN45 (a-c) and MKN74 (d-f) cells and EVs. a, d) Heatmap and dendrogram of microRNA profiles of GC EVs and cells in 2D and 3D cultures (Z-score normalized expression values). b, e) Venn diagrams showing the distribution of detected microRNAs. (c, f) Plots showing the number of total microRNAs and percentage of specific microRNAs detected in each condition (2 biological replicates). g, h) The top-10 microRNAs in cells (g) and EVs (h) identified by supervised hierarchical clustering according to un-paired t-tests, and subjected to transformation of the normalized expression intensities to z-scores.



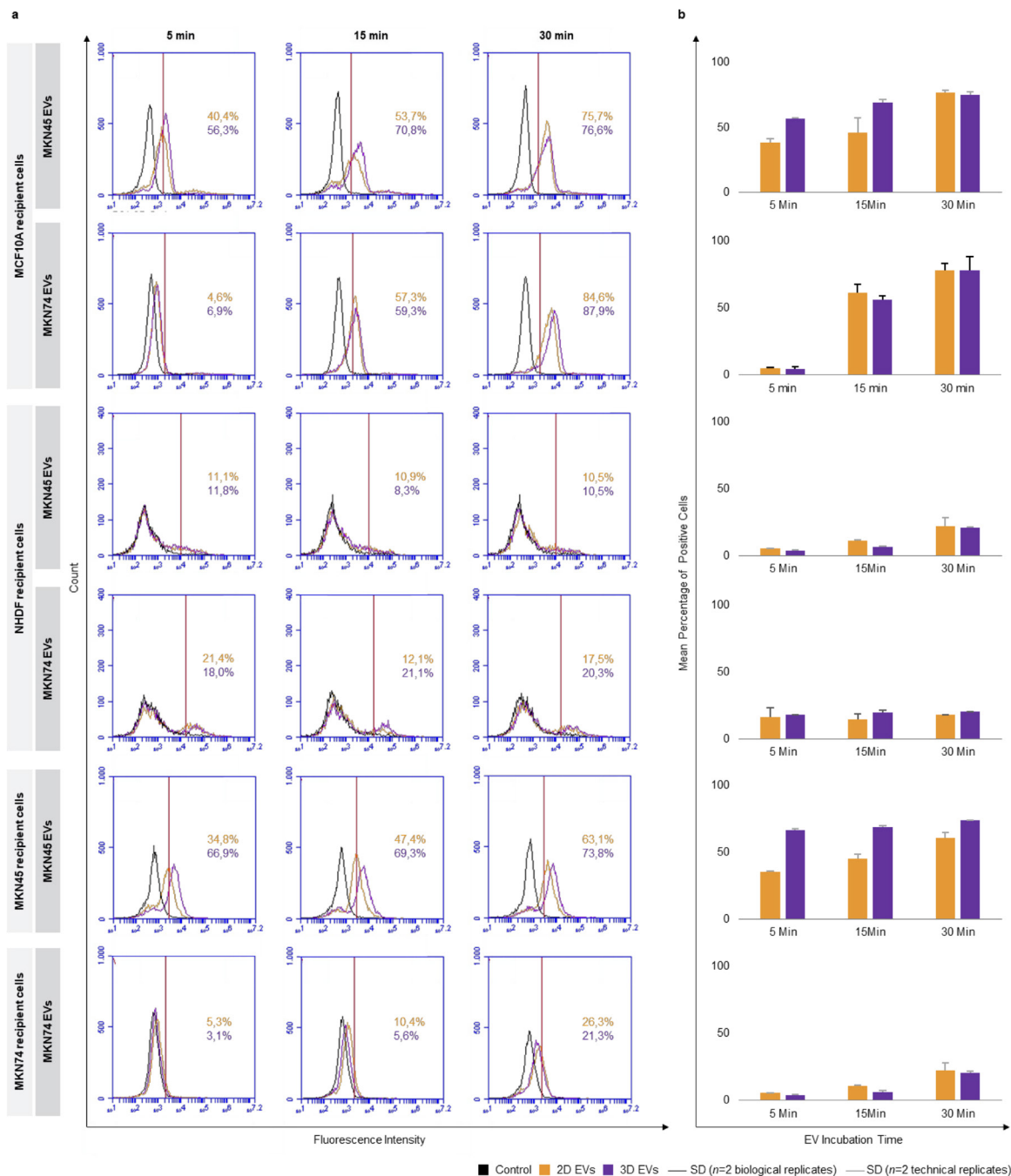
Supplementary Figure 4. Validation of the small RNA sequencing data by quantitative real-time PCR. a-c) Heatmap and dendrogram of 6 microRNAs detected in MKN45 and MKN74 EVs and cells in 2D and 3D cultures (Z- score normalized expression values). Analysis performed with data for both MKN45 and MKN74 GC cell lines (a), and individually for MKN45 (b) or MKN74 (c). d) Quantitative RT-PCR and small RNA sequencing data of the six microRNAs from MKN45 and MKN74 cells and EVs selected for validation. Each biological replicate is independently represented.



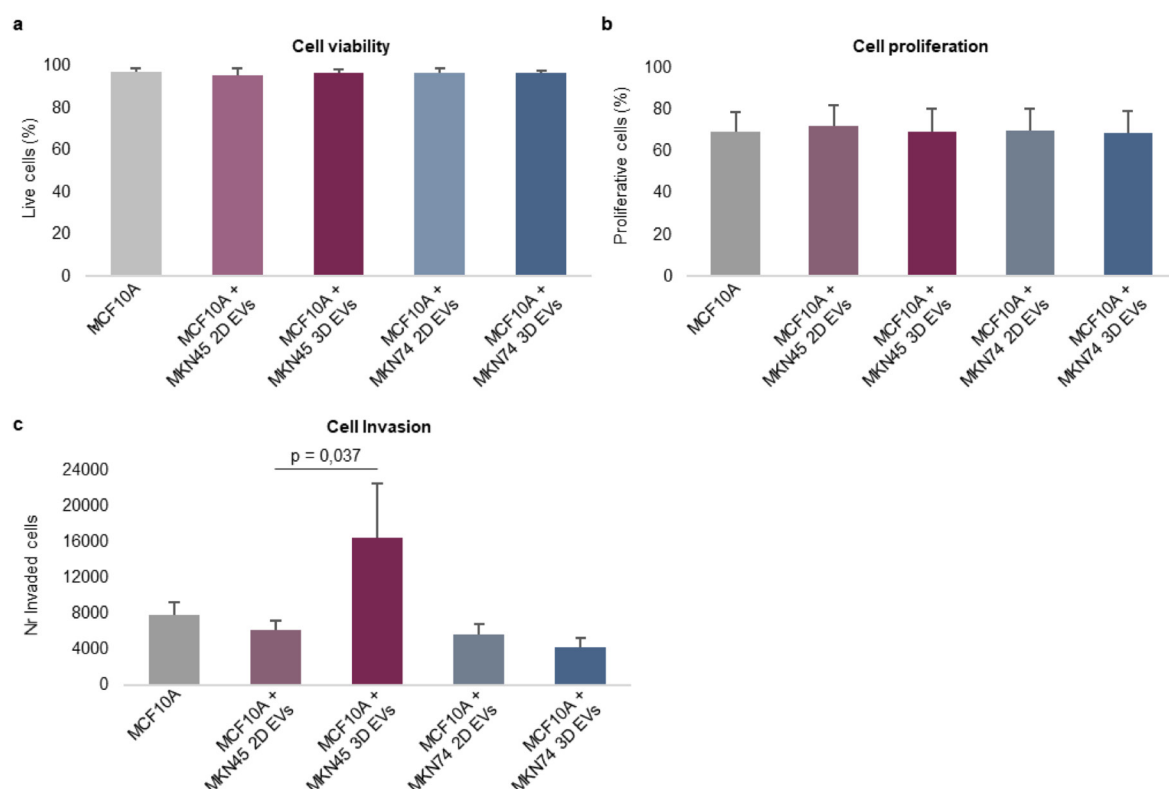
Supplementary Figure 5. GSEA analysis results. GSEA analysis of 164 microRNAs detected in EVs from 3D culture, showing pathways highly enriched in 3D EVs.



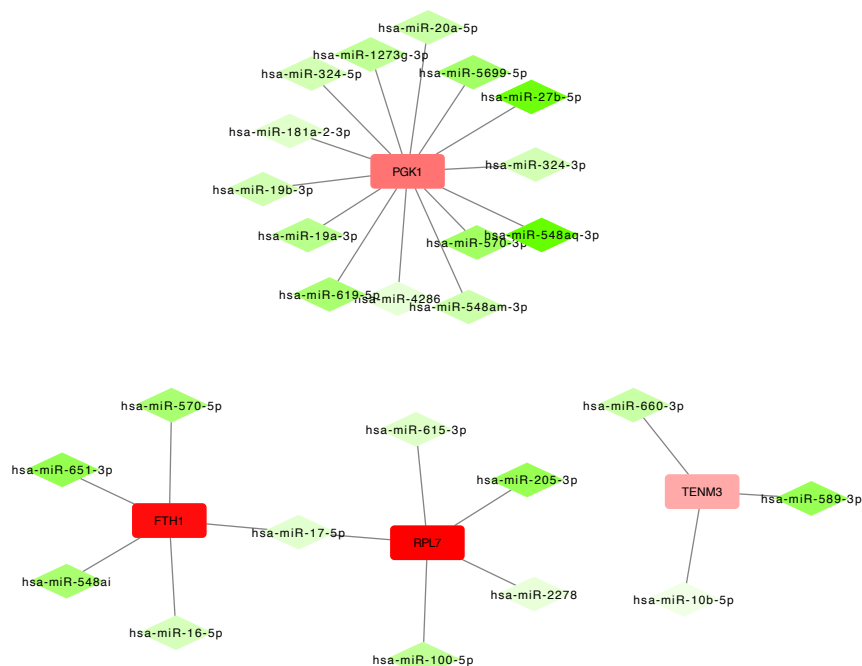
Supplementary Figure 6. Protein expression profiles of EVs isolated from 2D and 3D cultures. Heatmap and dendrogram of proteins detected individually in MKN45 (a) and MKN74 (b) EVs isolated from 2D and 3D cultures (Z- score normalized expression values).



Supplementary Figure 7. Association between cells and EVs isolated from 2D and 3D cultures. Representative histograms showing the fluorescence intensity of EVs associated with different recipient cells (MCF10A, NHDF, MKN45 and MKN74 cells). Black curves for the control, orange curves for association with 2D EVs and purple for association with 3D EVs (a). Graphs showing the mean percentage of positive cells, *i.e.* cells that are associated with fluorescently labelled EVs (b).



Supplementary Figure 8. Functional impact of EVs isolated from 2D and 3D cultures. Graphs showing cell viability (a), proliferation (b) and invasion (c) of MCF10A cells untreated and treated with EVs derived from 2D and 3D cultures of MKN45 and MKN74 cells. Results represent the mean $\pm$ standard deviation of three biological replicates. Data was analyzed with one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test.



Supplementary Figure 9. Protein set enrichment and integrative network analysis. Three representative clusters showing that microRNAs (rhombs) target specific proteins (rectangles), being de-regulated in opposite direction. All proteins in EVs are down-regulated (red) while the microRNAs targeting these proteins are up-regulated (green).

Supplementary Table 1. Technical information regarding the 2D and 3D culture systems used in this study

		3D cultures	2D cultures
Working unit		9.15 cm ² agarose matrix	175 cm ² flask
Volume per working unit (mL)		6	20
Total period of culture (days)		7	5
Period of culture in EV-depleted medium (days)		6	2
Number of cells at time of collection per working unit	MKN45	2,70x10 ⁶ ± 6,69x10 ⁵	1,77x10 ⁷ ± 8,36x10 ⁶
	MKN74	2,06x10 ⁶ ± 1,16x10 ⁶	1,88x10 ⁷ ± 6,15x10 ⁶
Number of EVs collected per working unit	MKN45	2,42x10 ⁹ ± 1,09x10 ⁹	8,49x10 ⁹ ± 1,91x10 ⁹
	MKN74	2,02x10 ⁹ ± 1,35x10 ⁹	6,09x10 ⁹ ± 1,87x10 ⁹
Number of EVs collected per mL	MKN45	4,04x10 ⁸ ± 1,82x10 ⁸	4,25x10 ⁸ ± 9,55x10 ⁷
	MKN74	3,37x10 ⁸ ± 2,25x10 ⁸	3,04x10 ⁸ ± 9,37x10 ⁷
Number of EVs collected per cell	MKN45	1019,59 ± 687,36	636,49 ± 374,97
	MKN74	1656,11 ± 1621,61	365,10 ± 165,67

Results represent the mean ± standard deviation of four biological replicates.

Supplementary Table 2. Total number of genes detected in each biological replicate according to specific small RNA classes

Small RNA Classes	Number of Annotated Genes	Total number of genes detected in each sample											
		MKN45						MKN74					
		Cells			EVs			Cells			EVs		
		2D		3D	2D		3D	2D		3D	2D		3D
		Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2	Rep1	Rep2
miRNA	2803	778	162	687	758	27	148	660	711	843	813	168	177
misc_RNA	1100	112	9	165	166	64	98	159	84	166	407	53	71
Mt_rRNA	3	3	2	3	3	1	1	3	3	3	3	0	1
ribozyme	6	1	0	2	0	0	0	0	1	0	0	0	0
rRNA	510	253	88	230	297	11	18	316	184	196	419	20	42
scaRNA	9	7	2	8	9	0	2	8	6	7	9	0	1
scRNA	1	1	0	1	0	0	0	1	1	1	0	0	0
snoRNA	234	96	23	89	104	4	9	92	88	100	123	3	4
snRNA	1801	511	162	440	641	15	23	576	319	362	920	16	36
Total	6467	1762	448	1625	1978	122	299	1815	1397	1678	2694	260	332
												229	411

Supplementary Table 3. Over-representation analysis of 10 microRNAs exclusively present in EVs from 3D culture

Category	Subcategory	Enrichment	p-value	expected	observed	miRNAs/precursors
Diseases (miRWalk)	Burkitt Lymphoma	over-represented	8,3173E-05	0,157895	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Organs (miRWalk)	Germinal Center	over-represented	0,00052901	0,0560748	2	hsa-miR-155-5p; hsa-miR-127-3p
Organs (miRWalk)	B-Lymphocytes	over-represented	0,00342595	0,476636	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Target genes (miRTarbase)	BCL6	over-represented	0,0045355	0,128571	2	hsa-miR-155-5p; hsa-miR-127-3p
Target genes (miRTarbase)	HK2	over-represented	0,0045355	0,128571	2	hsa-miR-155-5p; hsa-miR-143-3p
Target genes (miRTarbase)	MYO6	over-represented	0,0045355	0,128571	2	hsa-miR-155-5p; hsa-miR-143-3p
Diseases (miRWalk)	Lymphoma	over-represented	0,00553097	0,552632	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0042092 type 2 immune response	over-represented	0,00631842	0,15	2	hsa-miR-155-5p; hsa-miR-127-3p
Target genes (miRTarbase)	KRAS	over-represented	0,00838286	0,171429	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO002829 negative regulation of type 2 immune response	over-represented	0,0107243	0,192857	2	hsa-miR-155-5p; hsa-miR-127-3p
Target genes (miRTarbase)	FNDC3B	over-represented	0,0107243	0,192857	2	hsa-miR-155-5p; hsa-miR-143-3p
Diseases (miRWalk)	Lymphoma B-Cell	over-represented	0,0160689	0,236842	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0034968 histone lysine methylation	over-represented	0,0193664	0,257143	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0045579 positive regulation of b cell differentiation	over-represented	0,0193664	0,257143	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0004396 hexokinase activity	over-represented	0,0227713	0,278571	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0034405 response to fluid shear stress	over-represented	0,0227713	0,278571	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0042730 fibrinolysis	over-represented	0,0227713	0,278571	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0045334 clathrin coated endocytic vesicle	over-represented	0,0227713	0,278571	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0005536 glucose binding	over-represented	0,026431	0,3	2	hsa-miR-155-5p; hsa-miR-143-3p

Supplementary Table 3. Continued.

Gene Ontology (miRWalk)	GO0030195 negative regulation of blood coagulation	over-represented	0,026431	0,3	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0044027 hypermethylation of cpg island	over-represented	0,026431	0,3	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0051385 response to mineralocorticoid stimulus	over-represented	0,026431	0,3	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0050727 regulation of inflammatory response	over-represented	0,0275727	0,921429	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0000060 protein import into nucleus translocation	over-represented	0,0295902	0,942857	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO000302 response to reactive oxygen species	over-represented	0,0303409	0,321429	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO002634 regulation of germinal center formation	over-represented	0,0303409	0,321429	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0030275 Irf domain binding	over-represented	0,0303409	0,321429	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0046324 regulation of glucose import	over-represented	0,0303409	0,321429	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0061045 negative regulation of wound healing	over-represented	0,0303409	0,321429	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO002467 germinal center formation	over-represented	0,0344966	0,342857	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO0010757 negative regulation of plasminogen activation	over-represented	0,0344966	0,342857	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0032287 peripheral nervous system myelin maintenance	over-represented	0,0344966	0,342857	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0035022 positive regulation of rac protein signal transduction	over-represented	0,0344966	0,342857	2	hsa-miR-155-5p; hsa-miR-143-3p
Pathways (miRWalk)	WP272 Blood Clotting Cascade	over-represented	0,0359716	0,350365	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO0016461 unconventional myosin complex	over-represented	0,0388936	0,364286	2	hsa-miR-155-5p; hsa-miR-143-3p

Supplementary Table 3. Continued.

Gene Ontology (miRWalk)	GO0051602 response to electrical stimulus	over-represented	0,0388936	0,364286	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO002903 negative regulation of b cell apoptotic process	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO003886 dna cytosine 5 methyltransferase activity	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO006809 nitric oxide biosynthetic process	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO035024 negative regulation of rho protein signal transduction	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO050829 defense response to gram negative bacterium	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO051918 negative regulation of fibrinolysis	over-represented	0,0435274	0,385714	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO007281 germ cell development	over-represented	0,0437911	1,07143	3	hsa-miR-155-5p; hsa-miR-143-3p; hsa-miR-127-3p
Gene Ontology (miRWalk)	GO030194 positive regulation of blood coagulation	over-represented	0,0483936	0,407143	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO032228 regulation of synaptic transmission gabaergic	over-represented	0,0483936	0,407143	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO032757 positive regulation of interleukin 8 production	over-represented	0,0483936	0,407143	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO043589 skin morphogenesis	over-represented	0,0483936	0,407143	2	hsa-miR-155-5p; hsa-miR-143-3p
Gene Ontology (miRWalk)	GO051926 negative regulation of calcium ion transport	over-represented	0,0483936	0,407143	2	hsa-miR-155-5p; hsa-miR-143-3p

Supplementary Table 4. Over-representation analysis of 64 microRNAs detected in EVs from 3D culture, absent in EVs from 2D culture and present in both, cells growing in 2D and 3D cultures

Category	Subcategory	Enrichment	p-value	expected	observed	miRNAs/precursors
Target genes (miRTarbase)	PIP5K1A	over-represented	0,0100419	2,03571	5	hsa-miR-222-3p; hsa-miR-1226-3p; hsa-miR-132-3p; hsa-miR-615-3p; hsa-miR-744-5p
Organs (miRWalk)	Blood Platelets	over-represented	0,0128746	3,18692	7	hsa-let-7e-5p; hsa-miR-10a-5p; hsa-let-7d-3p; hsa-let-7c-5p; hsa- miR-151a-5p; hsa-let-7i-5p; hsa- miR-28-5p
Target genes (miRTarbase)	OTUD5	over-represented	0,0257678	1,62857	4	hsa-let-7e-5p; hsa-miR-339-5p; hsa-miR-1260b; hsa-miR-505-3p
Target genes (miRTarbase)	RPA1	over-represented	0,0257678	1,62857	4	hsa-let-7e-5p; hsa-miR-31-5p; hsa- miR-339-5p; hsa-miR-615-3p
Gene Ontology (miRWalk)	GO0048846 axon extension involved in axon guidance	over-represented	0,0409112	2,44286	5	hsa-let-7e-5p; hsa-miR-324-5p; hsa-miR-26b-5p; hsa-miR-326; hsa-miR-330-3p
Target genes (miRTarbase)	MCM2	over-represented	0,0409112	2,44286	5	hsa-miR-423-3p; hsa-miR-31-5p; hsa-miR-1226-3p; hsa-miR-615-3p; hsa-miR-500a-5p

Supplementary Table 5. GSEA Analysis of 164 microRNAs detected in EVs from 3D culture – top-20 hits

Category	Subcategory	Enrichment	p-value	observed	miRNAs/precursors
Diseases (miRWalk)	Neoplasms	enriched	4,03E-09	106	hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-27a-3p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-miR-320a; hsa-let-7f-5p; hsa-miR-20a-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-let-7e-5p; hsa-miR-183-5p; hsa-miR-365a-3p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-27b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
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					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-23b-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-126-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-324-5p; hsa-miR-185-5p; hsa-miR-133a-3p; hsa-miR-106a-5p; hsa-let-7d-3p; hsa-miR-135b-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-378a-3p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-429; hsa-miR-26b-5p; hsa-miR-92a-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-100-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-let-7c-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-489-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-127-3p; hsa-miR-151a-5p; hsa-miR-96-5p; hsa-miR-30a-3p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-199b-5p; hsa-miR-132-3p; hsa-miR-30c-1-3p; hsa-miR-330-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-28-5p; hsa-miR-148b-3p; hsa-miR-193a-5p; hsa-miR-301a-3p; hsa-miR-22-3p
					hsa-miR-181a-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-30d-5p; hsa-miR-34a-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-205-5p; hsa-miR-192-5p; hsa-let-7f-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-197-3p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-103a-3p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-149-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-200b-3p; hsa-miR-423-3p; hsa-miR-141-3p; hsa

Supplementary Table 5. Continued.

Diseases (miRWalk)	Shock	enriched	4,49E-04	2	203a-3p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-let-7d-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-769-5p; hsa-miR-7-5p; hsa-miR-146b-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-29b-3p; hsa-miR-155-5p; hsa-miR-181b-5p; hsa-let-7i-5p; hsa-let-7f-5p; hsa-miR-22-3p
Diseases (miRWalk)	Carcinoma Hepatocellular	enriched	5,49E-04	43	hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-223-3p; hsa-let-7b-5p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-30d-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-93-5p; hsa-miR-26a-5p; hsa-miR-193b-3p; hsa-miR-183-5p; hsa-miR-122-5p; hsa-miR-144-3p; hsa-miR-16-5p; hsa-miR-106b-5p; hsa-let-7g-5p; hsa-miR-23b-3p; hsa-miR-195-5p; hsa-miR-203a-3p; hsa-miR-186-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-185-5p; hsa-miR-125b-5p; hsa-miR-29b-3p; hsa-let-7c-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-210-3p; hsa-miR-181b-5p; hsa-miR-151a-5p; hsa-miR-199b-5p; hsa-miR-30c-1-3p; hsa-miR-124-3p; hsa-miR-199a-3p; hsa-miR-301a-3p; hsa-miR-22-3p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-27a-3p; hsa-miR-34a-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-205-5p; hsa-let-7f-5p; hsa-miR-20a-5p; hsa-miR-17-5p; hsa-miR-193b-3p; hsa-miR-103a-3p; hsa-miR-16-5p; hsa-miR-200b-3p; hsa-miR-141-3p; hsa-miR-30c-5p; hsa-miR-203a-3p; hsa-miR-31-5p; hsa-miR-126-3p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-222-3p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-145-5p; hsa-miR-185-5p; hsa-miR-7-5p; hsa-miR-339-5p; hsa-miR-146b-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-92a-3p; hsa-miR-29b-3p; hsa-miR-342-3p; hsa-miR-155-5p; hsa-miR-128-3p; hsa-miR-182-5p; hsa-miR-345-5p; hsa-miR-29a-3p; hsa-miR-181b-5p; hsa-miR-96-5p; hsa-miR-326; hsa-miR-1226-3p; hsa-miR-330-3p; hsa-miR-199a-3p; hsa-miR-22-3p
Diseases (miRWalk)	Breast Neoplasms	enriched	9,60E-04	48	hsa-miR-181a-5p; hsa-miR-21-5p
Diseases (miRWalk)	Insulin Resistance	enriched	1,12E-03	2	hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-34a-5p; hsa-miR-221-3p; hsa-miR-205-5p; hsa-miR-26a-5p; hsa-miR-122-5p; hsa-miR-16-5p; hsa-miR-106b-5p; hsa-miR-141-3p; hsa-miR-126-3p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-126-5p; hsa-miR-145-5p; hsa-miR-185-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-29b-3p; hsa-miR-143-3p; hsa-miR-330-3p
Diseases (miRWalk)	Colonic Neoplasms	enriched	1,21E-03	17	hsa-let-7b-5p; hsa-miR-21-5p; hsa-miR-34a-5p; hsa-miR-192-5p; hsa-miR-320a; hsa-miR-183-5p; hsa-miR-365a-3p; hsa-miR-106b-5p; hsa-miR-195-5p; hsa-miR-186-5p; hsa-miR-126-3p; hsa-let-7a-5p; hsa-miR-145-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-15b-5p; hsa-miR-143-3p
Diseases (miRWalk)	Recurrence	enriched	1,45E-03	14	hsa-miR-21-5p; hsa-miR-191-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-20a-5p; hsa-miR-26a-5p; hsa-miR-183-5p; hsa-miR-122-5p; hsa-miR-203a-3p; hsa-miR-29b-3p; hsa-miR-100-5p; hsa-miR-326; hsa-miR-124-3p
Diseases (miRWalk)	Glioblastoma	enriched	1,45E-03	24	hsa-miR-181a-5p; hsa-miR-21-5p; hsa-miR-34a-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-192-5p; hsa-miR-20a-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-24-3p; hsa-miR-19a-3p; hsa-miR-195-5p; hsa-let-7d-5p; hsa-let-7a-5p; hsa-miR-18a-5p; hsa-miR-222-3p; hsa-miR-7-5p; hsa-miR-146b-5p; hsa-miR-92a-3p; hsa-miR-128-3p; hsa-miR-326; hsa-miR-199b-5p; hsa-miR-124-3p
Diseases (miRWalk)	Carcinoma Non-Small-Cell Lung	enriched	1,75E-03	11	hsa-miR-21-5p; hsa-miR-221-3p; hsa-miR-451a; hsa-miR-16-5p; hsa-let-7g-5p; hsa-miR-31-5p; hsa-miR-222-3p; hsa-miR-145-5p; hsa-miR-185-5p; hsa-miR-15a-5p; hsa-miR-107
Diseases (miRWalk)	Lymphoma	enriched	2,96E-03	21	hsa-miR-181a-5p; hsa-miR-30b-5p; hsa-let-7b-5p; hsa-miR-21-5p; hsa-miR-200c-3p; hsa-miR-34a-5p; hsa-let-7f-5p; hsa-miR-20a-5p; hsa-miR-17-5p; hsa-miR-26a-5p; hsa-miR-16-5p; hsa-miR-200b-3p; hsa-let-7a-5p; hsa-miR-200a-3p; hsa-miR-375; hsa-miR-15a-5p; hsa-miR-29b-3p; hsa-miR-155-5p; hsa-miR-29a-3p; hsa-miR-143-3p; hsa-miR-127-3p
Diseases (miRWalk)	Nevus	enriched	3,19E-03	3	hsa-let-7b-5p; hsa-miR-205-5p; hsa-miR-193b-3p
Diseases (miRWalk)	Encephalitis	enriched	4,78E-03	5	hsa-let-7b-5p; hsa-miR-21-5p; hsa-miR-34a-5p; hsa-miR-26a-5p; hsa-miR-145-5p

Supplementary Table 5. Continued.

Diseases (miRWalk)	Cholangiocarcinoma	enriched	5,15E-03	4	hsa-miR-21-5p; hsa-miR-320a; hsa-miR-200b-3p; hsa-miR-141-3p
Diseases (miRWalk)	Stomach Neoplasms	enriched	5,90E-03	27	hsa-miR-223-3p; hsa-miR-23a-3p; hsa-miR-21-5p; hsa-miR-27a-3p; hsa-miR-34a-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-93-5p; hsa-miR-451a; hsa-miR-93-5p; hsa-miR-16-5p; hsa-miR-106b-5p; hsa-miR-126-3p; hsa-miR-150-5p; hsa-miR-146a-5p; hsa-let-7a-5p; hsa-miR-222-3p; hsa-miR-375; hsa-miR-106a-5p; hsa-miR-107; hsa-miR-25-3p; hsa-miR-15b-5p; hsa-miR-29b-3p; hsa-miR-182-5p; hsa-miR-106b-3p; hsa-miR-143-3p; hsa-miR-148b-3p; hsa-miR-22-3p
Diseases (miRWalk)	Leukemia	enriched	5,92E-03	37	hsa-miR-181a-5p; hsa-miR-223-3p; hsa-miR-21-5p; hsa-miR-34a-5p; hsa-miR-125a-5p; hsa-miR-221-3p; hsa-miR-93-5p; hsa-miR-320a; hsa-miR-26a-5p; hsa-miR-19b-3p; hsa-miR-122-5p; hsa-miR-24-3p; hsa-miR-16-5p; hsa-miR-106b-5p; hsa-miR-126-3p; hsa-miR-150-5p; hsa-let-7a-5p; hsa-miR-222-3p; hsa-miR-10a-5p; hsa-miR-200a-3p; hsa-miR-145-5p; hsa-miR-106a-5p; hsa-miR-135b-5p; hsa-miR-15a-5p; hsa-miR-125b-5p; hsa-miR-107; hsa-miR-92a-3p; hsa-miR-29b-3p; hsa-miR-155-5p; hsa-miR-128-3p; hsa-miR-29a-3p; hsa-miR-505-3p; hsa-miR-143-3p; hsa-miR-181b-5p; hsa-miR-326; hsa-miR-124-3p; hsa-miR-22-3p

Supplementary Table 6. Top-20 proteins mostly regulated in EVs from 3D versus 2D culture conditions.

Spalte1	median group 1	median group 2	stdv group 1	stdv group 2	fold change	log(2) fold change	t-Test raw p- Value	t-Test adjusted p-Value
TSPAN14	66,2	134,35	15,79	17,03	0,49	-1,02	0,0010822	0,29319
ADGRG1	11,9	171,55	27,3	38,33	0,07	-3,85	0,0013449	0,29319
PTGFRN	48,75	137,15	13,15	31,02	0,36	-1,49	0,0046935	0,47446
EPHA2	52,4	146,9	4,1	26,29	0,36	-1,49	0,0055448	0,47446
CD151	48,15	147,25	17,95	39,14	0,33	-1,61	0,0079419	0,47446
PGK1	129,8	82	18,22	8,38	1,58	0,66	0,0083176	0,47446
CD46	79,4	129,45	21,15	17,6	0,61	-0,71	0,0096387	0,47446
ITGA3	48,25	151,8	6,93	35,51	0,32	-1,65	0,010731	0,47446
KPNB1	82,9	113,05	12,28	10,8	0,73	-0,45	0,010915	0,47446
ITGB1	57,95	152,25	12,26	33,39	0,38	-1,39	0,011497	0,47446
ADAM10	85	117,95	13,25	16,62	0,72	-0,47	0,01197	0,47446
DLG1	57,3	136,55	13,95	39,74	0,42	-1,25	0,017437	0,60867
EML2	124	86,75	24,08	18,62	1,43	0,52	0,020416	0,60867
ITGA2	44,1	155,95	10,68	47,96	0,28	-1,82	0,020496	0,60867
ANXA7	76,3	125,35	12,07	19,29	0,61	-0,72	0,021508	0,60867
RPL7	131,7	62,2	43,47	16,28	2,12	1,08	0,027731	0,60867
F11R	62,8	125,85	7,93	37,48	0,5	-1	0,028809	0,60867
ITGB5	42,7	134,95	19,39	47,93	0,32	-1,66	0,030499	0,60867
LAMP1	59,75	120,7	15,69	42,41	0,5	-1,01	0,030733	0,60867
ATP1A1	61,65	143,6	6,16	37,51	0,43	-1,22	0,030785	0,60867

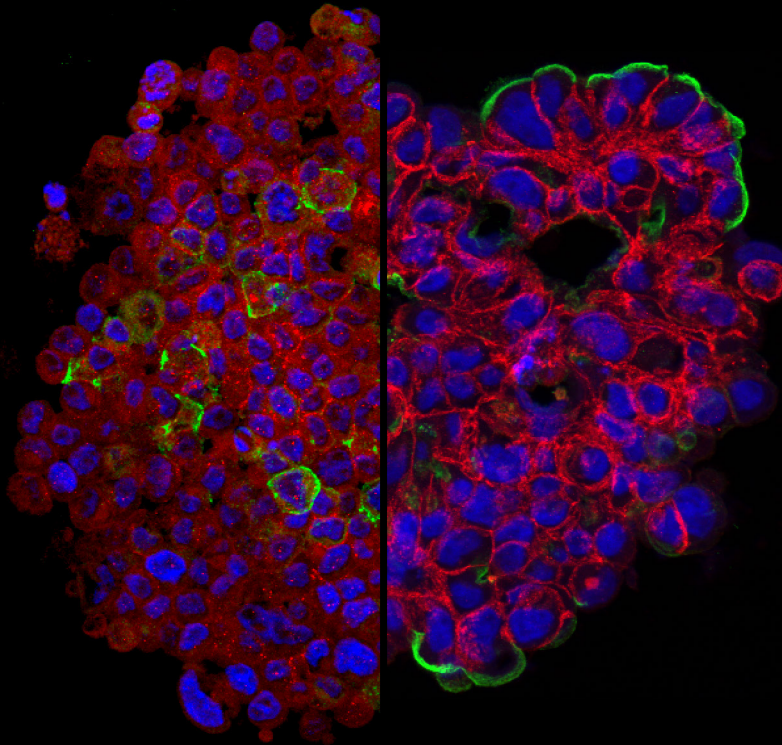
Supplementary Table 7. Detailed description of antibodies and TaqMan Advanced microRNA assays

Antibodies				
ID	Clone	Supplier	Dilution	Application
Integrin α 6	3750	Cell Signaling Technology	1:800	WB
Integrin β 1	D6S1W	Cell Signaling Technology	1:200	WB
EPHA-2	D4A2	Cell Signaling Technology	1:800	WB
GAPDH	G-9	Santa Cruz	1:500	WB
E-cadherin	C36	BD Biosciences	1:500	WB
p120 Catenin	H-90	BD Biosciences	1:500	WB
β -catenin	E-5	Santa Cruz	1:100	WB
Importin- β 1	E1F1G	Cell Signaling Technology	1:200	WB
Tubulin	DLN-15303	Dianova	1:500	WB
LAMP1	D4O1S	Cell Signaling Technology	1:800	WB
JAM-A	1H2A9	Santa Cruz	1:300	WB
Integrin α 3	C-18	Santa Cruz	1:400	WB
CD151	17327	Cell Signaling Technology	1:500	WB
Integrin α 2	AF1740	R&D Systems	1:200	WB
Ki-67	SP6	ThermoFisher Scientific	1:200	IHC
E-cadherin	24E10	Cell Signaling Technology	1:200	ICC & IHC
Mucin -1		collaborators		ICC & IHC
CD9	ALB6	Santa Cruz	3:20	IFC
CD81	5A6	Santa Cruz	3:20	IFC
Flotillin-1	H-104	Santa Cruz	3:20	IFC
Cytochrome c	7H8	Santa Cruz	3:20	IFC
TaqMan Advanced microRNA assays				
Assay name	Assay ID	Supplier		
hsa-miR-210-3p	477970_mir	ThermoFisher Scientific		
hsa-miR-27a-3p	478384_mir	ThermoFisher Scientific		
hsa-miR-183-5p	477937_mir	ThermoFisher Scientific		
hsa-miR-375	478074_mir	ThermoFisher Scientific		
hsa-miR-181b-5p	478583_mir	ThermoFisher Scientific		
hsa-miR-483-3p	478122_mir	ThermoFisher Scientific		
hsa-miR-30d-5p	478606_mir	ThermoFisher Scientific		
hsa-miR-152-3p	477921_mir	ThermoFisher Scientific		
hsa-miR-26b-5p	478418_mir	ThermoFisher Scientific		
hsa-miR-155-5p	477927_mir	ThermoFisher Scientific		

WB: Western Blotting; IHC: Immunohistochemistry; ICC: Immunocytochemistry; IFC: Imaging Flow Cytometry

Chapter 2

Mechanisms of gastric cancer metastatic organotropism mediated by extracellular vesicles



Extracellular vesicles promote gastric cancer metastasis to brown adipose tissue

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The role of extracellular vesicles (EVs) in the systemic dissemination of tumour cells has been widely studied, however such evidence remains scarce in gastric cancer (GC). This study addressed the contributions of GC-secreted EVs to the *in vivo* dissemination and colonization of intestinal- and diffuse-type GC cells.

To accomplish this, we performed an *in vivo* metastatic assay by priming immunodeficient mice with GC-EVs secreted by 2D and 3D cultures, followed by tail-vein injection of 2D and 3D GC cells. Haematoxylin-Eosin and Ki-67/keratin staining evaluated the resulting metastatic burden and proliferative rates, respectively. We also performed an integrative analysis of the small RNA and proteomic profiles of cells and EVs, to dissect putative targets and pathways involved in GC cell dissemination and colonization.

Our results confirmed distinct metastatic organotropism for intestinal- and diffuse-type GC cells. Whilst intestinal-type GC cells metastasized to the lungs, diffuse-type GC cells displayed simultaneous lesions at the lungs and in brown adipose tissue (BAT).

In particular, we observed a high prevalence of lung metastasis for intestinal-type GC cells cultured in 2D comparing with 3D counterparts. Moreover, diffuse-type GC cells cultured in 3D displayed faster disease progression and multiple metastasis foci in BAT, while 2D cells mainly metastasized to the lungs.

EVs led to a decreased ability of 3D intestinal-type GC cells to form lung metastasis, and potentiated the spread to BATs of both 2D and 3D diffuse-type cells. Interestingly, 3D EVs that enhance the ability of diffuse-type GC cells to form metastasis in BATs were enriched in microRNAs associated with BAT differentiation and activation.

Overall, this work unveils a role for EVs in GC metastasis, and provides evidence of putative regulators of GC metastasis in BAT.

Keywords

Extracellular vesicles
Gastric cancer
Brown adipose tissue
Metastasis
Liquid biopsies

Introduction

Metastasis is a multistep and highly inefficient process that allows tumour dissemination to regional or distant organs, mainly through lymphatics or the blood vasculature.¹ Gastric cancer (GC) patients are often diagnosed with locally advanced or metastatic disease, when tumours are unresectable, and the available therapeutic options are poorly effective.² In fact, the 5-year survival rates significantly decrease from 65% to 5% if, at the moment of the diagnosis, tumours have already spread to distant organs.³ GCs often disseminate early in the disease trajectory,^{4,5} and their metastatic organotropism is associated with the histological properties of the tumour. Whilst intestinal-type GCs often induce distant metastasis in liver and lung, diffuse-type GCs tend to disseminate through the peritoneum and to regional and distant lymph nodes.⁶

Extracellular vesicles (EVs) are small, membrane-enclosed vesicles that transport specific biomolecules, such as RNAs and proteins, from donor to recipient cells, that can be either localized in the surroundings or at distant sites.⁷ Moreover, EVs have been implicated in several steps of tumour progression and metastasis.⁸ As so, EVs derived from pancreatic adenocarcinoma and melanoma contribute to the pre-metastatic niche formation in lymph nodes and lungs.⁹⁻¹¹ Furthermore, EVs from tumour cells with distinct organotropism were shown to fuse preferentially with resident cells at their predicted destination, due to specific integrin profiles carried by EVs.¹² In GC, tumour-derived EVs carry epidermal growth factor receptor (EGFR) to liver stromal cells, which activates hepatocyte growth factor (HGF) through the suppression of miR-26a/b expression.¹³ HGF binds the c-MET receptor expressed by migrating GC cells, thus facilitating liver colonization. Nevertheless, the mechanisms underlying EV-mediated GC metastasis in other organs remains unclear. We have previously demonstrated that the cargo and functional effects of GC-secreted EVs (GC-EVs) is dependent on the tumour histology and cellular architecture.^{14,15} In this work, we hypothesized that these tumour intrinsic factors also modulate the role of EVs in GC metastasis. Hence, we pursued to unveil the contributions of GC-EVs to the *in vivo* dissemination and colonization of intestinal- and diffuse-type GC cells.

In the present study, we evaluated the *in vivo* dissemination of intestinal- and diffuse-type GC cells cultured in 2D and 3D conditions, which confirmed their distinct metastatic organotropism. Intestinal-type GC cells metastasized solely to the lungs, and higher metastatic burden was identified for cells cultured in 2D, as compared to 3D counterparts. Instead, diffuse-type GC cells displayed metastasis in the lung and in brown adipose tissue (BAT). Of notice, whilst diffuse-type GC cells cultured in 3D displayed faster disease progression and multiple metastasis foci in BAT, 2D cells mainly metastasized to the lungs.

We then assessed whether an initial priming with EVs produced by 2D and 3D cells would modulate the observed patterns of GC cell dissemination. Strikingly, EVs led to a decreased ability of 3D intestinal-type GC cells to form lung metastasis and potentiated the spread to BATs of both 2D and 3D diffuse-type cells. Further analysis on the content of 3D EVs, showed an enrichment of microRNAs associated with BAT differentiation and

activation. Ongoing integrative analysis of the small RNA and proteomic profiles of cells and EVs, will potentially unravel new targets and pathways involved in GC cell dissemination and colonization.

Results

To investigate the role of EVs in the *in vivo* dissemination of intestinal and diffuse-type GC cells, we conducted the following experimental workflow (Figure 1). Briefly, EVs isolated from 2D and 3D cultures of intestinal- or diffuse-type GC cells, were intravenously injected in nude mice for four weeks, followed by a single tail-vein injection of GC cells.

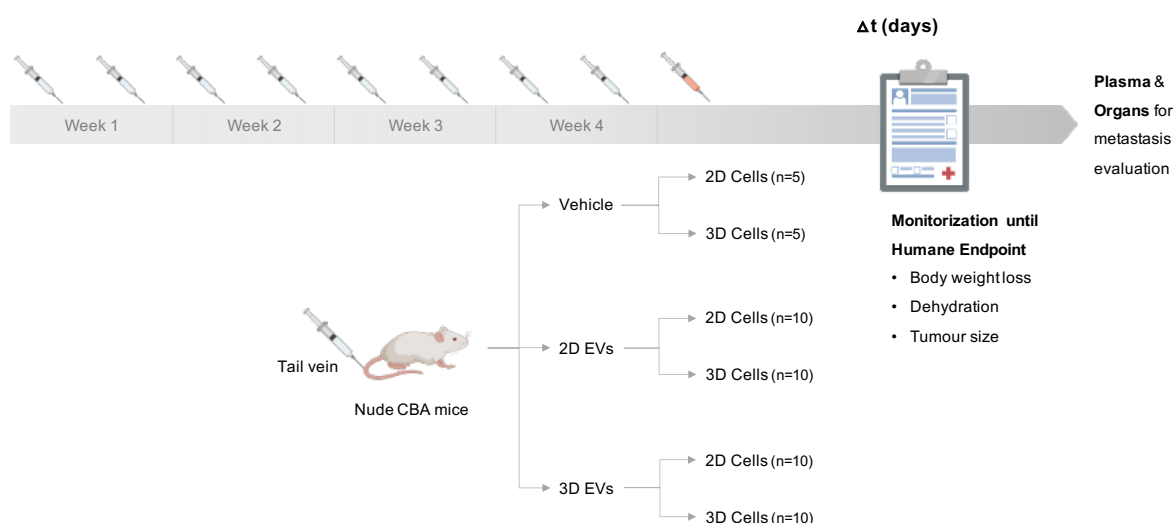


Figure 1. Treatment scheme in the *in vivo* metastatic assay. Tail vein injections of EVs isolated from 2D or 3D GC cultures were performed for 4 weeks, twice a week, followed by GC cells injection. Animals were euthanized when humane endpoint was achieved. Organs and peripheral blood were collected for further analysis.

EVs inhibit lung metastasis of intestinal-type GC cells

We observed that intestinal-type GC cells (MKN74) previously cultured in 2D and 3D conditions developed disease symptoms at similar timepoints, which were not altered upon treatment with EVs (Figure 2A). Of notice, some of these animals did not present disease symptoms during the experimental time (90 days). Moreover, a deep analysis of the metastatic lesions revealed a tropism exclusively to the lungs, though with differences in terms of size and lung occupancy (Figure 2B). Further, macroscopic evaluation of these lesions showed that 2D cells mostly develop extensive metastasis, which was not modified by the treatment with EVs, either collected from 2D or 3D cultures. In contrast, cells that had been previously cultured in 3D conditions, displayed only mild metastatic lesions. Interestingly, the ability to form metastasis was further reduced in animals treated with EVs, particularly those isolated from 3D cultures.

Overall, these results showed that 3D cell architecture modulates the ability of intestinal-type GC cells to metastasize to the lungs, and that EVs hampered lung colonization.

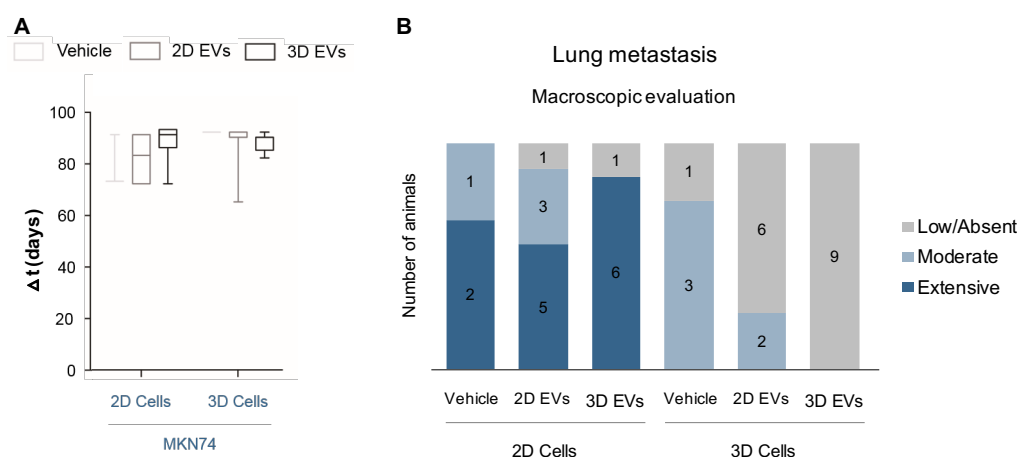


Figure 2. Intestinal-type GC cell *in vivo* dissemination. (A) Time until experimental endpoint. (B) Number of animals with extensive, moderate or low/absent lung metastasis, after macroscopic evaluation of the lungs.

EVs promote metastasis of diffuse-type GC cells to brown adipose tissue

Thereafter, we performed similar experiments using diffuse-type GC cells (MKN45) cultured in 2D or 3D conditions, and their corresponding EVs. In contrast to intestinal-type GC cells, 2D and 3D diffuse-type GC cells developed disease at different timepoints, and with distinct organotropisms (Figure 3A, 3B). Hence, mice injected with 3D cells achieved a humane endpoint faster than the group injected with 2D cells (Figure 3A). Moreover, 3D cells formed more and larger metastatic lesions that were distributed by different anatomic locations, as compared to the ones formed by 2D cells (Figure 3B). Interestingly, treatment with 2D and 3D EVs resulted in a marked increase of the metastatic burden of 2D and 3D cells (Figure 3A, 3B). In particular, treatments with EVs isolated from 3D cultures, potentiated the size, number and spread of metastatic lesions (Figure 3A, 3B). Next, the microscopy evaluation of these lesions revealed a close contact, or nearby, adipocyte tissue, mainly

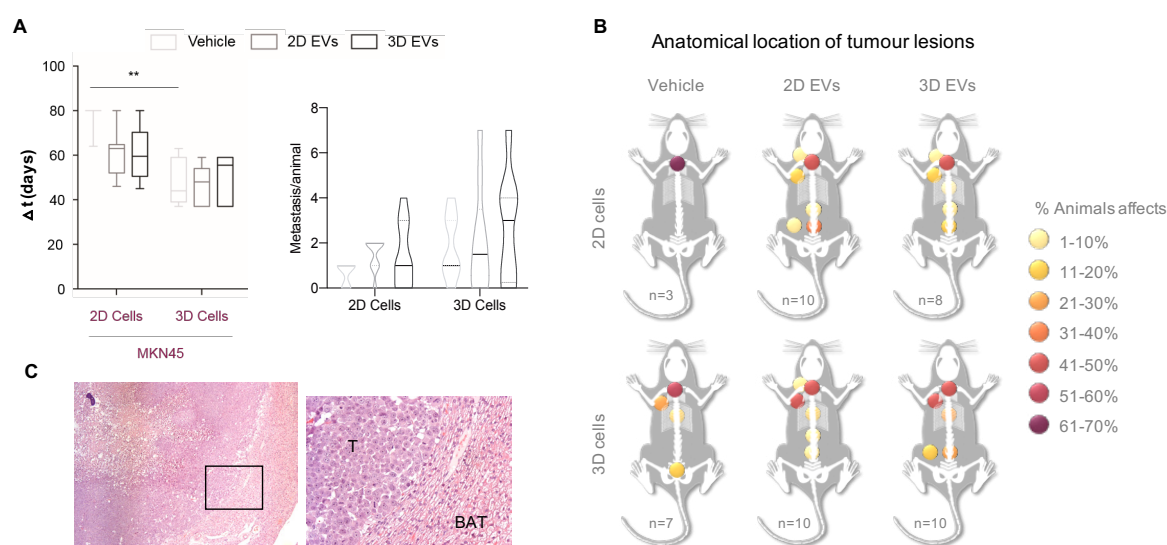


Figure 3. Diffuse-type GC cell *in vivo* dissemination. (A) Time until experimental endpoint and number of metastasis detected in each animal. (B) Anatomic location of diffuse-type metastasis and percentage of mice displays metastasis in each location. (C) Representative HE staining of a tumour metastasis (T) developing within BAT.

comprised by brown adipocytes (Figure 3C). Additional lung metastasis was found both in mice injected with 2D or 3D cells. These lesions are currently being subject of microscopic evaluation.

Altogether, these observations suggest that diffuse-type GC cells may have a particular tropism for brown adipocyte depots, which is fuelled by EVs.

EVs carry microRNAs involved in brown adipocyte differentiation and activation

To further dissect the putative role of EVs in the dissemination of diffuse-type GC cells, we performed a comparative analysis of the microRNAs expressed in 2D and 3D EVs from both intestinal- and diffuse-type GC. Interestingly, within the 37 microRNAs enriched in 3D EVs of diffuse-type GC cells (MKN45 3D EVs), 60% (22 out of 37) were involved in the regulation of adipocyte differentiation, conversion of white to brown adipocytes, and activation of brown adipocytes (Figure 4). Moreover, 24% of the microRNAs enriched in 3D EVs of diffuse-type GC (green arrows) displayed a strong and positive association with the aforementioned processes.

These results suggest that EVs may be active modulators of the adipose tissues that receive diffuse-type GC cells, thus facilitating metastasis to BATs.

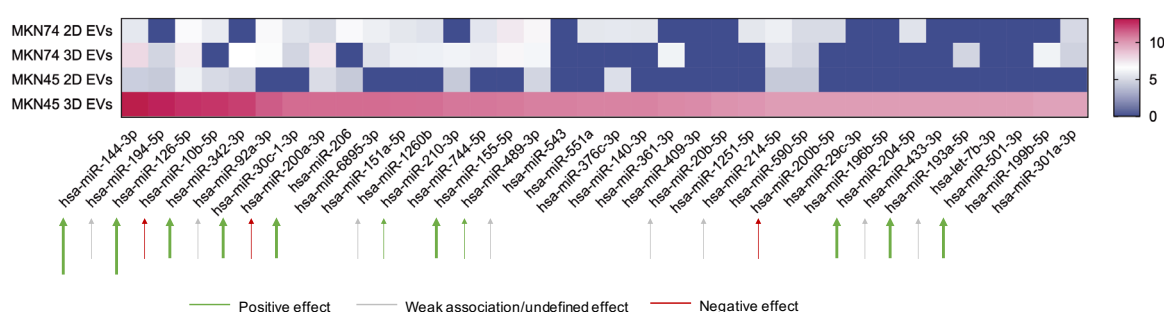


Figure 4. Heatmap of microRNAs enriched in diffuse-type GC EVs cultured in 3D conditions. Arrow identify microRNAs involved in adipocyte differentiation, conversion of white to brown adipocytes, and activation of brown adipocytes. Green and red arrows pinpoint microRNAs with positive and negative association with these processes, respectively. Grey arrows identify microRNAs with weak association or undefined effect.

Discussion

This study presents novel insights on the role of EVs in the dissemination of GC, particularly of the diffuse-type. Although preliminary, the data gathered in this work suggested that diffuse-type GC cells display a tropism to BATs, which is mediated and fueled by EVs.

Patient-derived studies have showed that intestinal-type GCs often metastasize to lung and liver, while diffuse-type GCs disseminate through the peritoneum and to lymph nodes.^{6,16} Concordantly, in this study, intestinal-type GC cells developed lung metastatic

lesions, whereas diffuse-type GC cells originated multiple and widely distributed tumour lesions. Interestingly, such anatomical locations corresponded to the previously identified human-like BAT and beige depots.¹⁷ Histological analysis of these lesions confirmed that diffuse-type GC cells colonized and grew in close proximity with brown adipose tissue.

In brown and beige adipocytes, uncoupling protein 1 (UCP1) renders the inner mitochondrial membrane 'leaky', which makes mitochondrial respiration remarkably inefficient.¹⁸ This event dramatically increases cellular respiration and the release of energy in the form of heat. Thus, activated brown/beige adipocytes require the uptake of free fatty acids and glucose from blood circulation to support fatty acid oxidation. Importantly, BAT activity and conversion of white to brown adipocytes (browning) has been correlated with cancer-associated cachexia, *i.e.*, the loss of adipose and skeletal muscle tissues that lead to a marked patient weight loss.¹⁹ Although the prevalence of BAT decreases drastically since birth, metabolically active BATs have been reported in healthy adult humans, both in visceral and subcutaneous locations.²⁰ Interestingly, visceral locations of human BATs included the greater omentum, transverse mesocolon, and periphery of solid organs like liver, spleen and pancreas.²¹ These locations overlap with the preferential site of diffuse-type GC metastasis, suggesting that our findings may closely mimic the human aspects of metastatic disease.

Notably, diffuse-type EVs enhanced the formation of BAT-associated lesions, thus suggesting the involvement of EVs in the tropism of diffuse-type GC. In fact, tumour-associated EVs have been previously identified as stimulators of beige/brown differentiation and important players in the metabolic reprogramming of stromal adipocytes that underlies tumour progression.^{22,23} To further explore the role on EVs and their cargo in GC cell dissemination and colonization we are currently performing an integrative analysis of microRNA and proteomic contents of EVs. Potential targets will be thereafter validated in EVs isolated from the plasma of mice with BAT lesions and from the plasma of GC patients.

Overall, this study suggests that BAT may be anchoring points for circulating diffuse-type GC cells, and that EVs may potentiate this colonization. Ongoing integrative analysis may unravel new targets and pathways involved in GC metastasis that may reveal useful for patient management.

Materials and Methods

Cell culture

Human GC cell lines MKN74 (intestinal-type) and MKN45 (diffuse-type) (Japanese Collection of Research Bioresources Cell Bank) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaille, France) and 1% penicillin–streptomycin (PS; Thermo Fisher Scientific, Waltham, MA, USA). All cells were maintained at 37°C in 5% CO₂ humidified

atmosphere.

EV-depleted medium was obtained by overnight ultracentrifugation at 100 000×g in a 70Ti rotor (Beckman Coulter, Fullerton, CA, USA) of RPMI medium supplemented with 20% FBS and 1% PS. This EV-depleted medium was filtered through a 0.22 µm filter and further diluted in the same amount of RPMI medium supplemented with 1% PS and without FBS, to reach a final 10% FBS concentration.

2D and 3D GC cultures were performed as previously described¹⁴. Briefly, cell monolayers were maintained in normal medium until 60–70% confluent, and then cultured in EV-depleted medium during 48 h. As for the 3D cultures, GC cells were seeded in agarose microwell arrays, grown in normal medium for 24 h and in EV-depleted medium for another 6 d. At the end, conditioned medium was collected for EV isolation. Cells growing as monolayers or spheroids were detached/dissociated using 0.5% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, MA, USA; 5 min, 37 °C), resuspended in normal medium and counted.

Isolation of EVs

EVs were isolated from cell culture medium by differential centrifugation, as previously described. Briefly, conditioned medium was subjected to centrifugation at 300×g (4°C, 10 min), and 2 000×g (4°C, 20 min) and filtered through a 0.22 µm filter. EVs were pelleted through a 100 000×g (4°C, 4 h) centrifugation in a SW32 rotor (Beckman Coulter, Fullerton, CA, USA). The EVs pellet was washed in PBS without Ca²⁺ and Mg²⁺ (Capricorn Scientific GmbH, Ebsdorfergrund, Germany), recovered by 100 000×g (4°C, 2 h) centrifugation, and resuspended in appropriate volumes of 0.9% NaCl.

Nanoparticle tracking analysis

The number and size of EVs was determined using Nanosight NS 300 system (Malvern, Worcestershire, UK). EVs suspension was diluted (1:200) in 0.9% NaCl to achieve a particle count ~1×10⁸–1×10⁹ particles/mL. Samples were acquired under controlled fluid flow with a pump speed set to 40. For each sample, three videos of 30 s, with more than 1000 detected tracks per video, were analyzed using the automatic functions of NTA 3.1 Build 3.1.54 software and a detection threshold fixed at 5. Mode and mean size were calculated from the three videos per biological replicate.

In vivo metastasis assay

Male nude mice (CBA, 6~8 weeks) were housed in a pathogen-free animal facility with access to water and food, and allowed to eat and drink ad libitum. All of the experimental procedures were performed in accordance with protocols approved by the ethical committee of the Instituto de Investigação e Inovação em Saúde (i3S).

To investigate the role of EVs in GC metastasis, 1×10⁶ 2D or 3D EVs were injected in the tail vein of nude CBA mice, twice a week for 4 weeks. Control mice were injected at the same timepoints, with the same volume of vehicle (0.9% NaCl). EVs injections were followed by a single cell injection of 1×10⁶ GC cells, also at the tail vein. Body weight

loss, dehydration and presence of tumour lesions were monitored. Humane endpoints were considered terminal. When mice were euthanized, all organs and blood were collected for further analysis. Organs were fixed in formalin, paraffin-embedded and stained with hematoxylin/eosin (HE) to evaluate metastases by microscopy. Blood was centrifuged at 2000×g (10 min, room temperature) to collect plasma, which was stored at -80°C until further analysis.

Proteomics

EV and cell pellets obtained from 2D and 3D cultures were processed for liquid chromatography–tandem mass spectrometry at the i3S Proteomics Scientific Platform. Protein identification was performed using the UniProt/SwissProt human database.

microRNA profiling and proteomics data integration sequencing experiments

Small RNA data, obtained in previous studies,¹⁴ was used to compare the expression of microRNAs in EVs cultured in 2D and 3D conditions. FPKM values were used for heatmap construction using GraphPad Prism software version 8.0. Integrative analysis of microRNA and proteomic profiles is currently being performed.

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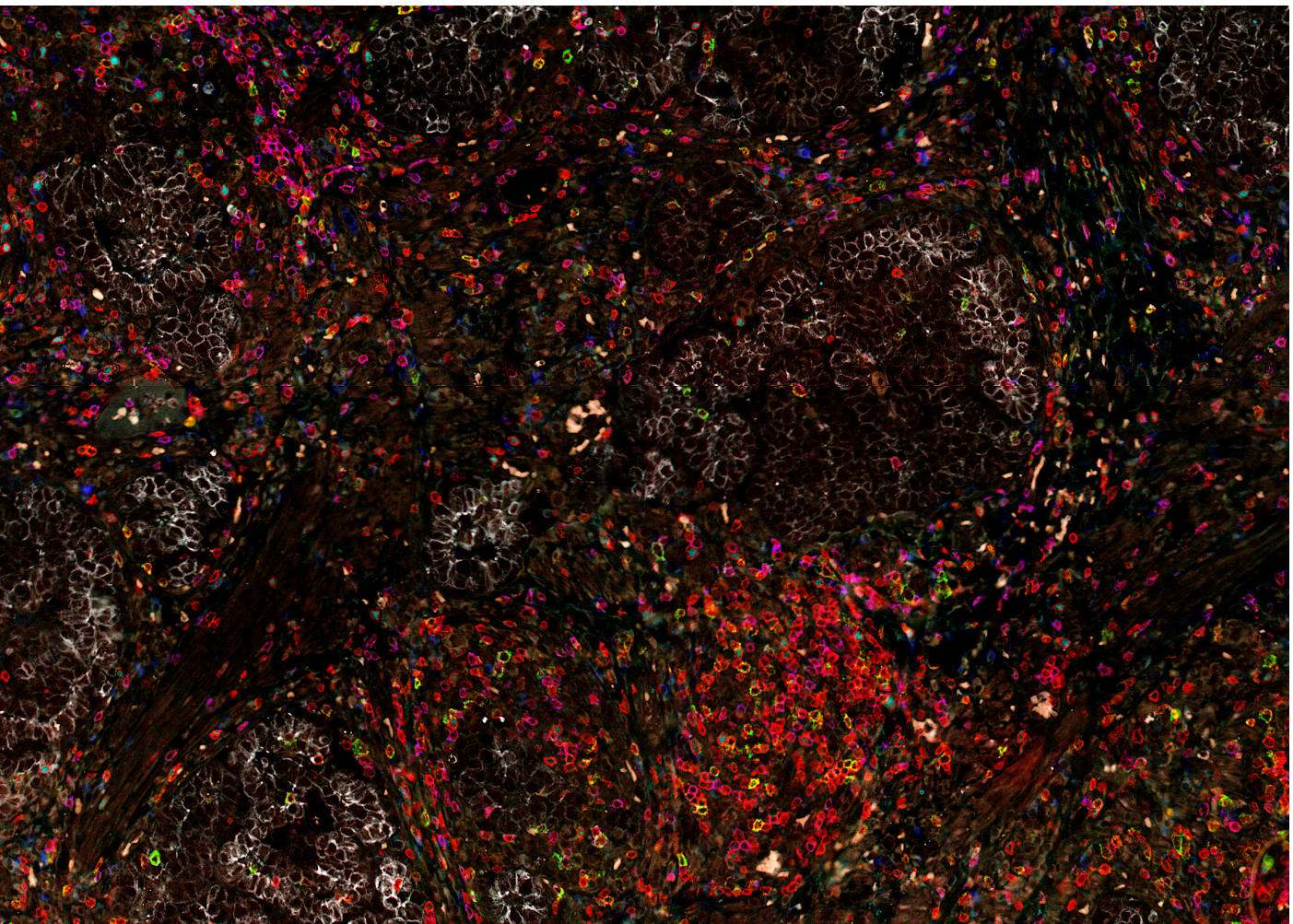
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Chapter 3

The role of tumour immune microenvironment in gastric cancer progression



Regulatory T cells fuel *de novo* expression of ILR2 α and proliferation of intestinal-type gastric cancer

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Regulatory T (Tregs) cells are abundant in the peripheral blood and among tumour-infiltrating lymphocytes of gastric cancer (GC) patients. Nevertheless, the role of Tregs in GC progression remains controversial. This study identifies a non-immunosuppressive mechanism through which Tregs directly modulate GC cells by promoting tumour growth. A comprehensive T cell immunophenotyping of 82 human GC cases, shows that Tregs located at the tumour nest are frequent in intestinal-type GCs but do not associate with increased levels of effector T cells. Moreover, 3D *in vitro* co-cultures reveal that Tregs preferentially infiltrate intestinal-type GC spheroids and, strikingly, induce the expression of IL2R α

and activation of MAPK signalling pathway in tumour cells, promoting spheroid growth. Accumulation of Tregs in intestinal-type GCs is increased at early stages and associates with the absence of vascular and perineural invasion. These findings hold insightful implications for therapeutic strategies targeting intestinal-type GCs and other tumours with similar immune context.

Keywords

Gastric cancer
Regulatory T cells
Immunophenotype
Tumour spheroids
ILR2 α

Introduction

Recent advances on the molecular aspects of gastric cancer (GC) have provided invaluable knowledge that led to the identification of new actionable targets and therapies.¹ Targeted treatments and immunotherapies are mainly offered to advanced cancer patients and have, so far, minimally improved GC prognosis, justifying why GC ranks as the third leading cause of cancer-related deaths worldwide.² Gastric tumours with high mutation load, microsatellite instability (MSI) or positive for Epstein–Barr viral infection are potentially immunogenic, and thus amenable for immunotherapy based on checkpoint inhibitors (e.g., anti-PD-1/PD-L1).^{3,4} Finding additional contexts for immuno-related therapies is an opportunity that is worth investigating further.

Regulatory T cells (Tregs) are CD4⁺T cells characterized by the surface expression of CD25/ILR2 α , and nuclear expression of the transcription factor FoxP3. Tregs are endowed with immunosuppressive activity that enforces peripheral tolerance and maintains immunological homeostasis.⁵ However, in cancer, the immunosuppressive environment promoted by Tregs withholds the antitumor immune response, hence promoting tumour progression and dissemination.^{6,7} In GC patients, peripheral blood and tumour-infiltrating lymphocytes are enriched in Tregs,^{8–12} which associates with increased tumour stage, poor prognosis and reduced patient survival.^{13–17} Nevertheless, other studies have shown that tumour infiltrating Tregs may also be associated with favourable prognosis, specifically in patients carrying tumours from the cardia or with MSI.^{18–20} Hence, the role of Tregs in GC progression remains poorly understood and highly controversial. Further, it is also unexplored in GC whether Tregs may promote tumour progression via non-immunological mechanisms, as described in other cancer models.^{21,22} Thus, we sought to explore the contribution of Tregs in GCs with distinct histology, and whether Tregs can directly impact tumour cells to promote GC progression.

To address this, we integrated the results of T cell immunophenotyping analysis with clinicopathological features of 82 GC patients. This analysis unveiled an enrichment of Tregs specifically in intestinal- and indeterminate-type GC, as compared to diffuse-type GC. Furthermore, our data suggests that a population of Tregs is maintained at the tumour nest of intestinal-type GC independently of the prevalence of effector T cells. Given these results, and to understand whether Tregs may actively modulate the phenotype of tumour cells, we established and explored 3D co-cultures of Tregs with intestinal- or diffuse-type GC cell lines. We found that Tregs actively infiltrate intestinal-type GC spheroids. Upon co-culture with Tregs, intestinal-type GC cells acquire expression of ILR2 α at the cell membrane, have increased activation of MAPK signalling pathway and spheroid growth. Furthermore, we found an enrichment of Tregs in early-stage intestinal-type GC, and in the absence of vascular and perineural invasion.

Altogether, our data suggests a direct effect of Tregs on tumour cells that may be particularly important in early stages of intestinal-type GC progression.

Results

Intestinal-type GC maintain a population of Tregs at the tumour nest independently on the prevalence of effector T cells

To investigate the immune T cell landscape within GC, we performed immunophenotyping of 82 patient samples. Each tumour section was stained simultaneously for a panel of seven markers, including five T cell-associated (CD3, CD8, FoxP3, PD-1, and CD103), cytokeratin to label epithelial tumour cells and DAPI for nuclei detection (Figure 1A, 1B). We focused the analysis on three major phenotypes, defined as follows: 1) Helper T cells, CD3⁺CD8⁻FoxP3⁻ cells (Figure 1C); 2) Cytotoxic T cells, CD3⁺CD8⁺ cells (Figure 1D); and 3) Tregs, CD3⁺CD8⁻FoxP3⁺ cells (Figure 1E). Cells expressing cytokeratin were excluded when building the T cell profiles. Poor performance of anti-IL2R α antibodies in the immunofluorescence staining hampered the analysis of IL2R α expression in tissue samples.

To disclose a potential association between T cell phenotypes and GC histology, we evaluated the density of each cell population according to GC histological types (Figure 1G; File S1). We observed that whereas the presence of helper and cytotoxic T cells only slightly changed across GC histological types, Tregs were significantly enriched in intestinal- and indeterminate-type GC, as compared to diffuse-type GC ($p=0.0175$ and $p=0.0028$, respectively; Figure 1G).

We further assessed whether these differences could be explained through a differential prevalence of MSI cases within each histotype. Whilst, 37% and 40% of intestinal- and indeterminate-type GC cases were MSI, only 13% and 18% of diffuse- and mixed-type GC had this phenotype (Figure 2A). Given these differences, we re-assessed the distribution of T cell populations considering both GC histotype and MSI status. We found that indeterminate-type MSS cases have more helper, cytotoxic cells and Tregs as compared to intestinal-type MSS cases ($p=0.0261$, $p=0.0349$ and $p=0.0026$, respectively); display increased numbers of helper T cells and Tregs in comparison to diffuse-type MSS ($p=0.0297$ and $p=0.0002$, respectively); and increased number of Tregs in comparison to mixed-type MSS ($p=0.0065$; Figure 2B-2D). Although, indeterminate-type MSS cases had increased number of Tregs as compared to MSI cases ($p=0.0444$; Figure 2D), the Treg/cytotoxic T cell ratio was not significantly changed (Figure 2E). Overall, these results suggested that indeterminate-type MSS cases elicit stronger immune responses, as compared to the other histotypes, and that the increased accumulation of Tregs was likely the result of an accumulation of effector T cell populations. As for intestinal-type GC, MSS cases displayed significantly lower numbers of cytotoxic T cells ($p=0.0014$), but not of Tregs, as compared to MSI cases (Figure 2C, 2D). This led to a higher Treg/cytotoxic T cell ratio in intestinal-type MSS cases comparing with MSI cases ($p=0.0414$; Figure 2E).

To further dissect this unbalanced ratio in intestinal-type GC, we characterized the distribution of T cell populations regarding their location at the stroma (S) or tumour nest (T) areas (Figure 2F-2I; Supplementary Figure 1). We observed that the increased Treg/

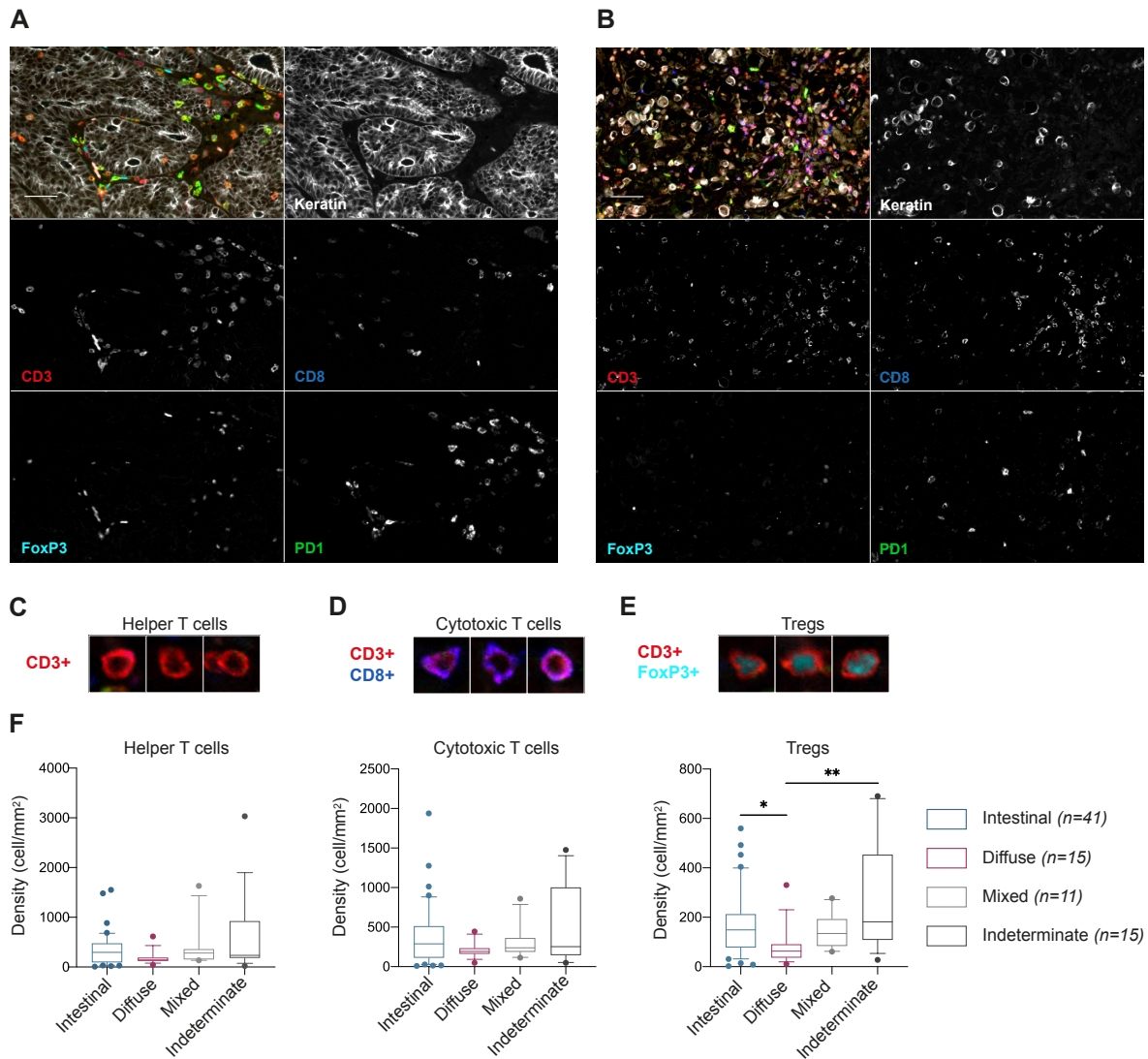


Figure 1. Characterization of the immune T cell landscape in GC tissue sections. (A-B) Representative composite and single-staining immunofluorescence images of the two main histological GC types, intestinal-type (A) and diffuse-type (B), obtained by multispectral imaging. Scale bar: 100 μ m. (C-E) Main T cell populations identified in the GC microenvironment based on the positivity for CD3, CD8, and FoxP3 cell markers. (G) Relative numbers of helper, cytotoxic T cells and Tregs, according to the histologic properties of the tumour: intestinal-type, $n=41$; diffuse-type, $n=15$; mixed-type, $n=11$; indeterminate-type, $n=15$ patients. Box and whiskers represent median $\pm$ 10–90 percentile. * $p<0.05$, ** $p<0.01$; Kruskal-Wallis test with Dunn's multiple comparison test.

cytotoxic T cell ratio in MSS tumours was maintained both at the stroma and tumour nest regions ($p=0.0274$ and $p=0.0109$, respectively; Figure 2F, 2G). Of notice, several MSS intestinal-type tumours showed particularly high numbers of Tregs comparing to the numbers of cytotoxic T cells, at the tumour nest (Figure 2G). Moreover, we observed that increased levels of cytotoxic T cells in MSI tumours were counterbalanced by increased numbers of Tregs at the stroma, but not at the tumour nest (Figure 2H, 2I). Hence, these data suggest that intestinal-type GCs, independently of MSI status, maintain a population of Tregs at the tumour nest that did not accompany the prevalence of cytotoxic T cells.

Altogether, these observations support the hypothesis that Tregs may have a non-immunosuppressive activity by directly affecting GC tumour cells with intestinal histology.

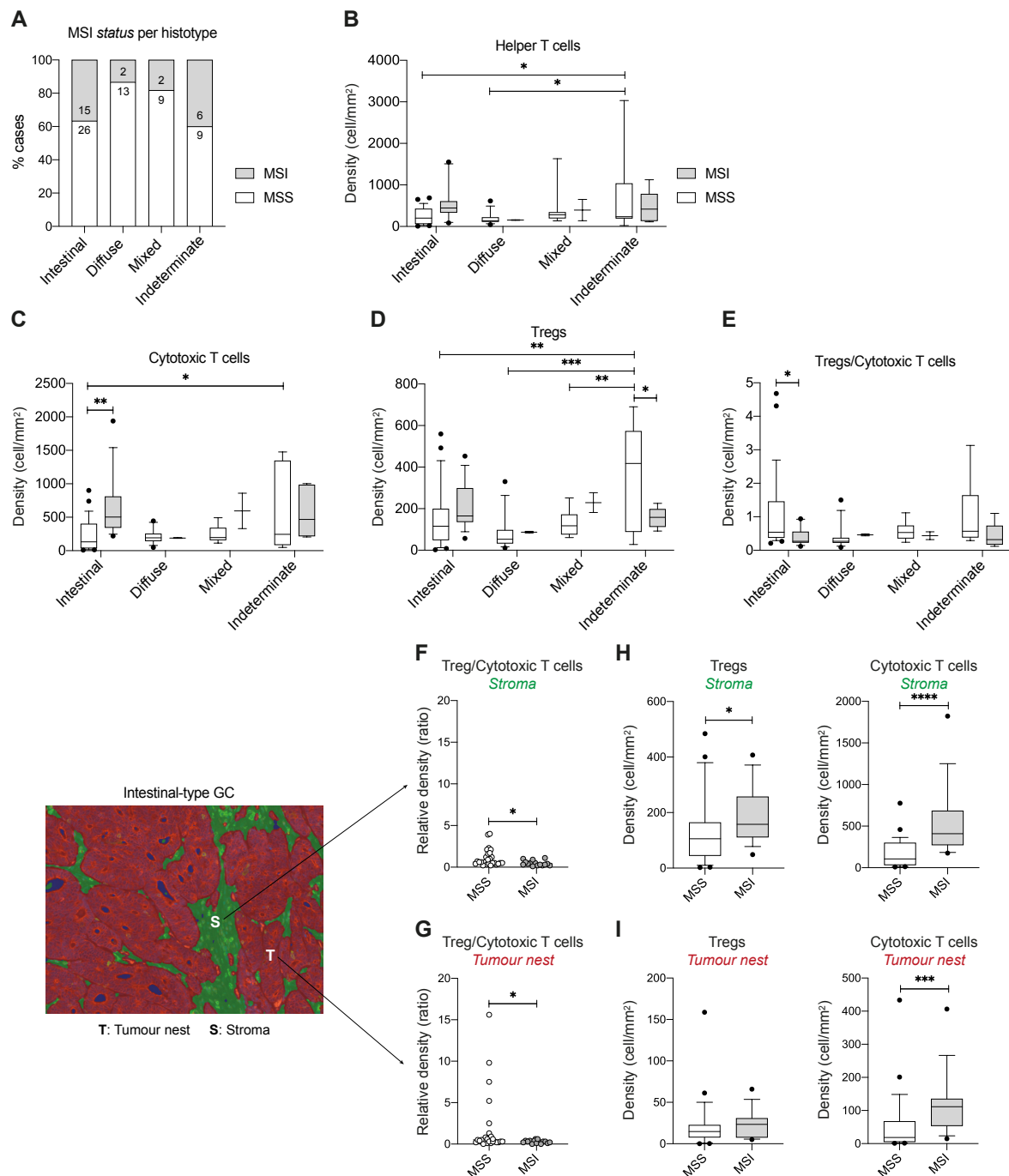


Figure 2. Distribution of T cell populations in MSS and MSI GC tumours. (A) Relative and absolute frequency of MSS and MSI cases for each GC histological type. (B-E) Relative numbers of helper (B), cytotoxic T cells (C), Tregs (D), and Tregs normalized to cytotoxic T cells (E), according to the MSI status and tumour histology. (F-I) Relative (F, G) and absolute (H, I) numbers of Tregs and cytotoxic T cells within the stroma (green; F, H) and tumour nest (red; G, I) areas of intestinal-type GC cases, according to MSI status. Box and whiskers represent median $\pm$ 10–90 percentile. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Two-way ANOVA with Tukey's/Sidak's multiple comparisons test (B-E) and Mann-Whitney U test (F-I).

Tregs actively infiltrate intestinal-type GC spheroids

To scrutinise the potential interactions between Tregs and tumour cells, we established direct 3D *in vitro* co-cultures of T cells, isolated from the peripheral blood of healthy donors, with GC cell lines (intestinal-type MKN74, and diffuse-type MKN45). Given the impossibility of using intracellular FoxP3 for cell sorting, we isolated CD3⁺CD4⁺CD127⁺ILR2 α ⁺ T cells, which are highly enriched in Tregs, from the peripheral blood of healthy donors (Supplementary Figure 2). As a control, CD3⁺CD4⁺CD127⁺ILR2 α ⁻ cells (naïve T cells) were also collected from the same donors.

T cells (either Tregs or naïve T cells) were added to GC spheroids recapitulating intestinal- or diffuse-type GC (Figure 3A). Co-cultures, imaged with time-lapse for 48 h, evidenced distinct Treg infiltration capacities depending on GC histology (Figure 3B, 3C). Whilst Tregs accumulated inside the intestinal-type GC spheroids (Figure 3B), they remained preferentially at the periphery of diffuse-type spheroids (Figure 3C). In both situations, the accumulation of Tregs was proportional to the number of Tregs in the co-culture (Figure 3B, 3C). As far as naïve T cells are concerned, accumulation either inside or at the periphery of spheroids was very low as compared to that of Tregs (Figure 3D, 3E). These observations support the findings from patients' tumours and reinforce specific crosstalk between Tregs and intestinal-type GC cells. Active infiltration of intestinal-type GC spheroids by Tregs was further validated through a four-angle analysis of the spheroids at distinct time-points (Figure 3F-3L; Supplementary Figure 3; Supplementary Video 1-3). This analysis revealed that only a small fraction of Tregs was actively infiltrating the spheroids (Figure 3F-3L), recapitulating the proportion of Treg infiltration observed in patients' intestinal tumours (Figure 1A).

Tregs induce ILR2 α expression at the membrane of intestinal-type GC cells

Next, we investigated whether GC cells could affect the phenotype of T cells and vice-versa. We started by analysing the expression of CD3, CD4, ILR2 α and FoxP3 T cell markers in cells collected from the co-culture conditioned media, and after dissociation of GC spheroids into single-cell suspensions. To assess the expression of these markers both in T cells (Tregs and naïve, stained with CTV) and in GC cells, we followed the gating strategy showed on Supplementary Figure 4. Both Tregs and naïve T cells maintained their original phenotype after 48 h of co-culture (Figure 4A), indicating that GC cells unlikely impact the expression of markers of Treg activation and immunosuppression, such as ILR2 α and FoxP3. Surprisingly, when co-culturing intestinal-type GC cells with Tregs, but not with naïve T cells, GC cells show *de novo* expression of membranous ILR2 α (Figure 4B, 4C), the α -chain of the IL2 receptor. This phenomenon was specific for intestinal-type GC cells, and the extent of expression was proportional to the number of Tregs in the co-culture (Figure 4B-4E). When intestinal-type GC spheroids were cultured in media supplemented with IL2 and increasing concentrations of anti-CD3/anti-CD28 beads, no ILR2 α induction was observed (Supplementary Figure 5A). Moreover, conditioned media from co-cultures did not elicit ILR2 α expression in tumour cells from spheroids that have not been previously exposed to T cells (Supplementary Figure 5B, 5C).

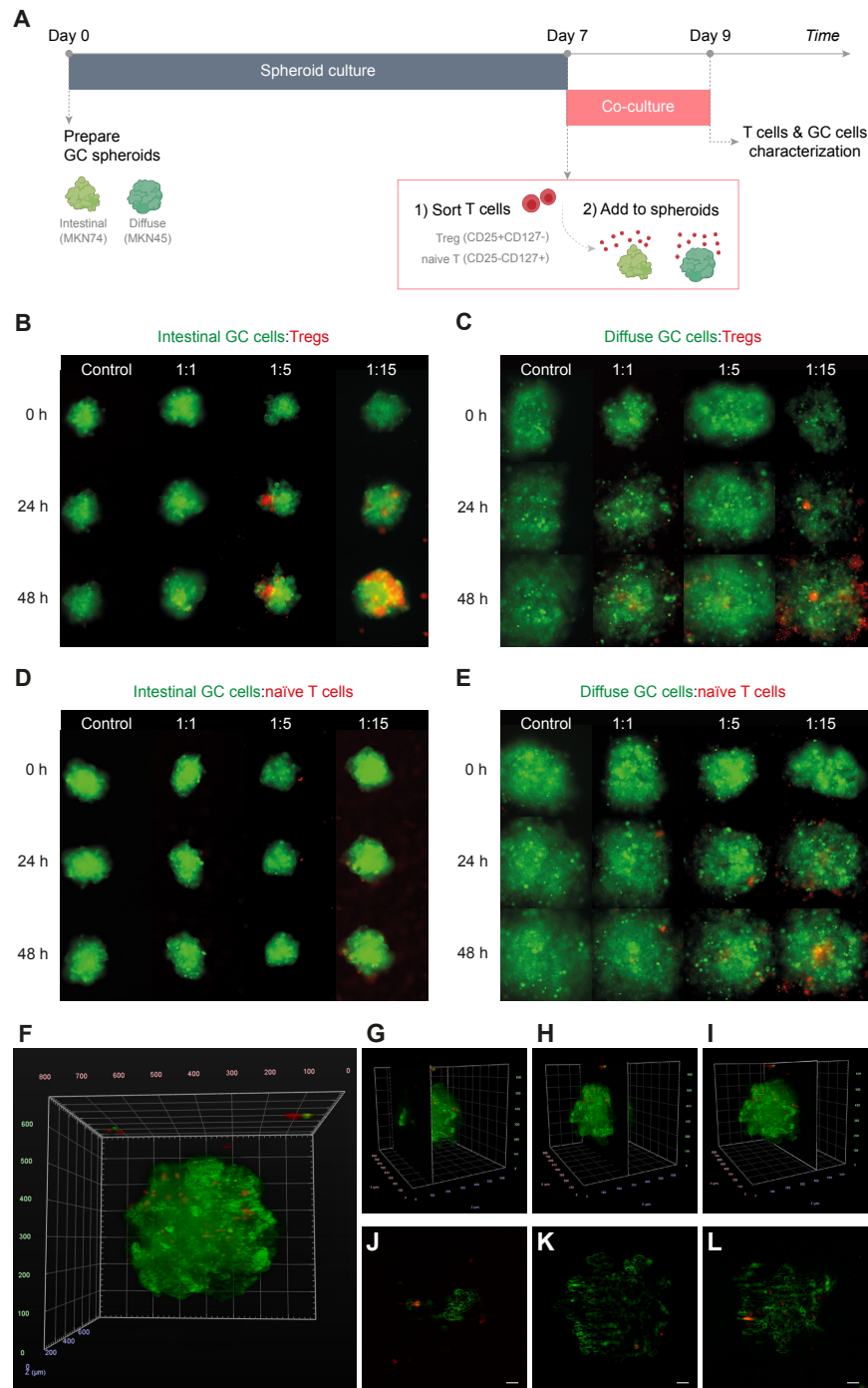


Figure 3. T cell infiltration of GC spheroids. (A) Schematic representation of the co-culture timeline. Spheroids (green) resembling intestinal- and diffuse-type GC were independently cultured for 7 days. Thereafter, Tregs and naïve T cells (red) were sorted from peripheral blood of healthy donors, based on the expression of CD3, CD4, CD127, and ILR2 α T cell markers, and added to GC spheroids. After 48h of co-culture, spheroids were dissociated for further characterization of both T cells and GC cells. (B-E) Monitorization of GC spheroids-T cell interactions during the 48h of co-culture by time-lapse microscopy. Control represents GC spheroids (green) that were not cultured with T cells (red). 1:1, 1:5 and 1:15 (GC cell:T cell) represent the increasing proportions of T cells to GC cells tested for (B) intestinal-type spheroids co-cultured with Tregs, (C) diffuse-type spheroids co-cultured with Tregs, (D) intestinal-type spheroids co-cultured with naïve T cells, (E) diffuse-type spheroids co-cultured with naïve T cells. (F-G) Light-sheet microscopy of 24h co-cultures of intestinal-type GC spheroids (green) and Tregs (red). (F) Co-culture 3D visualization after four-angle fusion of light-sheet microscopy images. (J-L) Lateral views of the 3D representation obtained from the light-sheet imaging data corresponding to the regions indicated in (G) ~124 nm, (H) ~290 nm, and (I) ~434 nm, to the entire co-culture. Scale bar: 50 μ m.

We further confirmed by imaging flow cytometry, that after co-culture with Tregs, intestinal-type GC cells displayed a 1.8-fold increase of the median membranous ILR2 α fluorescence intensity (3312 ± 72 a.u.), in comparison to GC cells cultured with naïve T cells (1839 ± 71 a.u.; Figure 4F, 4G).

Altogether, these results suggest that while GC cells do not seem to affect the phenotype of Tregs, their direct interaction induces expression of ILR2 α at the membrane of intestinal-type GC cells.

ILR2 α expression in intestinal-type GC cells associates with MAPK signalling pathway activation and spheroid growth

IL2 is a key cytokine in the regulation of immune cell activation and proliferation, particularly in Tregs and effector T cells. In immune cells, the high-affinity IL2 receptor, comprising the α -chain (CD25), β -chain (CD122) and γ -chain (CD132), initiates signal transduction via JAK1/3, leading to the activation of MAPK, PI-3K and STAT signalling pathways.^{25,26} We tested whether these pathways were activated in intestinal-type GC cells expressing ILR2 α , since, to the best of our knowledge ILR2 α expression has never been described in epithelial tumour cells. We observed that sorted ILR2 α^+ intestinal-type GC cells overexpressed total and phospho-ERK1/2 (Figure 5A, 5B; Supplementary Figure 6A), but STAT3 and Akt expression/activation were not detected (Supplementary Figure 6B). Accordingly, we observed that intestinal-type GC spheroids co-cultured with Tregs had increased growth, as a consequence of higher proliferation ($p=0.0021$; Figure 5C, 5D; Supplementary Figure 6C). Indeed, during the first 24 h of co-culture, GC:Treg spheroid growth rate particularly increased in the 1:5 condition in comparison to control spheroids, and to GC:naïve T cell co-cultures (Figure 5C). This observation indicates an early proliferative effect of Tregs over intestinal-type GC cells. In contrast, Tregs, but not naïve T cells, induced a reduction of diffuse-type GC spheroid growth after 24 h of co-culture as compared to control spheroids ($p<0.05$; Supplementary Figure 6D). The latter observations suggest that Tregs may have the opposite effect over diffuse-type GC spheroids, and likely through a distinct mechanism. Altogether, these results show that Tregs potentiate activation of the MAPK signalling pathway and intestinal-type GC spheroid growth.

Early-stage intestinal-type GCs are enriched in Tregs

Considering that Treg interaction with intestinal-type GC spheroids potentiates their growth, we hypothesized that the population of Tregs present at the tumour nest of intestinal-type GC could also trigger tumour growth. We addressed this hypothesis by analysing whether the number of Tregs in GCs was associated with tumour growth into the stomach wall (T stage). We detected higher density of Tregs in earlier T stages of intestinal-type GC, namely comparing tumours limited to the submucosa and muscularis propria with those growing throughout the stomach wall and into nearby organs or structures (pT2 vs pT4,

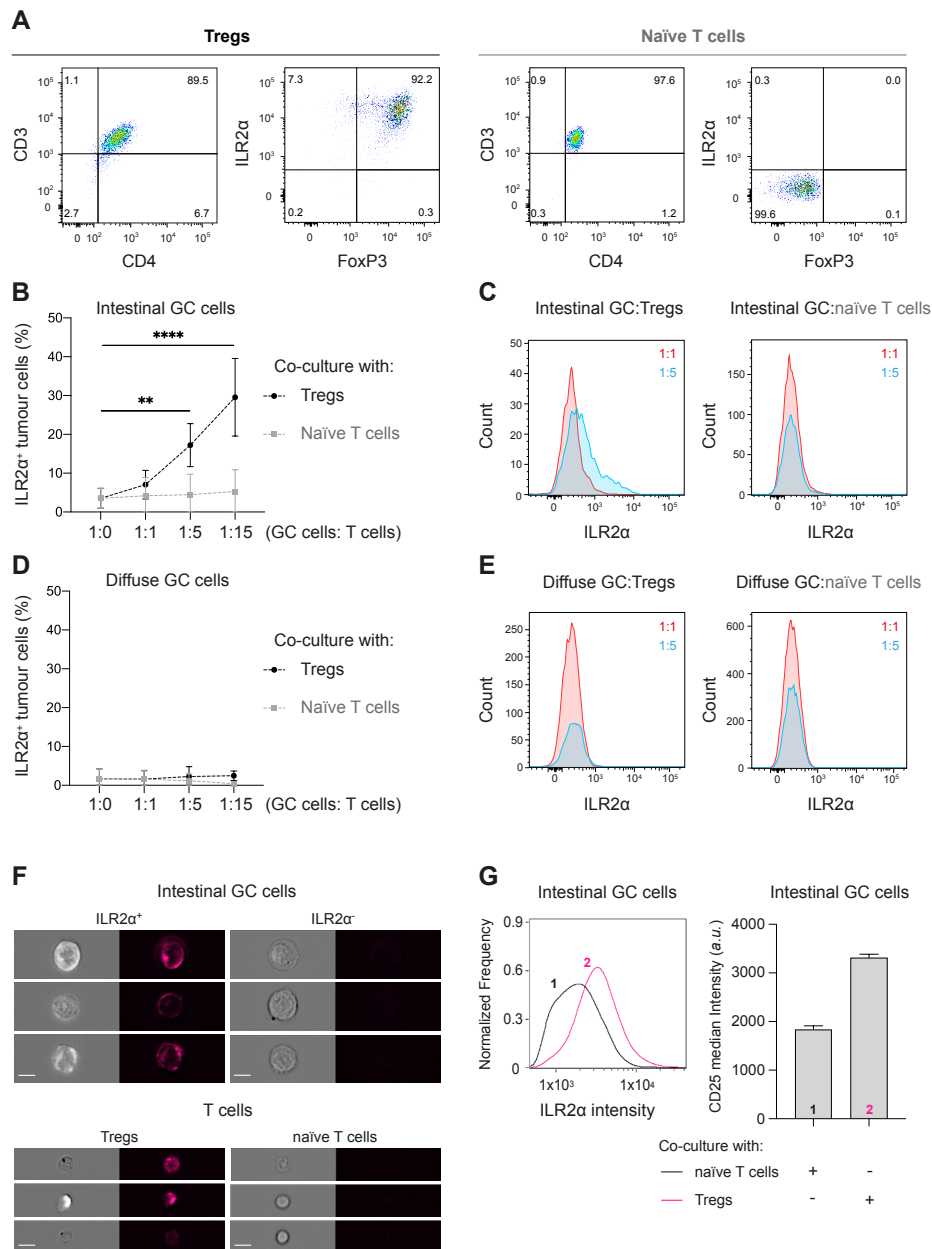


Figure 4. Characterization of GC and T cells after 48 h of co-culture. (A) Representative pseudocolour plots with the expression of CD3, CD4, ILR2α, and FoxP3 T cells markers by flow cytometry show that Tregs and naïve T cells maintain their characteristic phenotype after 48h of co-culture. (B-E) Quantitative expression of ILR2α in GC cells. (B, D) Plots showing percentage of ILR2α positive intestinal- (B) or diffuse-type GC (D) cells after 48 h co-culture with Tregs (black) or naïve T cells (grey). Data are shown as mean $\pm$ SD for co-cultures treated with T cells isolated from at least 6 healthy donors. ** $p < 0.01$, **** $p < 0.0001$. Two-way ANOVA with Dunnett's multiple comparisons test. (C, E) Representative histograms of ILR2α expression in intestinal- (C) or diffuse-type GC (E) cells after co-cultured at 1:1 (red) or 1:5 (blue) proportions with T cells. (F-G) Detection of membranous ILR2α expression by imaging flow cytometry. (F) Representative images of ILR2α expression in ILR2α positive (top left) and negative (top right) intestinal GC cells, as well as in Tregs (bottom left) and naïve T cells (bottom right). Scale bar: 10 μ m. (G) Representative histogram of ILR2α expression at the cell membrane (left graph) of intestinal-type GC cells co-cultured with naïve T cells (1-dark line) or Tregs (2-pink line) at 1:5 proportion and after 48 h of co-culture. Quantification of the median ILR2α intensity at the cell membrane (right graph). Graphs represent data from at least three independent experiments.

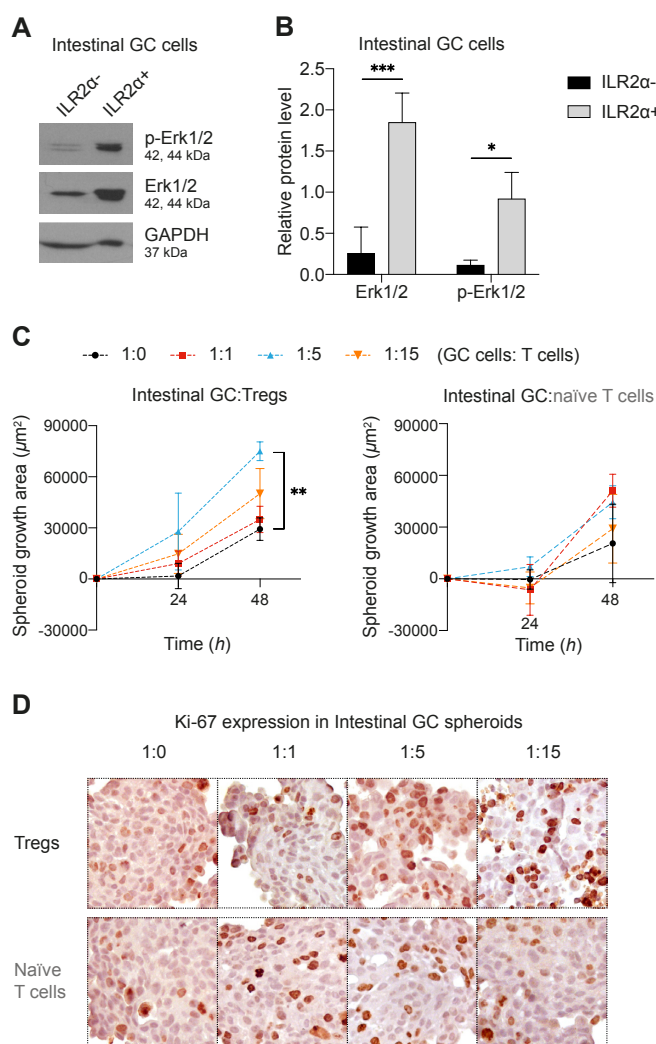


Figure 5. Spheroid growth after co-culture with T cells. (A-B) Western blot analysis for phosphorylated (p-ERK1/2) and total ERK1/2 protein levels in intestinal-type ILR2α positive and negative cells after 48 h co-culture with Tregs (1:5 proportion). (A) WB scans and (B) normalized expression represent three independent experiments. (C) Plots of growth area for intestinal GC spheroids co-cultured with Tregs (left graph) or naïve T cells (right graph) for 48 h. Control spheroids (dark) represent intestinal-type GC spheroids without T cells in co-culture. Co-cultures at 1:1, 1:5, and 1:15 proportions are represented in red, blue, and orange, respectively. Data shown mean ± SD of three independent co-cultures. ** $p < 0.01$. Two-way ANOVA with Dunnett's multiple comparisons test. (D) Representative images of Ki-67 nuclear expression (dark brown nuclei) in intestinal-type GC spheroids after 48 h of co-culture with Tregs or naïve T cells, at different 1:0 (control), 1:1, 1:5 or 1:15 proportions.

$p=0.0092$, Figure 6A; tumour nest: pT2 vs pT3, $p=0.0263$ and pT2 vs pT4, $p=0.0119$, Figure 6B; stroma: pT1 vs pT4, $p=0.0493$, and pT2 vs pT4, $p=0.0305$, Figure 6C). Additionally, a higher density of Tregs was also associated with the absence of vascular ($p=0.0049$; Figure 6D) and perineural invasion ($p=0.0263$, Figure 6E). Importantly, none of these associations were found when analysing helper or cytotoxic T cells (Supplementary Figure 7A-7C). Further, no association of T cell density was found with the presence/number of lymph nodes metastases (N), the presence of distant metastases (M), or with the TNM stage (Supplementary Figure 7D-7F).

These data suggest that the crosstalk between Tregs and intestinal-type GC cells is important at the early stages of tumour progression when the tumour is growing through the stomach wall into the muscularis propria, and before vascular and perineural invasion (Figure 6F).

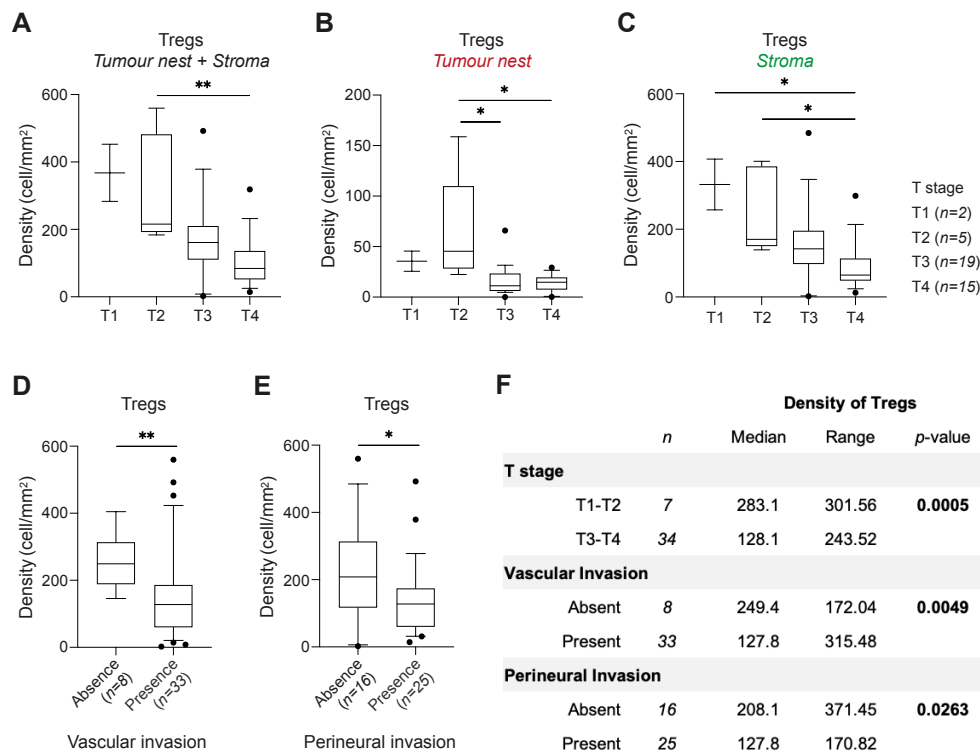


Figure 6. Distribution of Tregs in intestinal-type GCs and association with clinicopathological features. (A-C) Density of Tregs at tumour nest and stroma areas (A), tumour nest only (B), or stroma only (C), according to T stage: T1, $n=2$; T2, $n=5$; T3, $n=19$; T4, $n=15$. (D) Density of Tregs in intestinal-type GC with absence ($n=8$) or presence ($n=33$) of vascular invasion. (E) Density of Tregs in intestinal-type GC with absence ($n=16$) or presence ($n=25$) of perineural invasion. (F) Summary of the association of Treg density with clinicopathological features of intestinal-type GC. Box and whiskers represent median $\pm$ 10–90 percentile. $*p<0.05$, $**p<0.01$; Kruskal-Wallis test with Dunn's multiple comparison test (A-C) and Mann-Whitney U test (D-F).

Discussion

In this study, we combined single-cell immunophenotyping of GC samples, clinicopathological data, and 3D *in vitro* co-culture cell models to highlight and characterize a tumour-promoting, non-immunosuppressive role of Tregs in GC progression (Figure 7). According to our findings, intestinal-type GCs are enriched in Tregs that preferentially accumulate during the initial steps of tumour progression. Tregs that successfully infiltrate the tumour nest of intestinal-type GCs may directly contribute to tumour growth by inducing membranous expression of ILR2 α in tumour cells, which associated with MAPK pathway activation and cell proliferation.

Despite growing evidence of Treg enrichment in GC microenvironment, the role of Tregs in GC remains poorly understood. Studies showing that Tregs are already increased in non-neoplastic lesions, such as gastritis and peptic ulcers,^{27,28} suggest that Treg accumulation may be a consequence of inflammation and occurs before GC initiation. These findings have initially fostered our hypothesis that Tregs might differently contribute to the neoplastic development of tumours arising in distinct inflammatory contexts. To investigate this and considering recent data on the importance of studying the immunophenotype of GC,^{29,30} we performed a single-cell spatial analysis of the immune landscape of GC tumours with

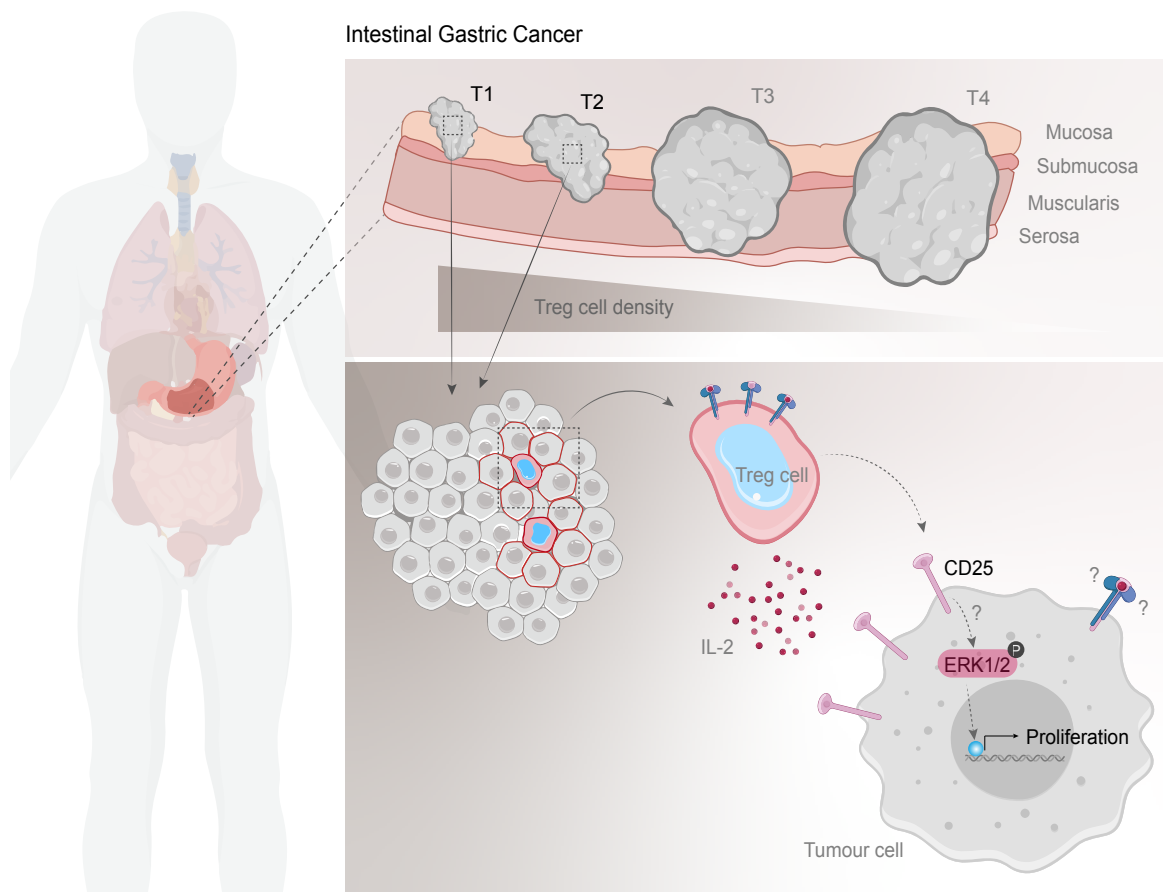


Figure 7. Schematic model of Tregs non-immunosuppressive role in intestinal-type GC. Early-stage intestinal GC, that do not present vascular and perineural invasion, display higher density of Tregs. *In vitro* results indicate that Tregs induce ILR2 α expression at the membrane of tumour cells, which associates with activation of MAPK signalling pathway, increased proliferation, and spheroid growth. Further studies are required to understand the mechanism behind ILR2 α expression in tumour cells and its implications in future IO therapies.

distinct histology. Our results showed that among the T cell populations analysed, Tregs were enriched in intestinal-type and indeterminate-type GC, which are often associated to chronic inflammation, in comparison to diffuse-type GC. This association of Tregs with inflammatory environments supports the proposed mechanisms of Treg accumulation in GC tumours based on CCL17/CCL22-mediated recruitment of Tregs or TGF- β -mediated conversion of naïve T cells into Tregs.³¹⁻³³ Another similarity between intestinal-type and indeterminate-type GCs was the increased proportion of MSI cases, which could elicit stronger anti-tumour immune responses.³⁴ Indeterminate-type MSS GCs showed elevated, though highly variable, T cell numbers that likely recapitulate the heterogenous nature of these tumours. Interestingly, in intestinal-type GCs, we observed an unbalanced ratio of Tregs and cytotoxic T cells, comparing MSS and MSI tumours. Such findings were more evident at the tumour nest of MSS tumours, where the number of Tregs greatly overdid the number of cytotoxic T cells. This excess of Tregs at the tumour nest fostered the hypothesis of a direct crosstalk between Tregs and intestinal-type tumour cells. Hence, we established direct co-cultures of T cells and 3D GC spheroids recapitulating the histological properties of intestinal- and diffuse-type GC.²⁴ This valuable tool allowed us to assess specific and active spheroid infiltration by T cells. As tumours can remodel their microenvironment,

modulating Tregs' phenotype or even converting naïve T cells into Tregs,⁷ we prepared co-cultures with T cells isolated from healthy donors, presumably without prior contact with tumour cells. We demonstrated that only a small fraction of Tregs infiltrates GC spheroids, even if high numbers of Tregs are added to the culture. Our 3D co-culture system not only closely mimics tumours from GC patients, but also their microenvironment context: 1) Treg density is higher outside than inside GC spheroids, which resembles the high T cell density at the GC stroma comparing to tumour nest; 2) Tregs, but not naïve T cells, infiltrate GC spheroids, recapitulating the increased Treg numbers observed in the tumour nest; 3) Tregs preferentially infiltrate intestinal-type GC spheroids, mimicking the histological differences observed between intestinal- and diffuse-type GC. Noteworthy is also the fact that intestinal-type GC spheroids display stronger homotypic cell-to-cell adhesion than diffuse spheroids,²⁴ which would hamper Treg infiltration. Furthermore, our study suggests that the interaction between Tregs and tumour cells has functional consequences, as intestinal-type cells acquire ILR2 α expression at the cell membrane, have stimulation of MAPK signalling pathway and increased spheroid growth. To our knowledge, this is the first study reporting ILR2 α de novo expression in epithelial tumour cells, which may hold important implications for the design of novel therapeutic strategies. Thus, it is critical to further understand the mechanisms that lead to ILR2 α expression in tumour cells and its molecular consequences. Our data suggests that ILR2 α is not passively transferred from Tregs to tumour cells, as tumour cells do not acquire other membrane Treg markers nor the cell tracer that was used to stain Tregs. Moreover, the conditioned medium of the Treg-intestinal tumour cell co-cultures was not sufficient to induce ILR2 α expression in other intestinal-type GC cells. Thus, it is unlikely that Treg-derived soluble factors or extracellular vesicles could actively foster or transfer ILR2 α to tumour cells. We expect that further studies focused on direct cell-cell interactions between Tregs and tumour cells, will enlighten the mechanisms underlying ILR2 α expression in tumour cells.

In immune cells, ILR2 α is known to bind IL2 and form heterodimers with the β - (CD122) and γ c- (CD132) chains of the IL2 receptor, to activate downstream signalling transduction.^{25,26} Hence, it would be important to address whether ILR2 α -tumour cells additionally express β - and γ c-chains of IL2R, and are responsive to IL2 signals. These experiments are crucial to determine the molecular effects of Tregs in tumour cells that should be further validated in tissue samples.

A deeper understanding on why, and under which circumstances, Tregs may induce expression of ILR2 α in tumour cells, may greatly impact the therapeutic strategies for intestinal-type GC patients and others presenting tumours with similar behaviour. This knowledge may also shed light on the contrasting effects of IL2-based tumour therapies.³⁵ Our results suggest that, under specific tumour contexts, therapies based on IL2 administration may favour tumour growth instead of just promoting immune T cell proliferation and survival. Additionally, tumour cells expressing ILR2 α at the membrane may compete with effector T cells for IL2, a tumour immune evasion strategy that, until now, was solely attributed to Tregs.⁷ Finally, innovative tumour therapies may arise directly from knowing that the

initial steps of tumour growth may be dependent/potentiated by ILR2 α -mediated signalling. Strategies targeting ILR2 α ³⁶ can be specifically directed to epithelial cells, which may reduce the secondary effects from unspecific targeting of Tregs.

Overall, our study suggests that during the early steps of intestinal-type gastric carcinogenesis, Treg cells accumulate within the tumour microenvironment and, likely through a contact-dependent mechanism, promote ILR2 α expression and stimulation of growth signalling pathways, such as MAPK pathway, in tumour cells. Our findings hold promising and relevant implications for future IO therapies and patient stratification in GC, which may potentially be expanded to other immune cold tumours.

Materials and Methods

Patient data

Tissue samples were obtained retrospectively from 82 GC patients who underwent gastric surgery with curative intent at Centro Hospitalar Universitário São João (CHUSJ; Porto, Portugal). Clinicopathological features were collected from medical and pathological records for further analysis (Table S1). Formalin-fixed paraffin-embedded (FFPE) specimens from 41 intestinal-type, 15 diffuse-type, 11 mixed-type, and 15 indeterminate-type GC tumours, according to Laurén histological classification, were included. For each case, the H&E-stained section used for histological classification was sequential to the one used for immunophenotyping analysis, to allow the association between the T cell immune profiling and tissue histological features. All cases were staged according to the eighth edition of the American Joint Committee on Cancer (AJCC) staging system. None of the patients included in this study was treated with neoadjuvant chemotherapy nor chemo-radiation therapy. The study was approved by the CHUSJ Ethics Committee (protocol 78/13).

Immunofluorescence staining, image acquisition, and analysis

T cell immunophenotyping of GC samples was performed using a seven-target immunofluorescence detection protocol on single FFPE tissue sections, following a previously described combination of tyramide signal amplification (TSA) with direct and indirect immunostaining.³⁶ Briefly, after an initial deparaffinisation of 4- μ m tissue sections with xylene and washing with ethanol, endogenous peroxidase was inactivated by blocking with 0.3% hydrogen peroxide in methanol (20 min; Merck Millipore, Burlington, MA, USA) and heat-induced antigen retrieval was performed with Tris-EDTA buffer (10 mM/1 mM, pH 9.0, 10 min). After cooling, slides were incubated with Superblock solution (30 min; Thermo Fisher Scientific, Waltham, MA, USA) to block non-specific binding. For TSA, slides were incubated with anti-PD-1 antibody (D4W2J, 1:2000 dilution, 60 min; Cell Signalling Technology, Danvers, MA, USA), followed by incubation with poly-horseradish peroxidase solution (30 min; Immunologic, Duiven, The Netherlands) and with Opal 520 reagent (1:100 dilution, 60 min; Perkin Elmer, Waltham, MA, USA). Before proceeding to

the indirect immunofluorescence step, microwave-induced antibody stripping was achieved by incubating slides in citrate buffer (10 mM, pH 6.0) at 90 W for 15 min. For the two-step immunofluorescence detection, slides were incubated with anti-FoxP3 (236A/E7, 1:25 dilution; Thermo Fisher Scientific), anti-CD8 (4B11, 1:50 dilution; Dako, Glostrup, Denmark) and anti-CD103 (EPR4166 2, 1:100 dilution; Abcam, Cambridge, UK) primary antibodies overnight, and with corresponding conjugated secondary antibodies (CF and Alexa Fluor dyes; Sigma–Aldrich, Saint Louis, MO, USA, and Thermo Fisher Scientific, respectively) for 60 min. Direct conjugated antibodies anti-CD3-AF594 (EP447E; Abcam) and anti-keratin-AF546 (AE1/AE3 and C11; Thermo Fisher Scientific and Cell Signalling Technology) were incubated for 5 h, after which slides were incubated with DAPI (1 μ M) for nuclear staining and mounted using Prolong™ gold antifade reagent (Cell Signalling Technology). Further details on the antibodies used can be found in Table S2.

A complete scan of the slides was obtained using Vectra 3.0 Automated Quantitative Pathology Imaging System (4 $\times$ magnification; Perkin Elmer) after which three regions of interest at the tumour core and another three at the invasive front were scanned with 20 $\times$ magnification (1340 $\times$ 1000 μ m images). Single-marker immunostainings were used to create the spectral libraries at the InForm Cell Analysis software (Perkin Elmer; Figure S7A). DAPI/keratin staining was used to segment images into tumour nest and stroma (Figure S7B). For cell segmentation, DAPI staining was used to segment nuclei, while CD3/CD8 staining was used to detect cell membrane (Figure S7C). The resulting cells were analysed based on the following phenotypes (Figure S7D): 1) T helper cells, which were CD3⁺CD8⁻FoxP3⁻ cells; 2) CD8 T cells, CD3⁺CD8⁺; 3) and Treg cells that were CD3⁺FoxP3⁺ but CD8⁻. We have also analysed the expression of CD103 (resident T cells) and PD-1 (activated cells) within these phenotypes (Figure S7E). Cells expressing cytokeratin were excluded when building T cell profiles. For each of the previous steps (*i.e.*, tissue segmentation, cell segmentation, and immunophenotyping), the InForm Cell Analysis software was manually trained before automated classification. Cell density (cells mm⁻²) was calculated by normalizing the cell counts by the tissue area of a given image.

Gastric Cancer 3D cell culture

Human GC cell lines MKN74 and MKN45 (intestinal- and diffuse-type GC, respectively; Japanese Collection of Research Bioresources Cell Bank) were seeded on 3D CoSeedis matrices (1 $\times$ 10⁵ cells mL⁻¹; abc biopply, Solothurn, Switzerland), as previously described,³³ and cultured in RPMI 1640 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France) and 1% penicillin-streptomycin (PS; Thermo Fisher Scientific), at 37 °C in 5% CO₂ humidified atmosphere, for 7 d to allow spheroid formation before co-culture with T cells.

Preparation of Treg and naïve T cell enriched samples

Human T cells were freshly isolated from anonymized healthy donor buffy coats, provided by Instituto Português do Sangue e da Transplantação (Porto, Portugal). The isolation of immune cells from healthy blood donors was approved by the CHUSJ Ethics Committee

(protocol 90/19) after each donor informed consent collection. Blood samples were diluted (1:3) in 2% FBS/PBS, layered on top of Histopaque®-1077 (Sigma-Aldrich), on SepMate™ tubes (Stemcell Technologies, Vancouver, BC, Canada), and centrifuged at 1200×g (10 min, with the brake on). Resulting peripheral blood mononuclear cells (PBMCs) were enriched for CD4⁺ T cells using MojoSort™ Human CD4 T Cell Isolation Kit (BioLegend, San Diego, SA, USA). According to the manufacturer instructions, PBMCs were filtered through a 70-µm cell strainer and incubated with CD4 magnetic Nanobeads (20 min, on ice). CD4⁺ T cells were separated and washed in 2% FBS/PBS using a DynaMag™-15 magnet (Thermo Fisher Scientific). Thereafter, CD4⁺ T cells were incubated with fluorescent conjugated antibodies anti-CD3-PE (OKT3, 1:400 dilution; Thermo Fisher Scientific), anti-CD4-FITC (OKT4, 1:400 dilution; Thermo Fisher Scientific), anti-IL2Rα-PE/Cy7 (BC96, 1:25 dilution; Thermo Fisher Scientific) and anti-CD127 APC/Cy7 (eBioRDR5, 1:50 dilution; Thermo Fisher Scientific) for 30 min. Fluorescence-activated cell sorting (FACS) of Tregs (CD3⁺CD4⁺CD127⁺IL2Rα⁺) and naïve T cells (CD3⁺CD4⁺CD127⁺IL2Rα⁻) was performed on FACS AriaII flow cytometer (BD Biosciences). Further details on the antibodies used can be found in Table S2.

Co-culture of GC spheroids and T cells

For the co-culture of GC spheroids and T cells, MKN74 (intestinal-type) and MKN45 (diffuse-type) GC spheroids were transferred to round-bottom 96-well plates, and Tregs/naïve T cells were added to the culture media at 1:1, 1:5 and 1:15 (GC cell:T cell) ratios. Co-cultures were maintained in RPMI 1640 with 25 mM HEPES (Lonza, Basel, Switzerland) supplemented with 10% FBS, 1% PS, 1% Sodium pyruvate (Sigma-Aldrich) and recombinant human IL-2 (10 ng mL⁻¹; PeproTech, London, UK). Treg cultures were further supplemented with anti-CD3/anti-CD28 MACS bead (0.5 beads per T cell; T Cell Activation Kit, Miltenyi Biotec, Bergisch Gladbach, Germany). As a control, MKN74/MKN45 single cultures (1:0; GC cell:T cell) were maintained in the same conditions as co-cultures.

Live cell imaging

To monitor T cell infiltration into tumour spheroids, MKN74 and MKN45 cell lines stably expressing mEmerald were used to form the GC spheroids, and sorted Tregs and naïve T cells were stained with CellTrace™ Far Red (CTFR) Cell Proliferation Kit (20 min, 37°C; Thermo Fisher Scientific) before co-culture. First, co-cultures were imaged using an automated fluorescence widefield HCS microscope (IN Cell Analyzer 2000; GE Healthcare, Chicago, IL, USA), equipped with a Plan-Fluor Nikon 20x/0.45 objective lens and a large-chip CCD camera (CoolSNAP K4). Emerald-GC spheroids were acquired in the FITC channel (Excitation/Emission: 490/525 nm; Exposure: 50 ms) and T cells in the Cy5 channel (Excitation/Emission: 645/705 nm; Exposure: 30 ms). Images were acquired at every 2 h for 60 h, under temperature-controlled conditions.

To further confirm Treg (CTFR) infiltration into intestinal-type GC spheroids (MKN74-Emerald), spheroids were transferred to a fluorinated ethylene propylene (FEP) microtube and imaged with a custom-built Digital Scanner Laser Light Sheet microscope (LSFM) equipped with a Nikon Plan-Fluor 10x/0.3 water-immersion objective lens in illumination

and detection plans. The fluorescence signals of the Emerald-MKN74 spheroids and CTFR-Tregs were recorded using a Hamamatsu Orca-Flash 4.0 V3 camera, after sample excitation with a 488 (1.0 mW) and 640 nm (1.0 mW) laser lines, respectively, with a 525/50 nm bandpass (BP) emission filter for emerald-spheroids and a 700/75 nm BP emission filter for CTFR-Tregs. The co-culture was imaged at three timepoints (16 h, 24 h, and 48 h) from four angles (90° rotation) every 16 min. Each view consists of multiple slices 1-µm apart covering the entire spheroid. Image reconstruction was performed using the arivis Vision4D v3.1.4 (arivis AG, Rostock, Germany).

Immunophenotype by conventional and imaging flow cytometry analysis

After 48 h of co-culture, conditioned media and spheroids were collected to analyse the phenotype of Tregs and naïve T cells. Before co-culture with GC spheroids, Tregs and naïve T cells were stained using CellTrace™ Violet (CTV) Cell Proliferation Kit (20 min, 37°C; Thermo Fisher Scientific), to allow further evaluation of T cell proliferation and to separate T cells (CTV+) from GC cells (CTV-) in the flow cytometry analysis. To prepare single-cell suspensions, spheroids were dissociated using 0.5% Trypsin-EDTA (5 min, 37°C; Thermo Fisher Scientific). Both T cells and GC cells were stained with anti-CD3-PE, anti-CD4-FITC, and anti-IL2Rα-PE/Cy7, as previously mentioned, and with Fixable Viability Dye eFluor™ 780 (1:1000 dilution; Thermo Fisher Scientific) to assess cell viability. Intracellular staining of FoxP3 was performed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific), following manufacturer's instructions. Briefly, cells were fixed with Fixation/Permeabilization Buffer (30 min; Thermo Fisher Scientific), blocked with human IgG (1 mg mL⁻¹, 15 min; Sigma-Aldrich) and stained with anti-FoxP3-APC (1:15, 30 min; Thermo Fisher Scientific). Data acquisition was performed on an LSRFortessa cytometer (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) and analysed using FlowJo v10 software (Becton, Dickinson & Company) following the gating strategy explained on Figure S3A.

Imaging flow cytometry was performed on single-cell suspensions derived from co-cultures of MKN74-GC spheroids with Tregs/naïve T cells and stained with anti-IL2Rα-PE/Cy7 antibody. Data was acquired on an imaging flow cytometer (ImageStreamX; Amnis Corporation, Seattle, WA, USA) equipped with INSPIRE software. Samples were excited with a 488 nm argon laser, and for each event, bright-field cell images were acquired (40× magnification) on channel 1, while IL2Rα signal was detected on channel 6 (Excitation/Emission: 496/774 nm). Laser power was not modified throughout the sample acquisition. Data analysis was performed using the IDEAS software (Amnis Corporation). Further details on the antibodies used can be found in Table S2.

Recovery of IL2Rα⁺ GC cells and Western Blot analysis

To analyse the effect of IL2Rα expression specifically on intestinal-type GC cells, MKN74-GC cells were stained with CellTrace™ Yellow (CTY) Cell Proliferation Kit (Thermo Fisher Scientific) before grown as spheroids, while Tregs and naïve T cells were stained with CellTrace™ Far Red (CTFR) Cell Proliferation Kit (Thermo Fisher Scientific) before co-culture. As previously, after co-culture, spheroids were dissociated, and the resulting single-

cell suspension was stained with anti-IL2R α -PE/Cy7 antibody. MKN74-GC cells (CTY⁺) positive or negative for IL2R α were sorted on the FACS Aria II flow cytometer (Becton, Dickinson & Company) and collected to FBS. According to the number of sorted cells, samples were resuspended in proportional volumes of radioimmunoprecipitation assay buffer (RIPA buffer) supplemented with protease and phosphatase inhibitors (Thermo Fisher Scientific) and cell lysis was allowed for 30 min, on ice. To enhance protein recovery, lysates were sonicated twice (10 sec, 20x, 50%) before centrifugation at 14000 rpm (15 min, 4°C) to recover cleared lysates. Equal amounts of protein lysate (25 μ L) from each biological replicate were subjected to western blotting. Primary antibodies targeting phospho-Stat3 (Tyr705, D3A7; Cell Signalling Technology), Stat3 (124H6; Cell Signalling Technology), phospho-Akt (Ser473, D9E; Cell Signalling Technology), Akt (Cell Signalling Technology), phospho-p44/42 MAPK (phospho-ERK1/2, Thr202/Tyr204; Cell Signalling Technology), p44/42 MAPK (ERK1/2; Cell Signalling Technology), and GAPDH (1E6D9; ProteinTech, Rosemont, IL, USA) were used, as well as corresponding ECLTM anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). Detected signals were quantified using Quantity One® Basic software (Bio-Rad, Hercules, CA, USA). Unprocessed scans of western blots are provided in Figure S8. Additional information on the antibodies used is detailed in Table S2.

Ki-67 staining on GC-spheroids

To investigate GC-spheroid proliferation after co-culture, spheroids were fixed with 2% paraformaldehyde (overnight; Merck, Darmstadt, Germany), stained with Gill's hematoxylin (10 min; Bio-Optica, Milan, Italy) and injected within a drop of 2.4% low melting point agarose (50°C; Lonza). After gelling (10 min at room temperature followed by 20 min on ice), the agarose structures were included in paraffin blocks and then sectioned into 3- μ m slides. Antigen retrieval was performed using citrate buffer (10 mM, pH 6.0, at 98 °C, 40 min; Abcam). Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxidase solution (20 min; Sigma-Aldrich) followed by incubation with anti-Ki-67 antibody (SP6, 1:200 dilution, 90 min; Thermo Fisher Scientific). After washing, slides were incubated with REAL EnVision Detection System (Dako) substrate buffer (30 min) and with DAB Chromogen (10 min).

Acknowledgements

The authors would like to thank Serviço de Imunohemoterapia of Centro Hospitalar Universitário de São João (CHUSJ), for kindly donating Buffy Coats and to Maria José Oliveira and Susana Santos for enabling this collaboration protocol.

The authors acknowledge the support of the Advanced Light Microscopy, Bioimaging, BioSciences Screening and Histology and Electron Microscopy i3S Scientific Platforms members of the national infrastructure PPBI - Portuguese Platform of Bioimaging (PPBI-POCI-01-0145-FEDER-022122), and the support provided by the Translational Cytometry

i3S Scientific Platforms. The authors also acknowledge the excellent assistance of Catarina Meireles, Emília Cardoso, Paula Sampaio, Maria Lázaro, André Maia, Rossana Correia and Nuno Mendes.

Author contributions

S.R., J.C. and C.O. were involved in the conceptualization and design of the study; S.R., G.G., I.G., G.M.A., J.C., F.C. and C.O. were involved in collecting material and clinicopathological data from GC patients; S.R., M.E.I. and N.F.C.C.M. were involved in the immunophenotype analysis; S.R., A.B., S.P.T., M.A., J.T.B and L.G. were involved in the establishment and characterization of co-cultures; S.R., J.C. and C.O analyzed the data; S.R., J.C. and C.O wrote the manuscript; C.O. sought institutional approval, funding, national and international collaborations, and is responsible as guarantor for the overall content of the manuscript. All authors critically reviewed and gave final approval of the manuscript.

Funding

This work was supported by grants: 1) “Institute for Research and Innovation in Health Sciences” (POCI-01-0145-FEDER-007274), by FEDER - Fundo Europeu de Desenvolvimento Regional funds through the COMPETE 2020 - Operacional Programme for Competitiveness and Internationalisation (POCI), PORTUGAL 2020, Portuguese funds through Portuguese Science & Technology Foundation (FCT) / Ministério da Ciência, Tecnologia e Inovação; 2) Project CANCER Ref. NORTE-01-0145-FEDER-000029 and DOCnet Ref. NORTE-01-0145-FEDER-000003), by Norte Portugal Regional Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF); 3) Project Ref. PTDC/BBB-ECT/2518/2014, by FCT; (4) FCT PhD Programmes and by Programa Operacional Potencial Humano (POCH), specifically by the BiotechHealth Programme (Doctoral Programme on Cellular and Molecular Biotechnology Applied to Health Sciences); 5) “The role of gastric cancer cell-derived extracellular vesicles”, by IPATIMUP Board of Directors; 6) GenomePT project (POCI-01-0145-FEDER-022184), by COMPETE 2020 - POCI, Lisboa Portugal Regional Operational Programme (Lisboa2020), Algarve Portugal Regional Operational Programme (CRESC Algarve2020), under the PORTUGAL 2020 Partnership Agreement, through ERDF, and by FCT; 7) Marie Skłodowska-Curie grant agreement No. 722148 to Train-EV project by European Union’s Horizon 2020 research and innovation programme; 8) Project CANCERSTEM funded by ERDF, POCI and FCT; 9) FCT fellowship PD/BD/128406/2017 to SR, and Junior Research contract to JC (regulated by the decree-law 57/2016 emended by law 57/2017). IPATIMUP integrates the i3S Research Unit, which is partially supported by FCT. NFCCdM has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement No. 852832).

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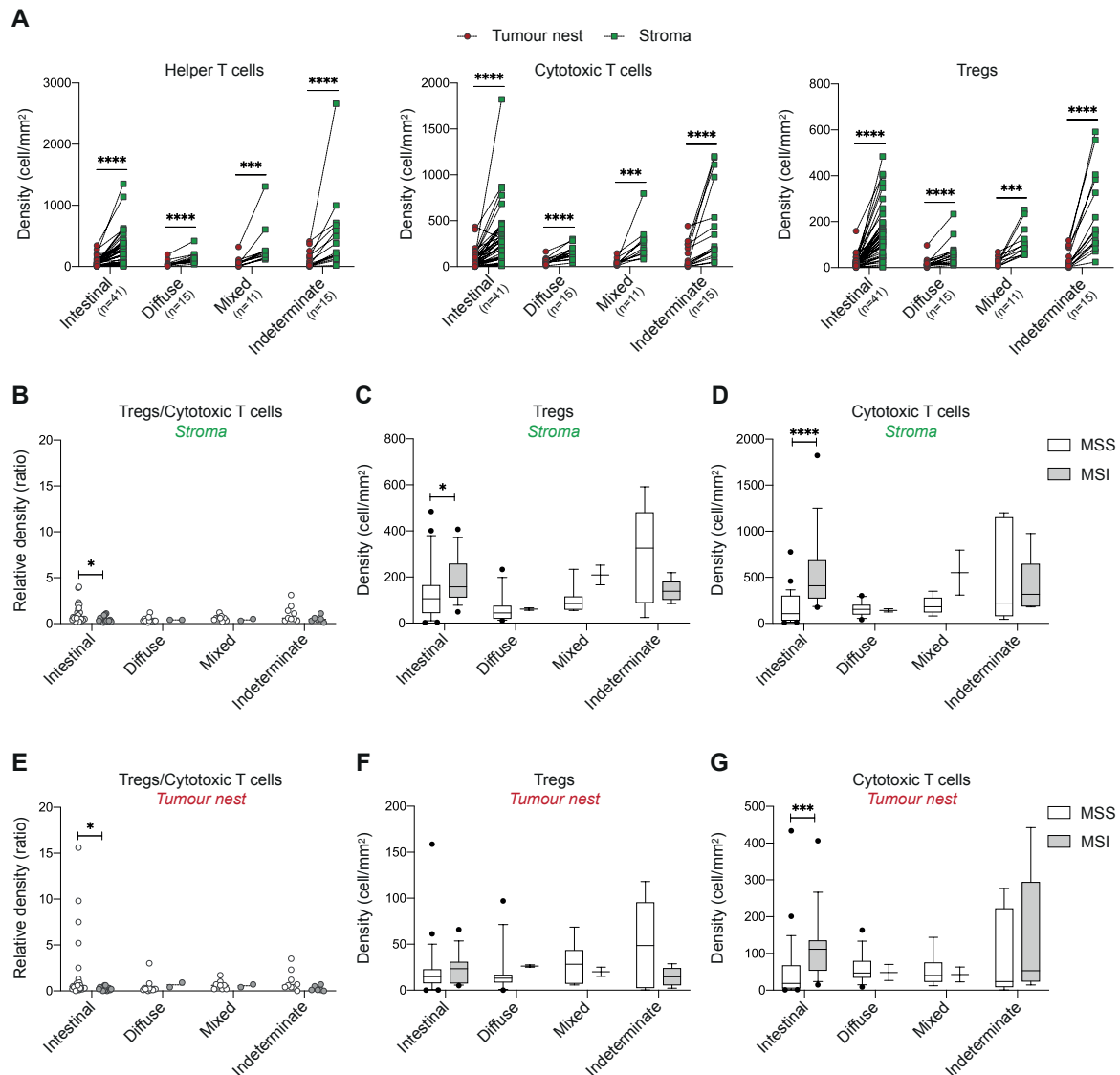
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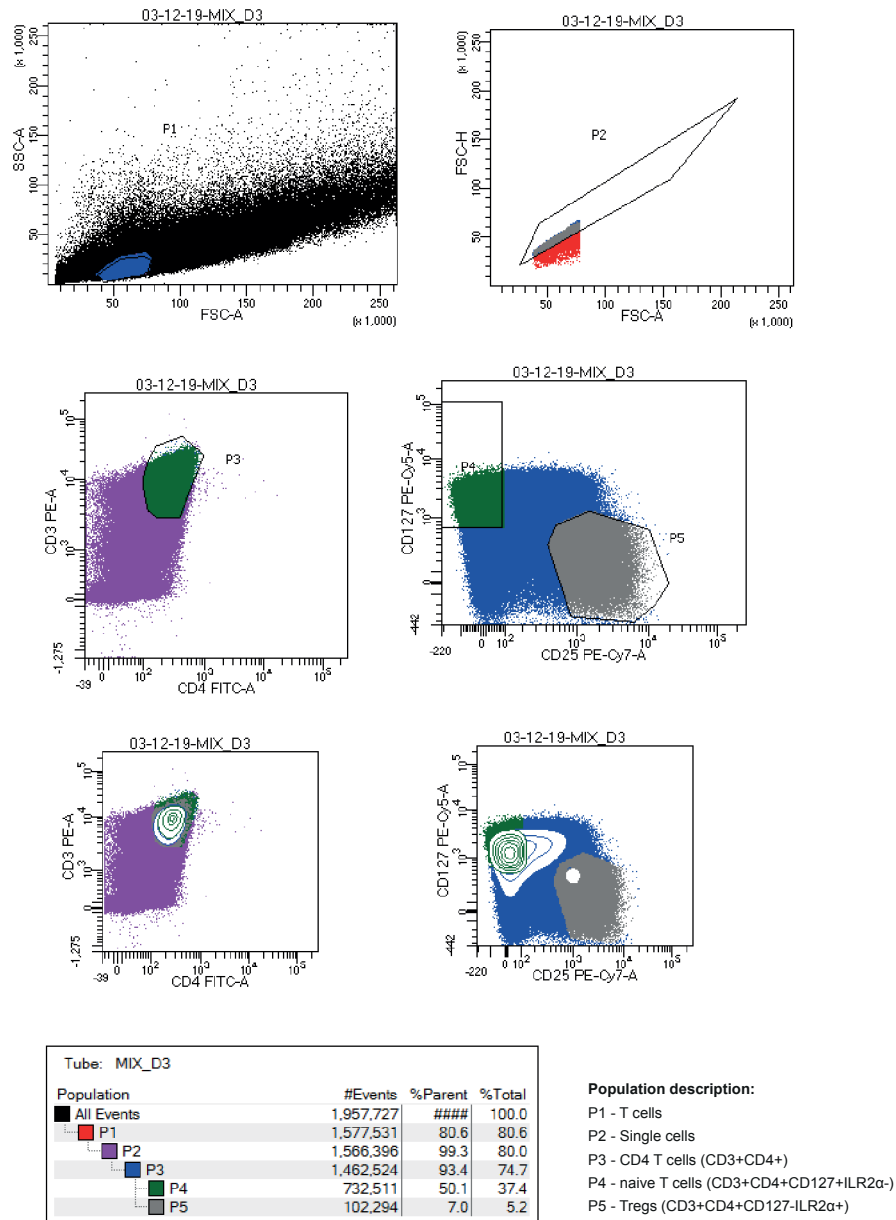
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Supplementary Information

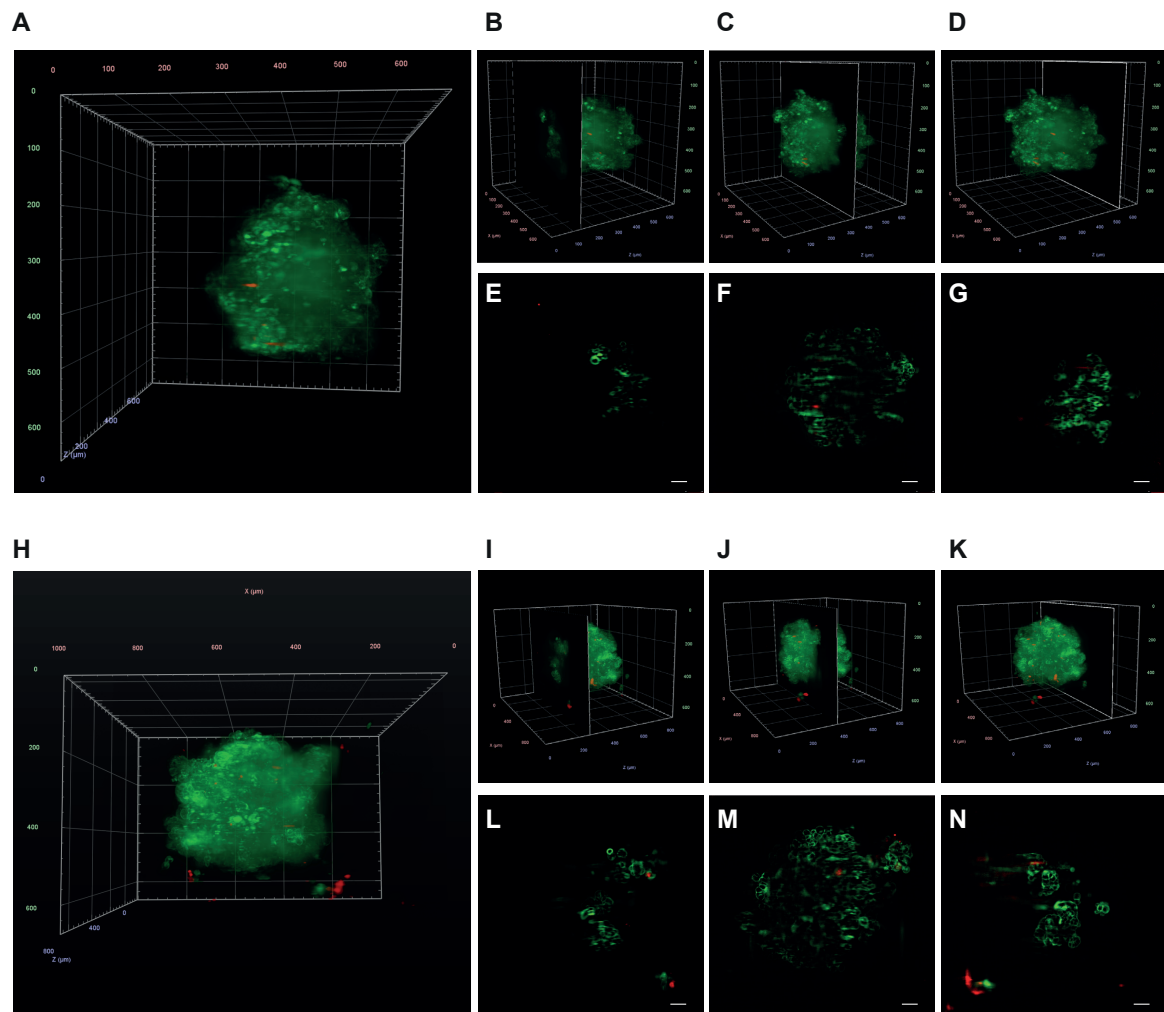
Supplementary Figures



Supplementary Figure 1. Distribution of T cell populations in MSS and MSI GC tumours. (A) Density of helper, cytotoxic T cells and Tregs within tumour nest (red) and stroma (green) areas, according to tumour histology. Each dashed line matches tumour nest and stroma areas of the same patient sample. * $p<0.05$, *** $p<0.001$, **** $p<0.0001$; Wilcoxon matched-pairs signed-rank test. (B-G) Relative (B, E) and absolute numbers of Tregs (C, F) and cytotoxic T cells (D, G) within the stroma (B-D) and tumour nest (E-G) areas of intestinal-type GC cases, according to MSI status and tumour histology. Box and whiskers represent median $\pm$ 10–90 percentile. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Mann-Whitney U test (F-I).



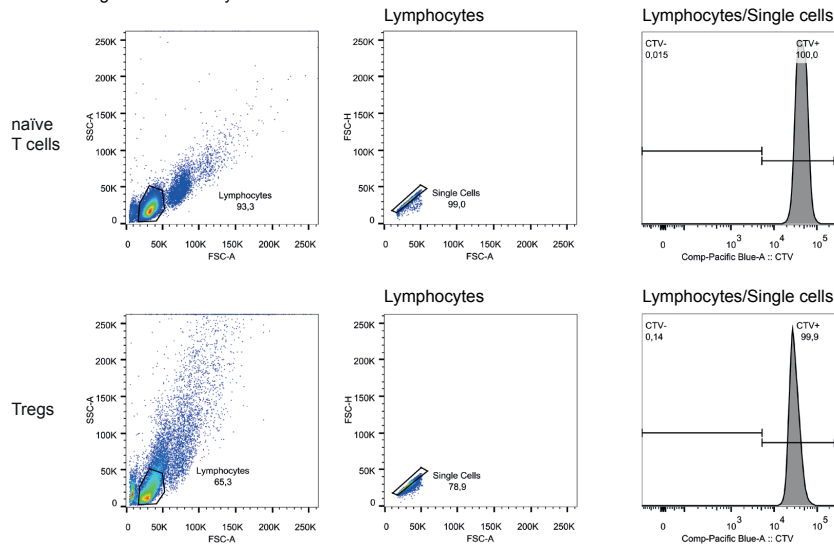
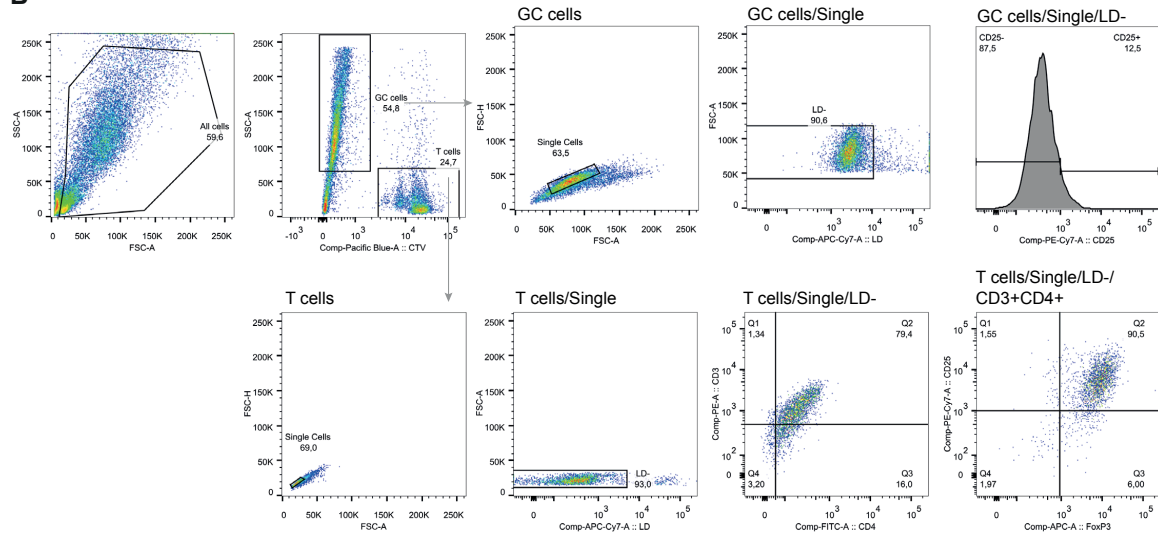
Supplementary Figure 2. Gating strategy of Tregs and naïve T cell sorting from peripheral blood of healthy donors. Firstly, T cells (P1) are gated from the general events acquired, and single cells (P2) are selected from this population. Then, CD3⁺CD4⁺ T cells are gated (P3) followed by the identification of naïve T cells, IL2Rα⁻ CD127⁺ (P4), and Tregs IL2Rα⁺CD127⁻ (P5).



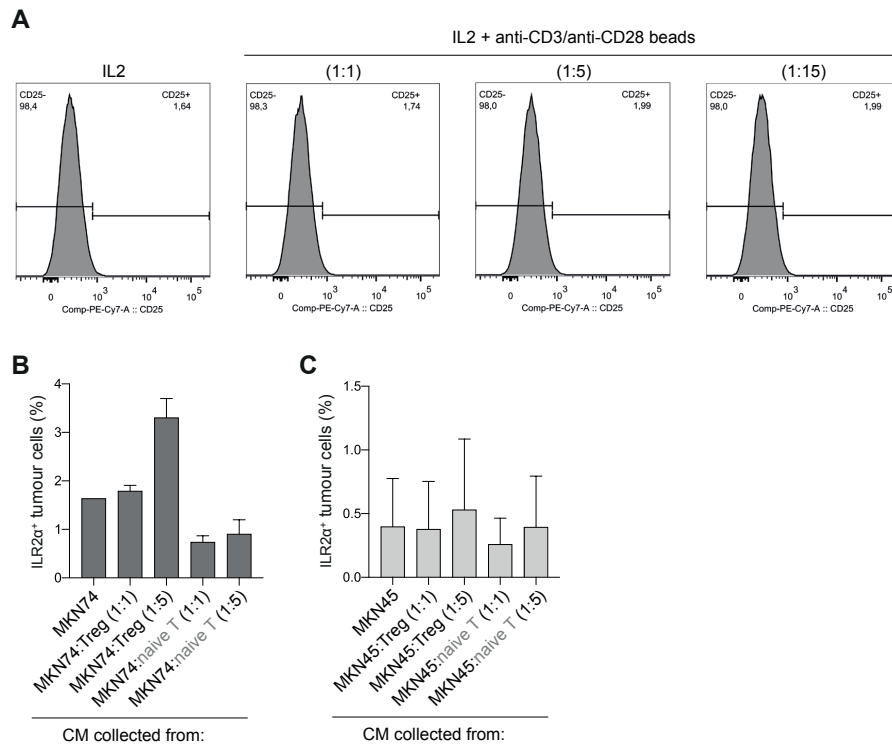
Supplementary Figure 3. T cell infiltration of GC spheroids by light-sheet microscopy. Co-cultures of intestinal-type GC spheroids (green) and Tregs (red) were imaged at 16 h (A-G) and 48 h (H-N). (A, H) 3D tomography of a four-angle fusion of the co-culture. Location of (B, I) section 1 (135 and 270 nm, respectively), (C, J) section 2 (348 and 392 nm, respectively) and (D, K) section 3 (552 and 678 nm, respectively) in relation to the entire co-culture. Images of cross-sections 1 (E, L), 2 (F, M) and 3 (G, N) show Tregs within GC spheroids. Scale bar: 50 μm .

A

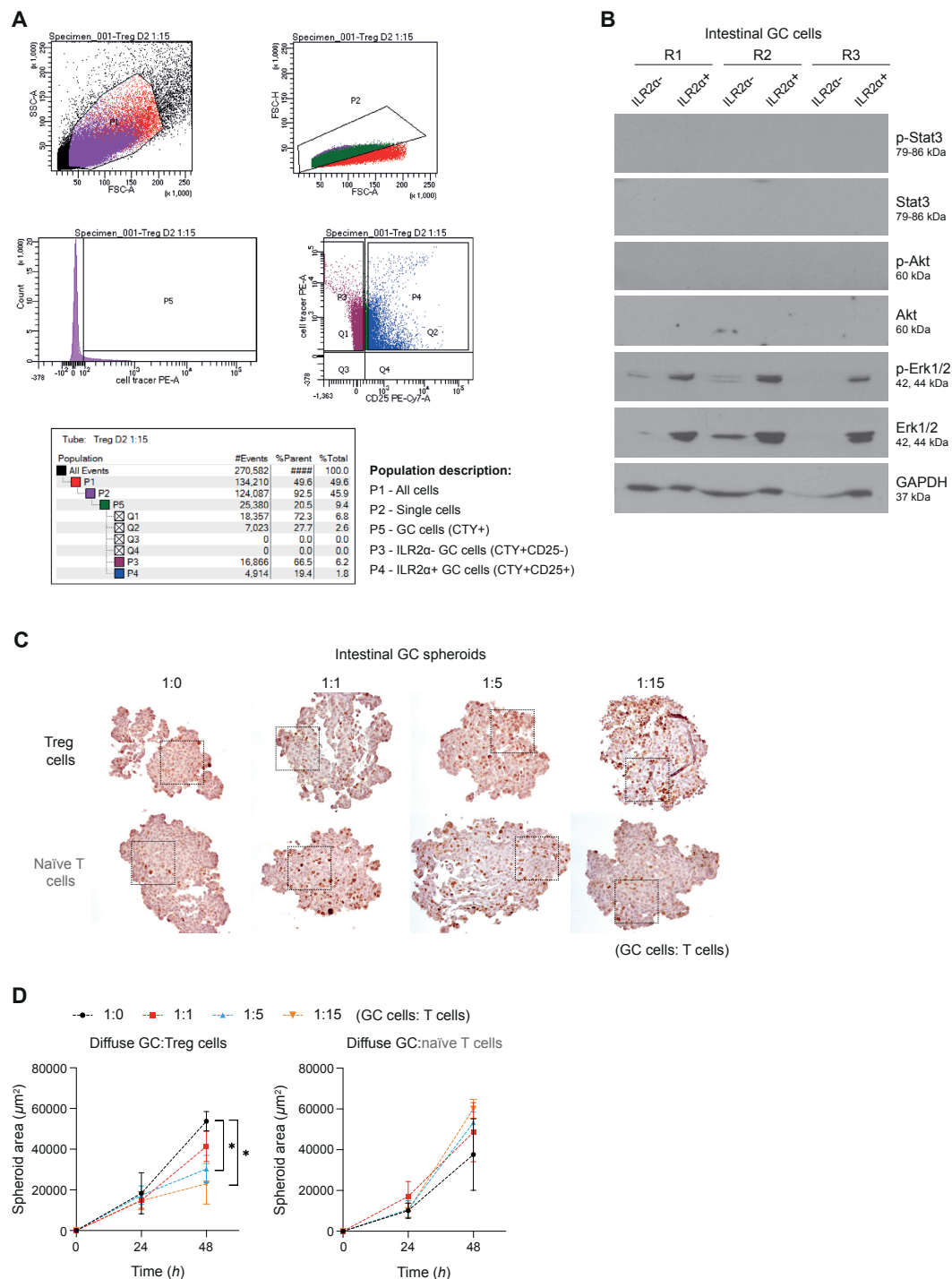
CTV staining of T cells at day 0

**B**

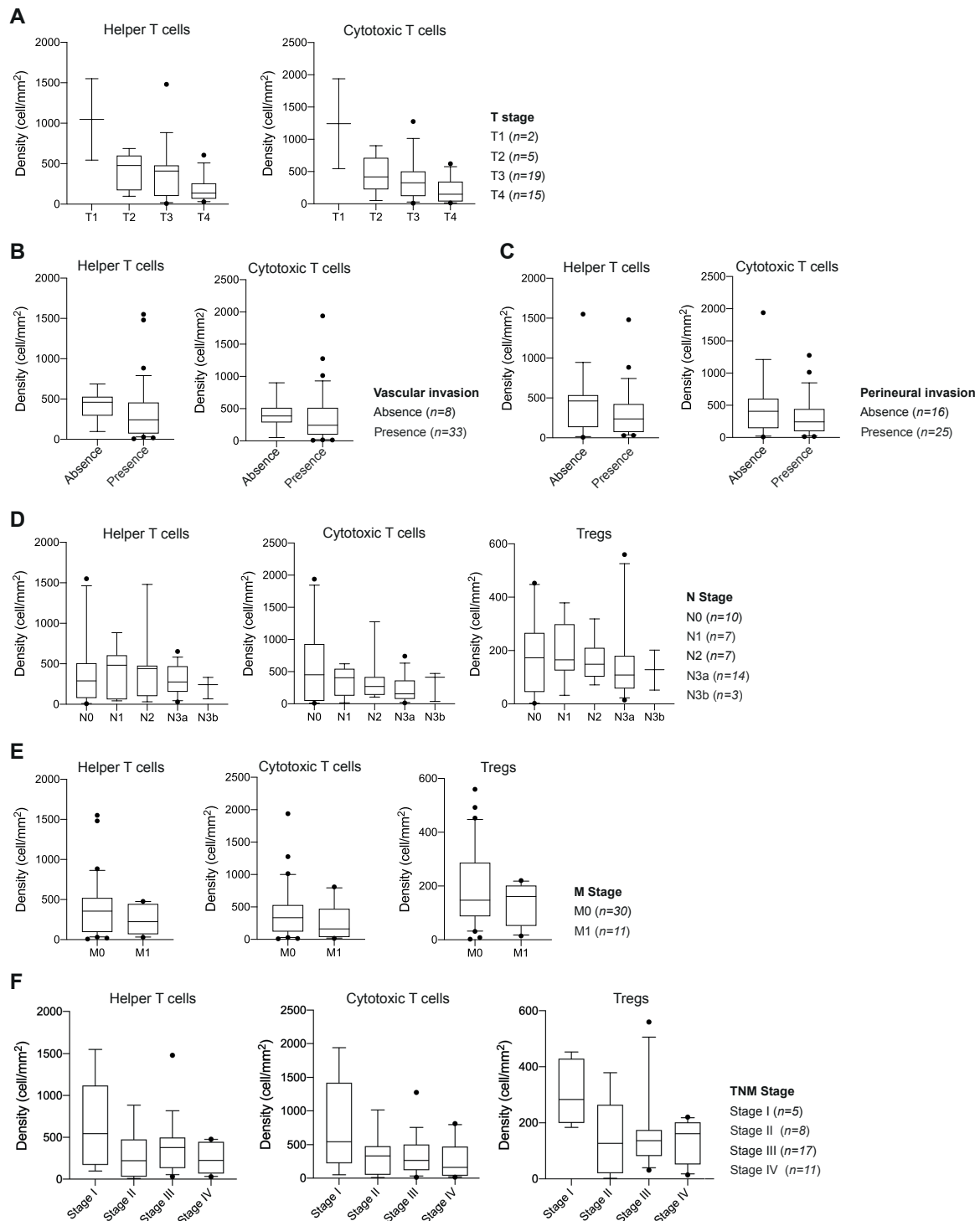
Supplementary Figure 4. Characterization of T cells before and after co-culture. (A) Detection of CTV on naïve T cells and Tregs before co-culture showing that all T cells were successfully stained. (B) Step-wise analysis of the flow cytometry data acquired after co-cultures. From the “All cells” gate, GC cells and T cells were separated according to their size (SSC-A) and intensity of CTV dye (which is only present in T cells) and analysed separately. Within each cell populations, gates on single and live (LD negative) cells were used to analyse the expression of CD3, CD4, IL2R α and FoxP3 markers in T cells and GC cells. In GC cells, IL2R α was the only marker with altered expression after co-culture.



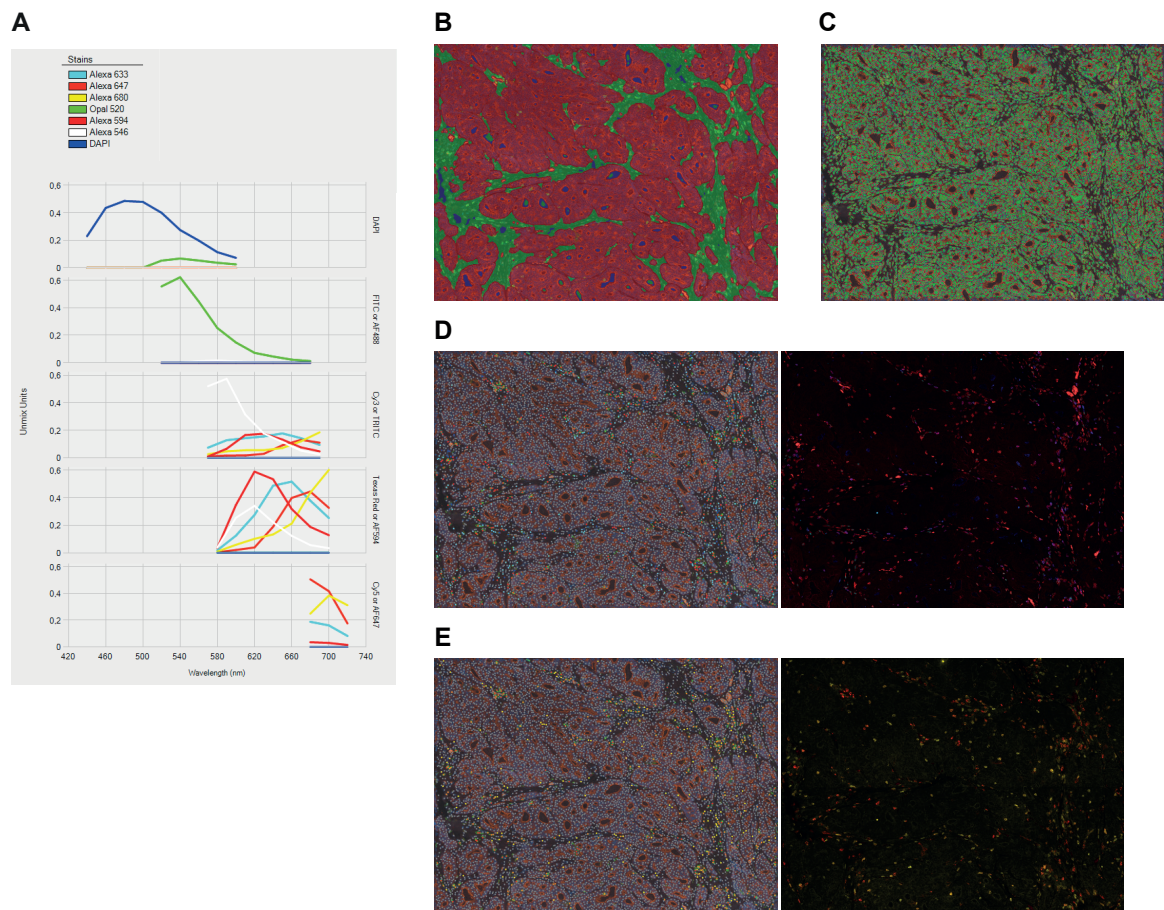
Supplementary Figure 5. Effect of IL2, anti-CD3/anti-CD28 beads, and co-culture conditioned medium in the expression of IL2R α in GC cells. (A) Histograms showing that IL2R α expression in intestinal-type GC cells is not altered by culture in IL2 only or IL2 with increasing concentrations of anti-CD3/anti-CD28 beads, the same concentrations used for the co-cultures with Treg cells. (C-D) Bar plots showing percentage of IL2R α positive intestinal-type GC (B) or diffuse-type GC (C) cells after 48 h co-culture with conditioned media collected from independent co-cultures with Tregs (dark grey) or naïve T cells (light grey). Black bars represent spheroids that were not exposed to conditioned media from co-cultures. Data shown as mean $\pm$ SD for co-cultures treated with T cells isolated from at least three healthy donors.



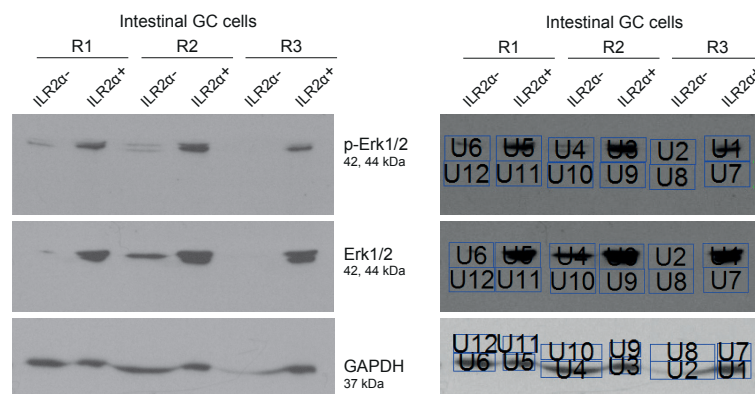
Supplementary Figure 6. GC cell phenotype after co-culture with T cells. (A) Gating strategy to analyse GC cells expressing IL2R α at cell membrane. Intestinal-type GC cells were gated on general events acquired (P1), and single cells (P2) are selected from this population. Then, Cell Tracer Yellow+ cells (only GC cells) were gated (P5) followed by the identification of IL2R α - (P3) and IL2R α + (P4) GC cells. (B) Western blot analysis for phosphorylated and total protein levels of Stat3, Akt and Erk1/2 in intestinal-type IL2R α - and IL2R α + GC cells after 48h co-culture with Tregs. Images show unprocessed scans for three biological replicates. (C) Whole spheroid image sections of Ki-67 nuclear expression in intestinal GC spheroids after 48 h of co-culture with Treg or naïve T cells, at different 1:0 (control), 1:1, 1:5 or 1:15 proportions. Selected region are shown by the dash boxes. (D) Plots of growth area for intestinal GC spheroids co-cultured with Treg (left graph) or naïve T cells (right graph) for 48 h. Control spheroids (dark) represent intestinal GC spheroids without T cells in co-culture. Co-cultures at 1:1, 1:5 and 1:15 proportions are represented in red, blue and orange respectively. Data shown as mean $\pm$ SD of three independent co-cultures. * p <0.05. Two-way ANOVA with Dunnett's multiple comparisons



Supplementary Figure 7. Distribution of T cell populations in intestinal-type GC patients and association with clinicopathological features. (A-C) Density of helper and cytotoxic T cells according to (A) T stage: T1, $n=2$; T2, $n=5$; T3, $n=19$; T4, $n=15$; (B) absence ($n=8$) or presence ($n=33$) of vascular invasion; and, (C) absence ($n=16$) or presence ($n=25$) of perineural invasion. (D-F) Density of helper, cytotoxic T cells and Tregs according to (D) N stage: N0, $n=10$; N1, $n=7$; N2, $n=7$; N3a, $n=14$; N3b, $n=3$; (E) M stage: M0, $n=30$; M1, $n=11$; and, (F) TNM stage: Stage I, $n=5$; Stage II, $n=8$; Stage III, $n=17$; and, Stage IV, $n=11$. Box and whiskers represent median $\pm$ 10–90 percentile.



Supplementary Figure 8. Step-wise multispectral imaging analysis of the immune T cell landscape in GC tissue sections. (A) Spectral library used for dye separation and image preparation at InForm cell analysis software. (B-E) Images illustrating the consecutive steps for image analysis in InForm software. (B) Tissue segmentation into tumour nest (red), and stroma (green). Areas without nuclei detection are represented in blue. (C) Cell segmentation based on nuclei and membrane staining. (D-E) Phenotyping and automatic cell count. In a first analysis cell phenotype was based on CD3, CD8 and FoxP3 expression (D) and in a second analysis, based on CD103 and PD1 expression (E).



Supplementary Figure 9. Western blot quantification for phosphorylated (p-ERK1/2) and total ERK1/2 protein levels in intestinal-type IL2R α ⁻ and IL2R α ⁺ GC cells after 48 h co-culture with Tregs. Images showing unprocessed scans for three biological replicates (left panel) and band quantification strategy (right panel).

Supplementary Tables

Supplementary Table 1. Description of patient information included in the analysed GC cohort

Patient gender	
Female	37 (45%)
Male	45 (55%)
Age of onset	
Mean	70
Standard deviation	12
Histological type (Laurén Classification)	
Intestinal	41 (50%)
Diffuse	15 (18%)
Mixed	11 (13%)
Indeterminate	15 (18%)
TNM Staging	
I	8 (10%)
II	13 (16%)
III	42 (51%)
IV	19 (23%)
pT stage	
T1b	3 (4%)
T2	8 (10%)
T3	36 (44%)
T4a	31 (38%)
T4b	4 (5%)
N Stage	
N0	16 (20%)
N1	11 (13%)
N2	16 (20%)
N3a	24 (29%)
N3b	15 (18%)
M stage	
M0	63 (77%)
M1	19 (23%)
Lymphatic Invasion	
Absent	8 (10%)
Present	74 (90%)
Vascular Invasion	
Absent	18 (22%)
Present	64 (78%)
Perineural Invasion	
Absent	26 (32%)
Present	56 (68%)
Microsatellite Instability	
MSS	57 (70%)
MSI	25 (30%)

Supplementary Table 2. Detailed description of antibodies

Application	Target	Clone	Dilution	Species	Isotype	Fluorochrome	Supplier	Detection method
<i>Immunofluorescence</i>	PD1	D4W2J	1:2000	Rabbit	IgG	Opal 520	Cell Signaling Technology	Opal detection
	FoxP3	236A/E7	1:25	Mouse	IgG1	CF633	Thermo Fisher Scientific	Indirect detection
	CD8	4B11	1:50	Mouse	IgG2b	AF647	Leica biosystems	Indirect detection
	CD103	EPR4166 2	1:100	Rabbit	IgG	AF680	Abcam	Indirect detection
	CD3	EP447E	1:50	Rabbit	IgG	AF594	Abcam	Conjugated antibodies
<i>Flow Cytometry</i>	Cytokeratin	AE1/AE3 + C11	1:50	Mouse	IgG1	AF546	Thermo Fisher Scientific and Cell Signaling Technology	Conjugated antibodies
	CD3	OKT3	1:400	Mouse	IgG2a	PE	Thermo Fisher Scientific	Conjugated antibodies
	CD4	OKT4	1:400	Mouse	IgG2b	FITC	Thermo Fisher Scientific	Conjugated antibodies
	CD127	eBioRDR5	1:50	Mouse	IgG1	PE/Cy5	Thermo Fisher Scientific	Conjugated antibodies
	CD25	BC96	1:25	Mouse	IgG1	PE/Cy7	Thermo Fisher Scientific	Conjugated antibodies
<i>Western Blotting</i>	FoxP3	PCH101	1:15	Rat	IgG2a	APC	Thermo Fisher Scientific	Conjugated antibodies
	phospho-Stat3	Tyr705, D3A7	1:1000	Rabbit	IgG	NA	Cell Signaling Technology	Indirect detection
	Stat3	124H6	1:1000	Mouse	IgG2a	NA	Cell Signaling Technology	Indirect detection
	phospho-Akt	Ser473, D9E	1:1000	Rabbit	IgG	NA	Cell Signaling Technology	Indirect detection
	Akt	NA	1:1000	Rabbit	NA	NA	Cell Signaling Technology	Indirect detection
	phospho-p44/42 MAPK	Thr202/Tyr204	1:1000	Rabbit	NA	NA	Cell Signaling Technology	Indirect detection
	p44/42 MAPK	NA	1:1000	Rabbit	NA	NA	Cell Signaling Technology	Indirect detection
	GAPDH	1E6D9	1:5000	Mouse	IgG2b	NA	Protein Tech	Indirect detection

Concluding Remarks

Recent advances on GC research nourished hopes of using complex 3D models as nuclear tools to improve the predictive value of *in vitro* findings. This thesis enclosed a bottom-up strategy to deconstruct GC into three essential elements: the 3D tumoral architecture; the intercellular communication mediated by EVs; and, the influence of Tregs, a prevalent cellular population in GCs. Through the individual analysis of these elements, this thesis enlightened crucial, albeit poorly understood, aspects of GC biology that may hold relevant clinical implications.

The discovery of glypican-1 as an EV-associated biomarker that detects early pancreatic cancer¹ has renewed the expectations on the game changing role of EVs in the early diagnosis of other cancers. Nevertheless, potential biomarkers identified for the early diagnosis of GC patients are scarce and require further validation.^{2,3} Hence, understanding the role of EVs in GC microenvironment, and the intrinsic and external factors modulating their cargo, may be of utmost importance to improve the clinical value of putative GC biomarkers. Data gathered in this thesis demonstrated that both the histological properties and the 3D cellular architecture impact the cargo and functional effect of EVs released by GC cells. To accomplish this, we developed a 3D model that could better recapitulate tumour morphology and organization. Importantly, this model was required to provide a feasible scalability to ensure enough EV-derived material for downstream applications. Notably, the established model not only provided a better representation of the tumour properties, as it proved to be even more efficient in producing EVs, as compared to traditional monolayers. This observation was validated by other studies, which reinforced that 3D cultures provide high yield EV production, associated with a more *in vivo* like phenotype.^{4,5} Moreover, when evaluating the metastatic potential of GC cells isolated from spheroids or monolayers, we observed distinctive metastatic burden and dissemination patterns. Of notice, 3D diffuse-type GC cells had an EV-mediated tropism towards brown adipocytes, which was not observed for the intestinal counterparts. Findings resulting from these experiments may provide crucial insights regarding the metastatic requirements of intestinal-type and diffuse-type GCs, as well as highlight a role for adipocytes in the dissemination of diffuse GCs, as previously shown in ovarian cancer.⁶ To further address this issue, we are currently analysing the small RNA and proteomic profiles of cells and EVs, as well as the metastatic lesions resulting from their dissemination. Putative biomarkers emerging from this analysis will be validated in EVs isolated from plasma of animals included in the *in vivo* metastatic assay, and from plasma of GC patients.

The results presented in this thesis indicate that, from the biological standpoint, EVs may have distinct roles in GC, thus fulfilling the distinct requirements of intestinal-type and diffuse-type GC biology and progression. From a clinical perspective, this minimal investigation may hold clinical implications for the differential diagnosis, follow-up or prognosis of GC patients with distinct tumour histological features.

Alongside, this thesis aimed at unveiling the contribution of external factors in the modulation of GC cell phenotypes and, consequently, on the communication mediated through EVs. Indeed, each component of the GC microenvironment could be held accountable for such modulation. However, given the importance of immune cells in the characterization of GC,⁷ and the poorly understood role of Tregs in GC biology, we decided to focus our study on these immune regulatory cells. The immunophenotyping of gastric tumours revealed a differential prevalence of Tregs in GCs with distinct histological properties, which was unique for this immune population. These results guided the hypothesis of a distinct role of Tregs in intestinal and diffuse-type GCs, which was further validated using the previously established 3D *in vitro* model. These direct co-cultures provided a unique view on the interactions between Tregs and GC cells, which led to the identification of a novel mechanism of tumour growth induced by Tregs. As so, this thesis also demonstrates that Tregs induce *de novo* expression of ILR2 α in GC cells, which was associated with enhanced MAPK activity and spheroid growth. These preliminary, albeit exciting, results require further validation in tumour samples, and a deeper analysis on the molecular mechanisms involved in ILR2 α expression in GC cells. If confirmed, this set of findings may spark new therapeutic strategies in GC; namely, the specific blocking of ILR2 α downstream effectors in epithelial GC cells. Notably, as previously demonstrated in other cancer models,^{8,9} our study evidenced a role of Tregs in tumour progression that was not related with the well-described effect on immunosuppression.

Overall, this dataset demonstrated that Tregs modulate the phenotype of GC cells, which is likely to alter the cargo of GC-EVs. Thus, the main question left unaddressed in this thesis was whether and how this interaction between Tregs and GC cells affects the signals conveyed by GC-EVs.

The beauty of science is that for each 'known unknown' that becomes a 'known known' several 'unknown unknowns' become 'known unknowns'. Thus, because of this thesis...

...we now know that:

- The message conveyed by GC-EVs has distinct functional impacts in recipient cells and is dependent on tumour histological properties.
- 3D cellular architecture induces upregulation of microRNAs, and downregulation of proteins in 3D EVs, leading to distinct functional effects on non-malignant recipient cells.
- 3D cellular architecture provides important clues for the *in vivo* dissemination of GC cells, particularly of the diffuse-type GC.
- EVs secreted by 3D cultures, potentiate the metastatic burden and tropism of diffuse-type GC cells towards brown adipocyte depots.
- EVs produced by 3D cultures of diffuse-type GC cells are enriched in microRNAs involved in adipocyte differentiation, conversion of white to brown adipocytes, and activation of brown adipocytes.
- Intestinal-type GCs are enriched in Tregs, as compared to diffuse-type GCs.
- Tregs induce de novo expression of IL2R α in intestinal-type GC cells, which associates with activation of MAPK signalling pathway and spheroid growth.

...we now know that we do not know:

- Whether the mechanisms involved in the biogenesis of EVs are dependent on the 3D cellular architecture.
- Why diffuse-type GC cells have a tropism to brown adipocytes, and how EVs mediate this dissemination.
- How, and in which circumstances, GC cells gain expression of ILR2 α .
- If other epithelial tumour cells may also express ILR2 α .
- If GC cells expressing ILR2 α are responsive to IL2 stimuli.
- What other consequences arise from ILR2 α expression in GC cells.

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