



HAL
open science

Investigating the function of macrophages in the awakening of dormant breast cancer cells

Kitti Jenovai

► **To cite this version:**

Kitti Jenovai. Investigating the function of macrophages in the awakening of dormant breast cancer cells. Molecular biology. Université Côte d'Azur, 2022. English. NNT : 2022COAZ6026 . tel-04043141

HAL Id: tel-04043141

<https://theses.hal.science/tel-04043141>

Submitted on 23 Mar 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

THÈSE DE DOCTORAT

Etude du rôle des macrophages dans le réveil des cellules tumorales mammaires dormantes

Kitti JENOVAI

Institut de Recherche sur le Cancer et le Vieillessement, Nice
CNRS UMR7284 / INSERM U1081 / UCA

Présentée en vue de l'obtention du
grade de docteur en
Sciences de la Vie et de la Santé
d'Université Côte d'Azur

Dirigée par : Dr. Cédric GAGGIOLI

Soutenue le 15 décembre 2022

Devant le jury, composé de :

Dr. Daniel Aberdam, Président du jury, Université Paris Cité

Dr. Veronique Maguer-Satta, Rapportrice, Université Claude Bernard Lyon

Dr. Mehdi Khaled, Rapporteur, Université Paris-Saclay

Dr. Carole Berruyer, Examinatrice, Université d'Aix-Marseille

Dr. Stoyan Ivanov, Examineur, Université Côte d'Azur

Dr. Cédric Gaggioli, Directeur de thèse, Université Côte d'Azur

Résumé

Etude du rôle des macrophages dans le réveil des cellules tumorales mammaires dormantes

Le cancer du sein est la tumeur maligne la plus fréquemment diagnostiquée chez les femmes dans le monde. En fonction du stade et du grade, les protocoles de gestions thérapeutiques du cancer du sein sont généralement efficaces, cependant, certaines cellules cancéreuses parviennent à échapper à ces tentatives et entrent en dormance. Les cellules cancéreuses dormantes sont des cellules viables et non prolifératives qui peuvent conserver ces propriétés pendant plusieurs années, voire des décennies, alors que le patient est considéré comme cliniquement guéri. Le réveil des cellules tumorales dormantes est un réel problème de santé, puisque les patientes présentant des récives ont un mauvais pronostic de survie. Dans le cancer du sein, tout comme lors du développement métastatique de ces mêmes tumeurs, les cellules cancéreuses évoluent au milieu d'un stroma dit « réactif », contenant de nombreux types cellulaires ainsi que des composants de la matrice extracellulaire (MEC). De nombreuses évidences expérimentales suggèrent que ces facteurs micro-environnementaux initient la réactivation des cellules cancéreuses dormantes, conduisant à une rechute du patient. Les macrophages sont l'un des principaux types de cellules favorisant le développement des tumeurs primaires et métastatiques dans le microenvironnement tumoral. Bien que les macrophages puissent favoriser le développement du cancer par divers mécanismes, leur implication dans l'échappement à la dormance n'a encore jamais été démontrée.

En utilisant la lignée cellulaire D2.OR - un modèle de dormance à la fois *in vitro* et *in vivo* - mes travaux expérimentaux démontrent pour la première fois que les interactions fonctionnelles entre les cellules cancéreuses dormantes et les macrophages entraînent le réveil des cellules cancéreuses. J'ai découvert que les macrophages entraînent le réveil des cellules tumorales dormantes via la sécrétion de facteurs paracrines et le remodelage de la MEC. En effet, mes travaux de thèse montrent que le remodelage de la MEC par un milieu conditionné dérivé des macrophages est suffisant pour induire le réveil des cellules dormantes, ce qui indique que des modifications de la MEC par les macrophages sont responsable du réveil des cellules tumorales. De plus, je démontre que les activités catalytiques des enzymes MMP9 et LOX sont essentielles dans ce processus.

Afin de mieux caractériser le rôle des macrophages dans le réveil des cellules cancéreuses, j'ai également cherché à savoir quelles sont les voies de signalisation activées dans les cellules D2.OR et qui sont responsables du réveil. Mes résultats montrent que le remodelage de la matrice par les macrophages entraînent la voie de signalisation intégrine- β 1/FAK/Src/Her2 dans les cellules tumorales, favorisant ainsi leur réveil. Cela aboutit à l'activation de la kinase ERK, permettant ainsi la transition de la dormance à un état prolifératif. Comprendre les mécanismes moléculaires qui régulent l'influence de l'inflammation sur le réveil des cellules tumorales dormantes est essentiel pour découvrir de nouvelles cibles thérapeutiques potentielles pour le développement de thérapies ciblées afin de prévenir la récive des patients.

Mots clés : Cancer du Sein, Microenvironnement, Dormance, Macrophages, Matrice extracellulaire, Signalisation

Abstract

Investigating the function of macrophages in the awakening of dormant breast cancer cells

Breast cancer is the most frequently diagnosed malignant tumor in women worldwide. Breast cancer therapeutic management protocols are usually considered to be successful, however, relapses are frequent as some cancer cells can manage to escape from these attempts by undergoing dormancy. Dormant cancer cells are viable, non-proliferative cells that preserve their status for years or even decades while the patient is considered as clinically free of cancer. However, when these cells awaken, patient relapse occurs with poor survival outcome. In breast carcinoma, cancer cells co-evolve with a reactive stroma containing extracellular matrix (ECM) components and cancer-promoting cellular elements. Evidence suggests that these microenvironmental factors can initiate the awakening of the dormant cancer cells, which can eventually lead to patient relapse. Macrophages are one of major tumor-promoting cell type within the tumor microenvironment. Although macrophages can support cancer development via various mechanisms, their direct involvement in dormancy escape has never been demonstrated.

Using the D2.OR cell line – a well-established model for cancer dormancy both *in vitro* and *in vivo* – we demonstrated for the first time that functional interactions between dormant cancer cells and macrophages result in cancer cell awakening. We found that macrophages mediate dormancy escape via the secretion of paracrine factors. Furthermore, we showed that priming the extracellular matrix with macrophage-derived conditioned media is sufficient to induce the awakening of dormant cells, indicating that macrophages mediate dormancy escape through ECM modifications. Moreover, we discovered that MMP9 and LOX enzyme activities have essential functions in this process.

Biochemistry and cellular biology analysis of the D2.OR intracellular signalling pathways revealed that signals from the ECM, following macrophage-mediated ECM remodelling, induce an integrin- β 1/FAK/Src axis that triggers Her2 receptor tyrosine kinase activation in a ligand-independent manner. This culminates to an ERK/MAP Kinase activation, thus promoting the transition from dormancy to a proliferative status.

Understanding the molecular mechanisms that regulate dormancy or the switch to a proliferative state is critical for discovering novel targets for the development of targeted therapies to prevent patient recurrence.

Key words: Breast cancer, Microenvironment, Dormancy, Macrophages, Extracellular matrix, Signalling

Acknowledgements

First, I would like to thank all the jury members for taking their time to participate in my doctoral defense. Specially, the Reporters, **Dr. Veronique Maguer-Satta** and **Dr. Mehdi Khaled** for carefully reading and judging my work. Along with them, I would like to thank the Examinators of this jury, **Dr. Carole Berruyer** and **Dr. Stoyan Ivanov** and the President of this jury, **Dr. Daniel Aberdam** for kindly accepting to be part of my PhD defense.

Cédric, I am really grateful, that I had the opportunity to join your team for my PhD. It was a great experience and I learned a lot in these 3 years. I would like to thank you for your scientific advises and it was a pleasure to perform every “you know what would be a funny experiment ” idea. ☺

I am also grateful for the members of my thesis follow-up committee: **Dr. Sophie Tartare-Deckert** and **Dr. Eva Faurobert** for their interest in my work and for their advises.

I would like to say thank you for all the current and previous members of the **TSI team**.

Jean, I am happy that I had the opportunity to work in the same team as you. I am grateful for all your scientific advises. It was amazing to see how you do science (in every sense). I still don't understand how you can see through the mess that sometimes surrounds you and still have the clearest and best ideas ever. I guess it's fair to say: “It's unbelievable but believable at the same time”.

Enora, it is hard to put into words how grateful I am for all the help that you gave me. Without you, I might still be filling out the paperwork to enrol in the École Doctorale. Thank you for all the phone calls to the bank, all the “Can I have a stupid question?”, all the days spent together in the TC trying to master transferring spheroids to some horrible Spotify playlist. I will always look back at our days together in the lab with a smile on my face. I'm happy that I could work together with a friend. ☺

Chloe, you are a wonderful person with a great heart. I will never forget your laugh, which sometimes I could hear even from the other side of the floor. I loved sharing the office with you.

Alexandra, I wish you all the best for finishing your PhD and after, the sky is the limit. ☺ I'm happy that I get to know you and thanks for being my constant lunch buddy (at noon, sharp).

Isa, thank you for being there for this group and organising, ordering, and keeping everything in your hands. I am glad that over time we have developed our common language, literally. ☺

Pascal, thanks for your advises and for teaching me several techniques in the lab. It was a pleasure to see you arrive every morning with a huge smile on your face and asking me: “What’s up girl?”.

JP, I loved that you were even more strict on the lunch rules than me. I will always try to wear red on Tuesdays.

Julia, Bruno. Working in this building allowed me to meet plenty of decent people. And you guys... are among the best ones. Thanks for all the fun we had together.

Victor, the bioinformatician specialised in Table of Contents. Thank you for always being there whenever I needed to complain about even the tiniest things and special thanks for helping me re-initiate TGIF.

TGIF committee (Sol, Martin, Aurelien): Thank you all for keeping this tradition alive. I made a lot of friends during these events. Hopefully it will continue and help others as well. Also, TGIF wouldn’t be the same without some of the regular visitors: **Dominic, Simona, Sabrina, Eirini, Roser, Noura** and **Clément** thanks for passing by my office and ask if I’m ok. I liked working on the same floor with you.

I would like to say thank you for all the members of the Institute. I’m happy to get to know you, especially: **Marco, David, Audrey, Julie, Iryna, Rita, Naz, Miguel, Nicolas, Mounir, Ludo**.

I would also like to mention my friends outside the lab and previous colleagues who always listened with interest as I told them about my lab work: **Lilla, Dia, Kitti, Sára, Denise, Évi, Sophie, Angi, Lili, Móni, Nóri**.

Apa, Anya, soha nem leszek képes meghálálni azt a rengeteg segítséget, törődést, szeretetet és bizalmat amit tőletek kaptam, de mindig igyekezni fogok. Szeretlek titeket.

Köszönet illeti a családom további tagjait is, **Gábort, Balázst, Evelint, Nórit, Zsoltit** és **Zsozsót**, amiért a háttérből támogattak.

Dávid, a legnagyobb köszönet azonban téged illet, nem csak azért mert elviseltél ebben az időszakban, de azért a rengeteg segítségért is, amit tőled kaptam. Boldog vagyok, hogy a hátralévő életünket együtt élhetjük le. Nekünk a legjobb. ☺

Table of contents

Résumé	2
Abstract	4
Acknowledgements	6
List of abbreviations	12
Introduction	16
1. Breast cancer.....	18
1.1. Epidemiology.....	18
1.2. Risk factors	20
1.2.1. Non-Modifiable Factors.....	20
1.2.1.1. Aging.....	20
1.2.1.2. Family history	20
1.2.1.3. Genetic mutations	21
1.2.1.4. Reproductive factors	21
1.2.1.5. Estrogen	21
1.2.2. Modifiable risk factors.....	22
1.3. Prevention and screening	24
1.4. Breast cancer classification.....	24
1.4.1. Luminal Breast Cancer	26
1.4.2. HER2-Enriched Breast Cancer	27
1.4.3. Basal-Like/Triple-Negative Breast Cancer.....	27
1.4.4. Claudin-Low Breast Cancer.....	28
1.4.5. American Joint Committee on Cancer Classification.....	28
1.5. Breast cancer treatment strategies.....	30
1.5.1. Surgery	30
1.5.2. Chemotherapy	30
1.5.3. Radiation Therapy.....	31
1.5.4. Endocrinal Therapy.....	31
2. Metastasis.....	33
2.1. Cell fate of the disseminated cancer cells	35
3. Dormancy.....	37
3.1. Genetic and epigenetic differences influencing dormancy	39
3.2. Micrometastasis dormancy	41

3.2.1. Angiogenic-dormancy.....	41
3.2.2. Immune-mediated dormancy	43
3.3. Cellular dormancy.....	45
3.3.1. Microenvironmental regulation of cellular dormancy	48
3.3.1. 1. Extracellular matrix and dormancy.....	48
3.3.1. 2. Integrin-mediated awakening.....	48
3.3.1. 3. Discotin domain receptor 1-mediated awakening.....	51
3.3.1. 4. Wnt-mediated awakening	51
3.3.2. Inflammation and dormancy	54
3.3.3. Cellular factors in dormancy escape.....	56
3.3.3.1. Natural killer cells and dormancy	56
3.3.3.2. CD4+ and CD8+ T-cells and dormancy	56
3.3.3.3. Pericytes and dormancy	57
3.3.3.4. Fibroblasts and dormancy	57
3.3.3.5. Neutrophils and dormancy	58
3.3.3.6. Macrophages and dormancy	59
4. Functions of macrophages in the metastatic cascade.....	64
4.1. Different macrophage phenotypes	64
4.2. Macrophage functions in cancer cell invasion and intravasation	67
4.3. Macrophages in epithelial to mesenchymal transition of cancer cells.....	70
4.4. Macrophages at the pre-metastatic site	71
4.5. Macrophages in extravasation and early colonization of cancer cells.....	72
5. Interactions between macrophages and the extracellular matrix	76
5.1. Mechanisms of ECM remodelling.....	76
5.2. ECM-mediated macrophage infiltration in the tumor.....	79
5.3. Macrophages as shapers of the extracellular matrix	81
5.3.1. Matrix metalloproteases.....	81
5.3.2. Lysyl oxidases.....	82
5.3.3. Serine proteases	82
5.3.4. Cathepsins	83
5.3.5. Secretion of matrix components	84
Results	86
Objectives	88
Summary of the results	90

Manuscript	92
Discussion.....	140
Bibliography	158
Collaborations	182
1 st article:.....	184
2 nd article:.....	188

List of abbreviations

ADAM: A Disintegrin And Metalloproteinase
AJCC: American Joint Committee on Cancer Classification
APMA:4-Aminophenylmercuric acetate
APPs: Acute-phase proteins
BAPN: β -Aminopropionitrile
BCS: breast-conserving surgery
bFGF: basic fibroblast growth factor
BHLHB3: basic helix-loop-helix protein 3
BMDM: bone marrow-derived macrophage
BRCA1: breast cancer associated gene 1
BRCA2: breast cancer associated gene 2
BRIP1: BRCA1-interacting helicase
CAFs: Cancer-associated fibroblasts
CCL2: chemokine C-C-motif ligand 2
CCL5: chemokine C-C-motif ligand 5
CHEK2: Checkpoint Kinase 2
CSF1: colony-stimulating factor 1
CSF-1R: colony-stimulating factor 1 receptor
CL: Claudin-low
CM: conditioned media
CRP: C-reactive protein
CXCL-12: C-X-C Motif Chemokine Ligand 12
DDR1: discoidin domain receptor 1
DKK1: Dickkopf WNT signalling pathway inhibitor 1
DTCs: Disseminated cancer cells
ECM: extracellular matrix
EDPNs: Early DTC pre-metastatic niches
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor

EMT: epithelial to mesenchymal transition
EpCAM: epithelial cell adhesion molecule
ET-1: endothelin-1
ER: estrogen receptor
EVs: extracellular vesicles
FAK: focal adhesion kinase
FGF: fibroblast growth factor
FGF-2: fibroblast growth factor-2
FZD: Wnt receptor Frizzled-7
GM-CSF: granulocyte macrophage colony-stimulating factor
GPI: glycosyl-phosphatidylinositol
HER2: human epidermal growth factor receptor 2
HGF: hepatocyte growth factor
HNSCC: head and neck squamous cell carcinoma
HRP: hormone replacement therapy
HSPGs: heparan sulphate proteoglycans
IL-1: interleukin-1
IL-1 β : interleukin 1 β
IL-6: interleukin 6
IRF7: interferon regulatory factor 7
JAK2: Janus kinase 2
LOX: lysyl oxidase
LOXL1: Lysyl oxidase like 1
LOXL2: Lysyl oxidase like 2
LOXL3: Lysyl oxidase like 3
LOXL4: Lysyl oxidase like 4
Ly6G: locus of the lymphocyte antigen 6 complex
MAMs: metastasis-associated macrophages
M-CSF: macrophage colony-stimulating factor
MDSCs: myeloid-derived suppressor cells
MIF-1: macrophage migration inhibitory factor 1

MMP2: matrix metalloproteinase 2
MMP7: matrix metalloproteinase 7
MMP9: matrix metalloproteinase 9
NETs: neutrophil extracellular traps
NF κ B: nuclear factor-kappa B
NSAID: non-steroidal anti-inflammatory drug
NSCLC: non-small cell lung cancer
NR2F1: nuclear receptor subfamily 2 group F member 1
OSM: oncostatin M
PA: plasminogen activator
PALB2: partner and localizer of BRCA2
PDAC: pancreatic ductal adenocarcinoma
PDGF: platelet-derived growth factor
PKC α : protein kinase C α
PLOD: procollagen-lysine 2-oxoglutarate 5-dioxygenase
PR: progesterone receptor
PTMs: post-translational modifications
p38: p38 mitogen-activated protein kinase
RBC: Red blood cell
RONS: reactive oxygen and nitrogen species
ROR: Risk of Recurrence
SCID: severe combined immunodeficient
Sox 2: SRY-Box Transcription Factor 2
Sox 9: SRY-Box Transcription Factor 9
STAT3: Signal Transducer and Activator of Transcription 3
TAMs: tumor-associated macrophages
TF: transcription factor
TME: Tumor-microenvironment
TNF: Tumor necrosis factor
TM4SF1: tetraspanin transmembrane 4 L six family member 1
TRAIL: TNF-related apoptosis-inducing ligand

TSP: Thrombospondin

TNBC: Triple-Negative Breast Cancer

uPa: urokinase plasminogen activator

uPAR: urokinase plasminogen activator receptor

VCAM-1: vascular cell adhesion protein-1

VEGF: vascular endothelial growth factor

VEGFR2: VEGF receptor 2

WASP: Wiskott-Aldrich syndrome protein

WBC: White blood cell

WHO: World Health Organization

Introduction

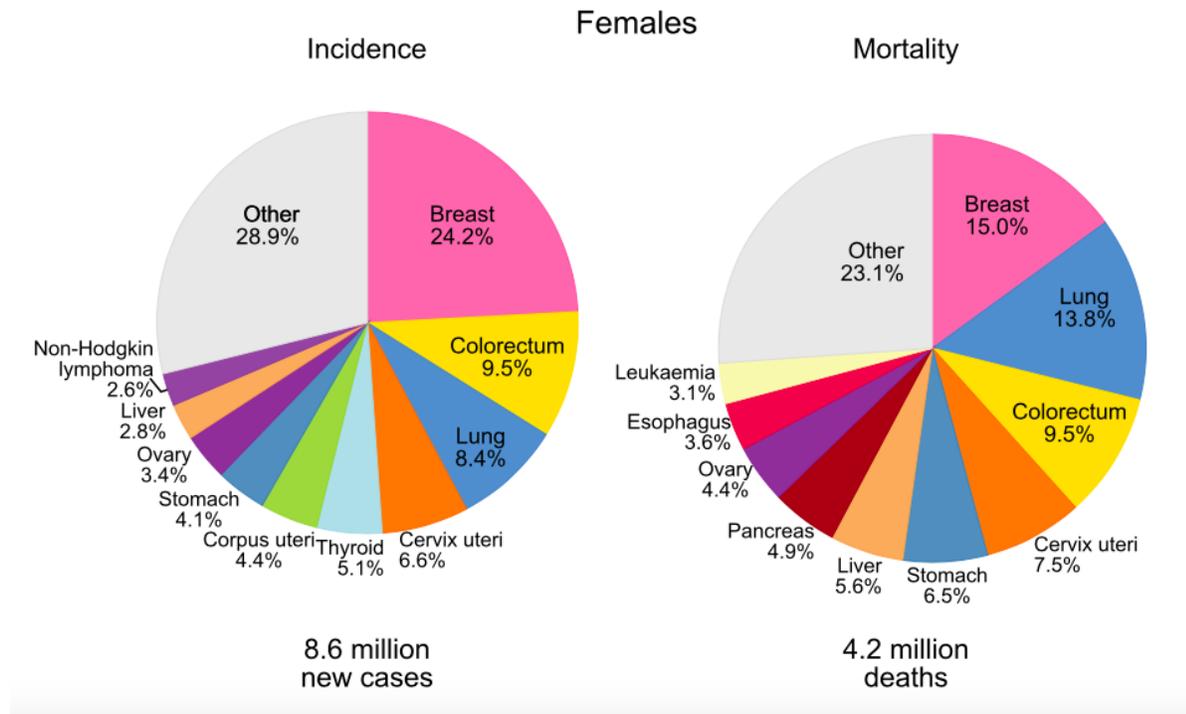


Figure 1. Distribution of incidence and mortality of the 10 most common cancers among women in 2018

The area of the pie chart reflects the proportion of the total number of cases or death.

Adapted from Globocan 2018 (Bray et al., 2018)

Breast cancer

Epidemiology

Breast cancer is the most frequently diagnosed cancer in women. The global incidence rate of breast cancer is increasing every year by ~3,1%. While in 1980 a total of 641,000 patients were diagnosed with breast cancer, this number increased to 1,6 million by 2010 (Bray et al., 2018). In 2018 ~2,1 million new breast cancer cases were diagnosed, whereby 626,679 women with breast cancer deceased.

Although lung cancer is the most commonly diagnosed cancer in both sexes combined, it is closely followed by breast cancer (Bray et al., 2018). Breast cancer is predominantly a female disease, as only 0,5 - 1% of all breast cancer is diagnosed in men (Ruddy & Winer, 2013). Based on a study in 2018, breast cancer accounts for the largest percentage of all cancers diagnosed in women (24,2 %), followed by colorectum (9,5 %) and lung cancer (8,4%). Additionally, breast cancer not only leads the incidence list, but also accounts for the largest proportion (15%) of cancer-related death among women (Bray et al., 2018) (Figure 1).

The incidence rates vary worldwide. Generally, more breast cancer cases are identified in high-income regions, compared to lower income regions, likely due to the well-developed health care systems and the availability of medical equipment. However, a higher percentage of cases diagnosed in high-income regions are identified at an early stage of breast cancer leading to increasingly improved survival outcomes (Torre et al., 2016).

Risk factors

Non-Modifiable Factors

Aging

The main risk factor for developing breast cancer is aging. About 50% of breast cancers are diagnosed between the age of 50 and 69 and about 28% are diagnosed after age 69. Therefore, about 80% of all breast cancer cases affect people over the age 50. Indeed, the 10-year probability to develop breast cancer is around ~1,5% at age 40, which increases to ~3% at age 50 and reaches about 4% at age 70, thus generating a cumulative lifetime risk of ~13,2%, meaning that 1 in 8 women are affected by this disease (Benz, 2008).

Even though aging affects each person differently, organs which are susceptible to develop cancer also undergo general age-related alterations. A fundamental question under continuous debate is whether these typical aging-related changes assist to the tumorigenic process. As an example, after the second decade of life, ovarian size and function gradually decrease in non-pregnant women and breast glandular mass progressively decreases and is replaced by a combination of collagenous stroma and adipose tissue (Milanese et al., 2006). Also, in the normal breast, estrogen receptor (ER) expression starts a progressive >3-fold rise beginning at early thirties which expression change plateaus by age 60. Contrarily, the average level of expression of estrogen-inducible proteins in the normal breast does not vary significantly with age (van Landeghem et al., 1985). Even though a growing body of evidence suggests a link between these aging-related changes and breast cancer development, the exact molecular mechanism needs to be further investigated.

Family history

Almost one-fourth of all breast cancer cases has a family history. Women, who has a first-degree relative (mother or sister) with breast cancer have a 1,75-fold increased risk to develop this disease, compared to women with no family history of breast cancer. Additionally, the risk of breast cancer raises significantly as the number of affected first-degree relatives increases and the risk may even be higher if the affected relatives are younger than 50 years old (Brewer et al., 2017).

Genetic mutations

Breast cancer-related gene mutations, like those in breast cancer associated gene 1 and 2 (BRCA1 and BRCA2) are partly responsible for the inherited susceptibility for the disease. Both genes encode tumor suppressor proteins which are involved in DNA damage repair. Deficiency of BRCA1 causes aberrant centrosome duplication, dysregulation of the cell cycle checkpoints, genetic instability and ultimately apoptosis (Deng, 2006). Whereas, BRCA2 is known to interact with RAD51 and DMC1 to regulate DNA double-strand break repair (J. S. Martinez et al., 2016; Sánchez et al., 2017). Both BRCA1 and BRCA2 mutations show an autosomal dominant pattern of inheritance. A meta-analysis revealed that the average cumulative risk of developing breast cancer by the age 70 was 57% or 49% for women carrying either BRCA1 or BRCA2 mutations, respectively (S. Chen & Parmigiani, 2007). Breast carcinogenesis has been also linked to a proportion of other DNA repair genes, such as ATM, Partner and localizer of BRCA2 (PALB2), BRCA1-interacting helicase (BRIP1) or Checkpoint Kinase 2 (CHEK2) (Meijers-Heijboer et al., 2002; N. Rahman et al., 2007; Renwick et al., 2006; Seal et al., 2006).

Reproductive factors

Reproductive factors can also affect the probability of breast cancer development. As an example, the length of the reproductive period (from first menarche until menopause) correlates with the risk of breast cancer development. Therefore, both early menarche and late menopause, as well as late age at first pregnancy are considered to elevate the risk for breast cancer. Based on studies, each year delay in menopause increases the risk by 3% to develop breast cancer, whereas, each year delay in menarche decreases the risk by 5% (Horn & Vatten, 2017).

Estrogen

Breast cancer risk is linked to both endogenous and exogenous estrogens. In premenopausal women, the endogenous estrogen is mainly produced by the ovary, therefore ovariectomy can lower the risk of breast cancer (Key, 2013).

The primary sources of exogenous estrogen are the oral contraceptive pills and the hormone replacement treatments (HRT). Since the 1960s, oral contraceptives have been extensively used and their formulations have been improved to minimize side effects, however the relative odds ratio is still greater than 1.5 (Veisy & Viga, 2018). Exogenous estrogen or other hormones are administered to menopausal or postmenopausal women as part of HRT. According to the Million Women Study, there is a 1.66 relative risk for those who use HRT compared to those who have never used it (“Breast Cancer and Hormone-Replacement Therapy in the Million Women Study,” 2003). Since these findings about the adverse effects of HRT were released in 2003, the incidence rate of breast cancer has reduced by about 7% due to the decreased use of HRT (Thods et al., 2006).

Modifiable risk factors

Around 20% of all breast cancer cases can be associated with modifiable risk factors, like obesity, increased dietary fat intake, the lack of physical activity or elevated alcohol consumption (Danaei et al., 2005).

It has been observed that alcohol consumption can increase the level of hormones associated to estrogen and subsequently stimulate the estrogen receptor pathways. According to a meta-analysis of 53 epidemiological studies, drinking 35 to 44 grams of alcohol per day can raise the risk of breast cancer by 32% (Hamajima et al., 2002).

Smoking also represents an increased risk factor for breast cancer. It has been shown that the tobacco-derived carcinogens can be delivered to breast tissue, thereby increasing the likelihood of oncogene and tumor suppressor gene mutations (Catsburg et al., 2015).

On the other hand, exercise was shown to decrease both cancer incidence and recurrence. Sheinboim and collaborators showed that exercise can trigger a metabolomic reprogramming with increased nutrient demand, which limits the available nutrient source for the tumor, thus prevents the formation of metastatic colonies (Sheinboim et al., 2022).

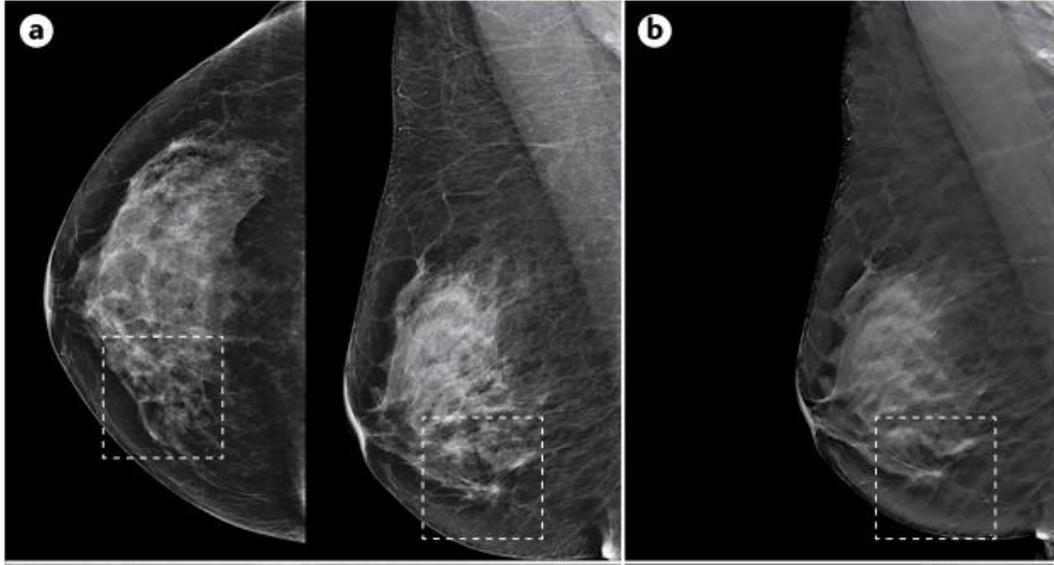


Figure 2. Breast cancer imaging via Mammography

Routine breast cancer screening identified a lesion of the right breast of 53-year-old postmenopausal women, who had no family history of breast cancer.

***a.** cranio-caudal view (left) and mediolateral oblique view (right)*

***b.** mediolateral view*

Adapted from (Harbeck et al., 2019)

Prevention and screening

Population screening for breast cancer with mammography is a secondary prevention method aiming to discover the malignancy at an early stage, hence effective treatment may be provided. Mammography is a non-invasive and accurate screening method which implies a low dose X-ray imaging of the breast (Figure 2). Randomized studies have collectively shown strong evidence that population screening considerably lowers breast cancer mortality by a relative risk of 20% for individuals who are subjected to routine screenings by mammography (Marmot et al., 2012).

It is widely recommended that women who have a higher than average risk of developing breast cancer (i.e. due to genetic mutations) undergo risk-tailored screening, which includes more regular controls or even the use of technologies other than mammography. For example, the suggested screening method for BRCA1 and/or BRCA2 mutation carriers and women at significantly elevated lifetime risk of breast cancer is MRI scanning in addition to standard mammography (Saslow et al., 2007).

When combined with mammography, new technologies such as tomosynthesis (3D mammography), contrast-enhanced mammography and gamma imaging show improved cancer detection rates, however, among these, tomosynthesis has the strongest body of evidence, making it the most promising candidate technic for breast cancer screening in the future (Marinovich et al., 2018).

Breast cancer classification

Breast cancers vary in terms of their clinical symptoms, behaviour and anatomy. Numerous classifications have been developed on the basis of histological and molecular characteristics. Based on histological characteristics, the World Health Organization (WHO) identifies at least 18 distinct breast cancer types (Hanby & Walker, 2004). However, this classification might be misleading as 40-80% of cases belongs to “invasive ductal carcinoma of no special type” (NST) , a category which contains tumors that cannot be classified to either histological subtype (Weigelt et al., 2008).

Breast cancer can be classified into molecular subtypes based on the levels of mRNA gene expression, regardless of histological subtypes. Perou and collaborators identified four molecular subtypes using microarray gene expression data on 38 breast cancers: Luminal, HER2-enriched, Basal-like, and Normal Breast-like (Perou et al., 2000). Additional research enabled the division of the Luminal group into two subgroups: Luminal A and B (Prat & Perou, 2011). Then, over 300 primary tumors were comprehensively profiled (at DNA, RNA, and protein levels) and grouped into biologically similar tumor types for The Cancer Genome Atlas Project (TCGA). Based on mRNA gene expression levels, a consensus classification had been established to differentiate four major intrinsic subtypes of breast cancer: Luminal A, Luminal B, HER2-enriched, and basal-like (Koboldt et al., 2012). Additionally, a combined investigation of human and mouse mammary cancers revealed the 5th intrinsic subtype—claudin-low breast cancer—in 2007 (Herschkovitz et al., 2007) (Figure 3A).

In order to detect intrinsic breast cancer subtypes and to calculate a Risk of Recurrence (ROR) score for breast cancers, a test called PAM50 which measures the expression of 50 selected genes was created. This test has 93 percent accuracy in reliably classifying specific breast cancers into the major intrinsic subtypes. Globally, PAM50 is increasingly being used in therapeutic settings (Gnant et al., 2014).

In addition to this, the human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), progesterone receptor (PR) and the proliferation marker Ki67 are the key proteins that are used in the clinical practice to differentiate the intrinsic subtypes (Figure 3B).

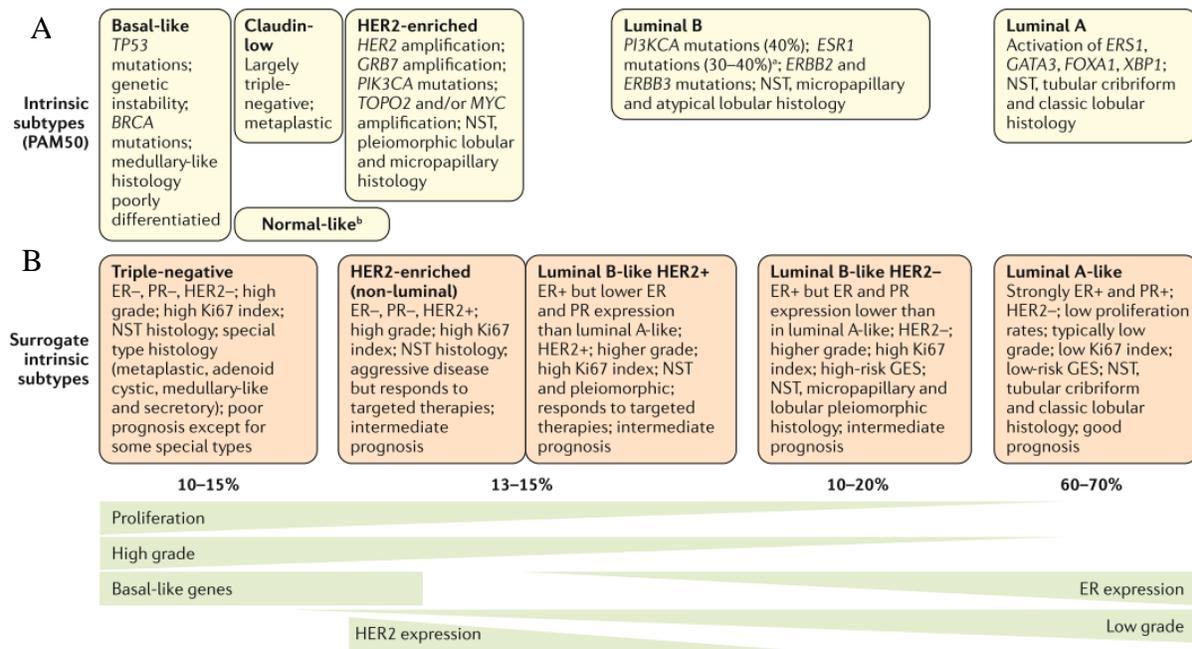


Figure 3. Breast cancer classifications

- Breast cancer intrinsic subtypes and main characteristics, based on a 50-gene expression signature (PAM50).*
- Breast cancer surrogate intrinsic subtypes and main characteristics, generally used in clinics, rely on histology and immunohistochemistry expression of characteristic proteins.*

Adapted from (Harbeck et al., 2019)

Luminal Breast Cancer

The majority (70%) of breast cancer cases in western populations are luminal breast cancers, which are ER-positive tumors (Howlander et al., 2014). Luminal-like tumors are classified into Luminal A and B subtypes, which have variable clinical outcomes. ER expression profile is used to differentiate between luminal and non-luminal breast cancers, however, it is expressed equally in both A and B subtypes. A and B subtypes can be further separated from each other based on proliferation-related markers (e.g., Ki67 index).

Luminal A tumors generally express estrogen receptor (ER) and/or progesterone receptor (PR), but do not express human epidermal growth factor receptor 2 (HER2). The ER-regulated transcription factors in this subtype activate genes whose expression is typical in the luminal epithelium which lines the mammary ducts (Weigelt, Geyer, et al., 2010). Luminal A cancers are low-grade, slow-growing tumors with low expression of proliferation-related genes. The clinical prognosis for Luminal A breast cancer is the most favorable (Eroles et al., 2012).

Luminal B tumors are higher grade and have a poorer prognosis compared to Luminal A tumors. Luminal B breast cancers are ER positive and might be PR negative and potentially HER2 positive. Furthermore, Luminal B tumors are also characterized by high expression of genes related to cell proliferation (e.g., MKI67 and AURKA) (Ades et al., 2014).

HER2-Enriched Breast Cancer

10-15% of breast cancers are HER2-enriched. It is distinguished by a high expression of HER2 and a lack of ER and PR. Instead of expressing luminal and basal gene and protein clusters, this subtype predominantly expresses genes and proteins associated to proliferation, such as ERBB2/HER2 and GRB7 (Danaei et al., 2005). HER2-enriched breast cancer grows more rapidly than the luminal tumors and prior to the development of HER2-targeted therapies, this subtype had the poorest prognosis. Important to note that HER2-enriched subtype is not equivalent with HER2-positive breast cancer, since many ER-positive/HER2-positive tumors are entitled as Luminal B group.

Basal-Like/Triple-Negative Breast Cancer

Breast tumors classified as ER-negative, PR-negative, and HER2-negative constitute the heterogeneous group known as Triple-Negative Breast Cancer (TNBC). They account for around 20% of all breast cancer cases. TNBC is more prevalent in women under 40 and in African-American women (Plasilova et al., 2016). TNBC make up the vast majority (about 80%) of breast cancers triggered by BRCA1 germline mutations, while 11–16% of all TNBC carry BRCA1 or BRCA2 germline mutations. TNBC has a biologically more aggressive behaviour than other cancer types and is frequently associated with the worst prognosis (Newman et al., 2015).

Although the labels “TNBC” and “basal-like” have been used indiscriminately, not all TNBC are basal type. TNBCs may be categorized into six subtypes based on gene expression profiling: basal-like (BL1 and BL2), luminal androgen receptor (LAR), mesenchymal stem-like (MSL), immunomodulatory (IM), mesenchymal (M), and unspecified group (UNS) (D. Y. Wang et al., 2019). However, further studies are required to fully understand the clinical implications of the subtyping of TNBC and how it affects therapy outcomes.

Claudin-Low Breast Cancer

7–14% of all invasive breast cancers are claudin-low (CL) breast cancers (Weigelt, Baehner, et al., 2010). Low expression of genes involved in cell-cell adhesion, such as claudin 3, 4, and 7, occludin, and E-cadherin defines the CL subtype. On the contrary, the epithelial-mesenchymal transition (EMT) genes and stem cell-like gene expression patterns are highly expressed in these malignancies (Prat et al., 2010). Claudin-low breast tumors characterized as mainly ER-negative, PR-negative and HER2-negative cancers with poor prognosis.

American Joint Committee on Cancer Classification

The American Joint Committee on Cancer (AJCC), has published an anatomy based staging approach which is widely accepted worldwide. It incorporates grading, immunohistochemistry biomarker and anatomical development analysis to estimate the likely prognosis of patients with breast cancer. Initially it relied on only anatomical features as tumor size (T), nodal status (N), and metastases (M), but the eighth edition of the AJCC staging manual (2018) describes a new prognostic staging method for breast cancer, that also takes into account molecular markers (ER, PR, HER2, grade, multigene assays) (Amin et al., 2017).

The Elston-Ellis modification of the Scarff-Bloom-Richardson grading system (Bloom & Richardson, 1957) is the most popular histological grading system for breast cancer (ELSTON & ELLIS, 1991). The morphologic characteristics of a tumor, including (a) tubule development, (b) mitotic count, (c) variability, and the size and shape of cellular nuclei are evaluated to establish the tumor grade. Each aspect is rated with a score ranging from 1 (most favorable) to 3 (least favorable). Compared to earlier staging that was solely based on anatomical characteristics of the

disease, the updated version of breast cancer staging by biological markers improved the outcome prediction (Weiss et al., 2018).

Breast cancer treatment strategies

Surgery

The removal of breast malignant tissues can be accomplished through either a mastectomy or a breast-conserving surgery (BCS), which are the two main surgical techniques for breast cancer management. BCS, also known as partial or segmental mastectomy, lumpectomy, wide local excision, or quadrantectomy enables the removal of the malignant tissue while simultaneously preserving the undamaged, healthy breast tissue. It is frequently used in combination with oncoplasty, a form of plastic surgery. Mastectomy includes the complete removal of the breast and is frequently followed by subsequent breast reconstruction. Despite the fact that BCS appears to be far more advantageous for patients, those who have undergone this procedure frequently display a tendency to eventually require a total mastectomy (Morrow et al., 2001). However, the main benefits of breast conserving surgery include improved cosmetic outcomes, a decreased psychological trauma and a lower incidence of postoperative problems (G. A. Rahman, 2011). The European Society for Medical Oncology (ESMO) provides guidelines for treating patients with early breast cancer that rely on the tumor size, surgical feasibility, clinical profile and the intention of the patient to retain the breast (Cardoso et al., 2019).

Chemotherapy

Chemotherapy is a systemic therapy for breast cancer that can be either neoadjuvant or adjuvant. While neoadjuvant treatment aims to decrease the size of large tumors to enable surgery, adjuvant therapy is typically applied after surgical excision of the tumor. Neoadjuvant chemotherapy can be administered intravenously or orally and is used to treat small tumors with poor prognostic molecular subtypes (such as HER2-enriched or TNBC), inflammatory and locally advanced breast cancers. Carboplatin, cyclophosphamide, 5-fluorouracil/capecitabine, taxanes (e.g. paclitaxel, docetaxel) and anthracyclines (e.g. doxorubicin, epirubicin) are being used in combination with one another as part of the treatment. Since distinct molecular breast cancer subtypes respond differently to preoperative chemotherapy, choosing the right medication is crucial (Rouzier et al., 2005). Despite the fact that chemotherapy is thought to be effective, it

frequently causes a number of side effects, such as hair loss, nausea/vomiting, diarrhea, mouth sores, fatigue, an increased risk of infection, bone marrow suppression combined with leucopenia, anemia and easier bruising or bleeding. Less frequently, but still potential side effects include cardiomyopathy, neuropathy, hand-foot syndrome and impaired mental functions. Menstrual cycle irregularities and reproductive difficulties may also emerge in younger women (Łukasiewicz et al., 2021).

Radiation Therapy

Breast cancer is often treated locally with radiotherapy following surgery and/or chemotherapy. It is done to ensure that all malignant cells are eliminated, thereby reducing the chance of a breast cancer recurrence. Additionally, radiation treatment is especially beneficial for breast cancer patients who have metastatic or inoperable disease as a palliative care (Jonathan Yang & Ho, 2013). The choice of radiation therapy depends on the type of previous surgery or specific clinical circumstances. The most common radiation approaches include breast radiotherapy (always used after breast cancer), chest-wall radiotherapy (often used after mastectomy), and "breast boost" (high-dose radiotherapy following breast conserving surgery). The most frequent side effects of radiation therapy are irritation and darkening of the exposed skin, fatigue and lymphoedema. However, radiation therapy is strongly associated with an increase in overall patient survival rates and a decreased chance of recurrence (Joshi et al., 2007).

Endocrinal Therapy

Patients with the Luminal-molecular subtype of breast cancer may receive hormonal therapy as either a neoadjuvant or adjuvant treatment. The blocking of ERs with hormone therapy is frequently employed as one of the prospective therapeutic methods since ER expression is a highly prevalent feature in breast cancer patients. Endocrinal treatment attempts either to reduce estrogen levels or to inhibit the ability of estrogen to stimulate breast cancer cells. Selective estrogen receptor modulators (SERMs) (e.g. tamoxifen, toremifene) and selective estrogen receptor degraders (SERDs) (e.g. fulvestrant) are treatment regimens that block ERs, whereas aromatase inhibitors (AIs) are therapies that attempt to reduce the level of estrogen (letrozole,

anastrozole, exemestane) (Tremont et al., 2017). Although over 50% of hormonoreceptor-positive breast cancers under such treatment develop gradually resistance to hormonal therapy, mortality rates are lower when chemotherapy is combined with endocrine therapy (Drăgănescu & Carmocan, 2017).

Metastasis

The metastasis formation is a complex multi-step cascade where cancer cells spread from the primary lesion to a distant organ. To metastasize, cancer cells must go through a specific sequence of events, including escape from the primary tumor, local invasion, followed by intravasation into the bloodstream, survival in the circulation and finally trans-endothelial migration into a new site, also referred as extravasation and metastatic seeding (Pantel & Brakenhoff, 2004; Valastyan & Weinberg, 2011) (Figure 4.).

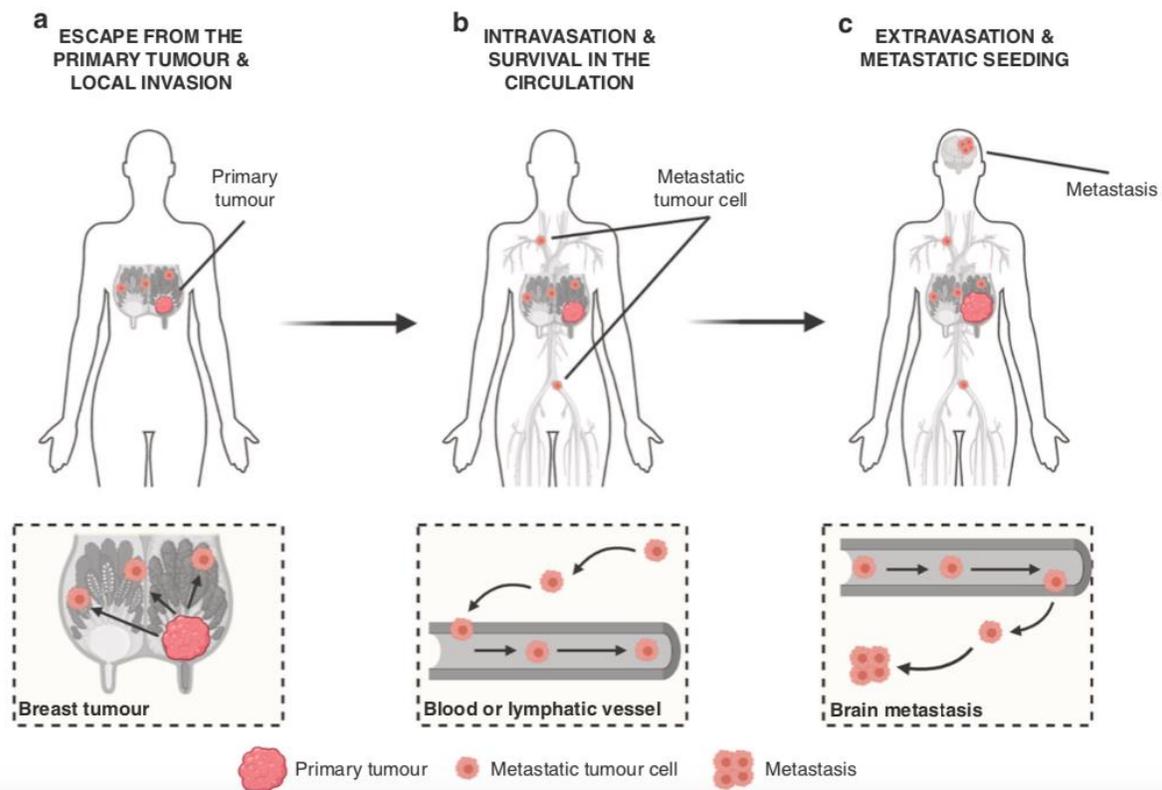


Figure 4. The multistep mechanism of breast cancer metastasis

- Some cancer cells in the primary breast tumor are escaping the tumor mass to locally invade nearby tissues.*
- Trans-endothelial migration of metastatic tumor cells into the bloodstream (intravasation) and survival in the circulation.*
- Trans-endothelial migration into a new site (extravasation) and colonization of a distant tissue. The brain is shown here as the most lethal metastatic location for breast cancer patients.*

Adapted from: (Riggio et al., 2021)

More than 90% of breast cancer-related fatalities are caused by metastasis, frequently as a result of the impairment of a crucial organ function (Chaffer & Weinberg, 2011). *De novo* metastatic disease can emerge when metastases are already present at the time of the initial diagnosis. However, metastases often appear only following the curative therapy as a form of relapse or recurrence (Seltzer et al., 2020). It has been reported that in addition to lymph nodes, breast cancer cells typically spread to the bones, lungs, liver and brain. Furthermore, clinical observations suggest that distinct types of breast cancer exhibit diverse organ tropisms (Lorusso & Rüegg, 2012) (Figure 5.)

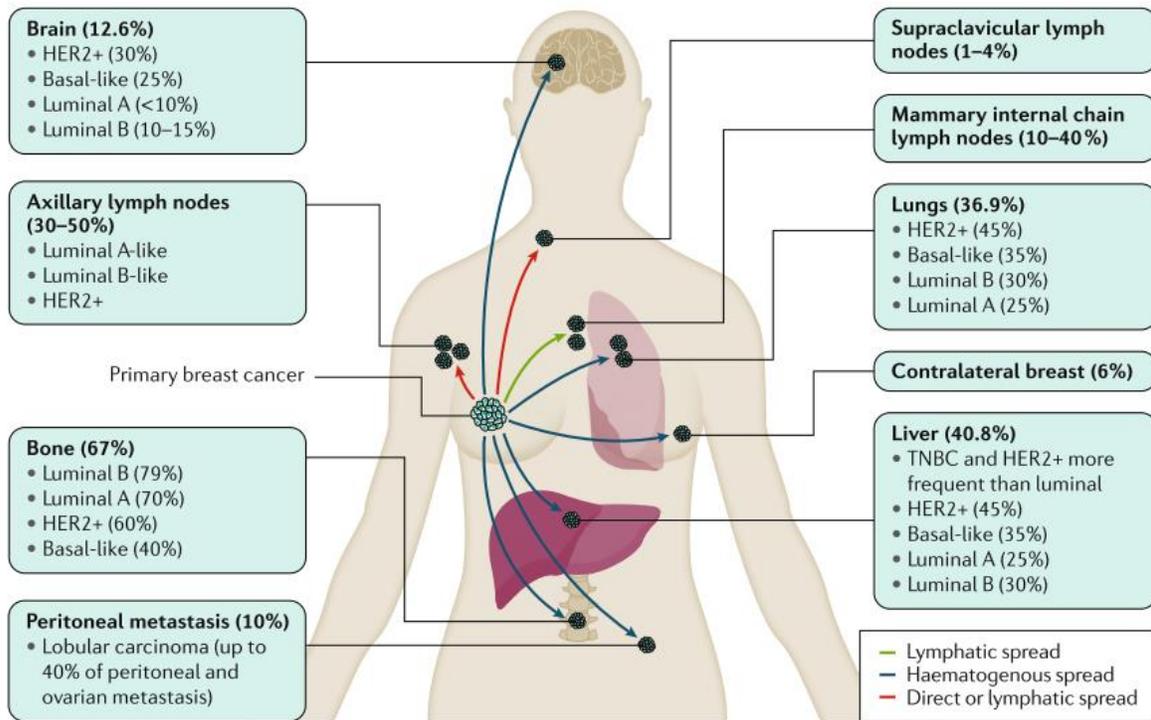


Figure 5. The most common breast cancer metastatic locations

Breast cancer cells metastasize mainly to the bone (67%), liver (40,8%), lungs (36,9%), the axillary lymph nodes (30-50%) and to the brain (12,6%). Depending on the genetic subtype, breast cancer approaches diverse distant metastatic sites. As an example, the axillary lymph nodes are mainly affected by Luminal A and B subtype, while the liver is more frequently metastasized by HER2+ and Triple negative breast cancer subtypes

Adapted from: (Harbeck et al., 2019)

Cell fate of the disseminated cancer cells

Disseminated cancer cells (DTCs) are tumor cells, that are physically separated from the primary tumor and managed to spread to a distant location. It is thought that cancer cells that have escaped from the primary tumor and settled in secondary sites are the cause of metastatic relapse (Klein, 2009). However, the metastatic cascade is extremely ineffective. According to estimations, around 99,98 percent of disseminated tumor cells perish before developing into metastasis (Gupta & Massagué, 2006). Therefore, colonization is considered as the rate-limiting phase of the metastatic process (Vanharanta & Massagué, 2013).

Sir Stephen Paget introduced the "seed-and-soil" theory in 1889, which postulated that disseminated tumor cells (seeds) induce metastasis once they reach a tissue (soil) that permits them to survive and proliferate. Three important points were emphasised: first, metastases can only develop in particular organs with biologically appropriate microenvironments, second, metastases consist of both tumor and host cells and third, metastasis is the result of a close communication between DTCs (the seeds) and the surrounding microenvironment (the soil) (Paget, 1989). After more than 130 years, Paget's ground-breaking observations are still accurate, supporting the idea that the fate of DTCs is determined by a delicate equilibrium between cell-extrinsic and cell-intrinsic variables (Langley & Fidler, 2011).

It is well known that certain cell-extrinsic determinants provided by the 'soil' affect the metastatic success, such as seeding in close proximity to blood vessels, the formed pre-metastatic niche by primary tumors as well as the activity of the immune system (locally and systemically) (Peinado et al., 2017; Shiao et al., 2011). A few noteworthy cell-intrinsic cues that control the "seeds" are the ability to self-renewal and adapt to new environments, as well as genetic and epigenetic plasticity (Valastyan & Weinberg, 2011). Disseminated tumor cells therefore can ultimately face several fates once they reach the target organ: metastatic outgrowth, cell death (spontaneous or immune-mediated) or **cancer dormancy** (Figure 6).

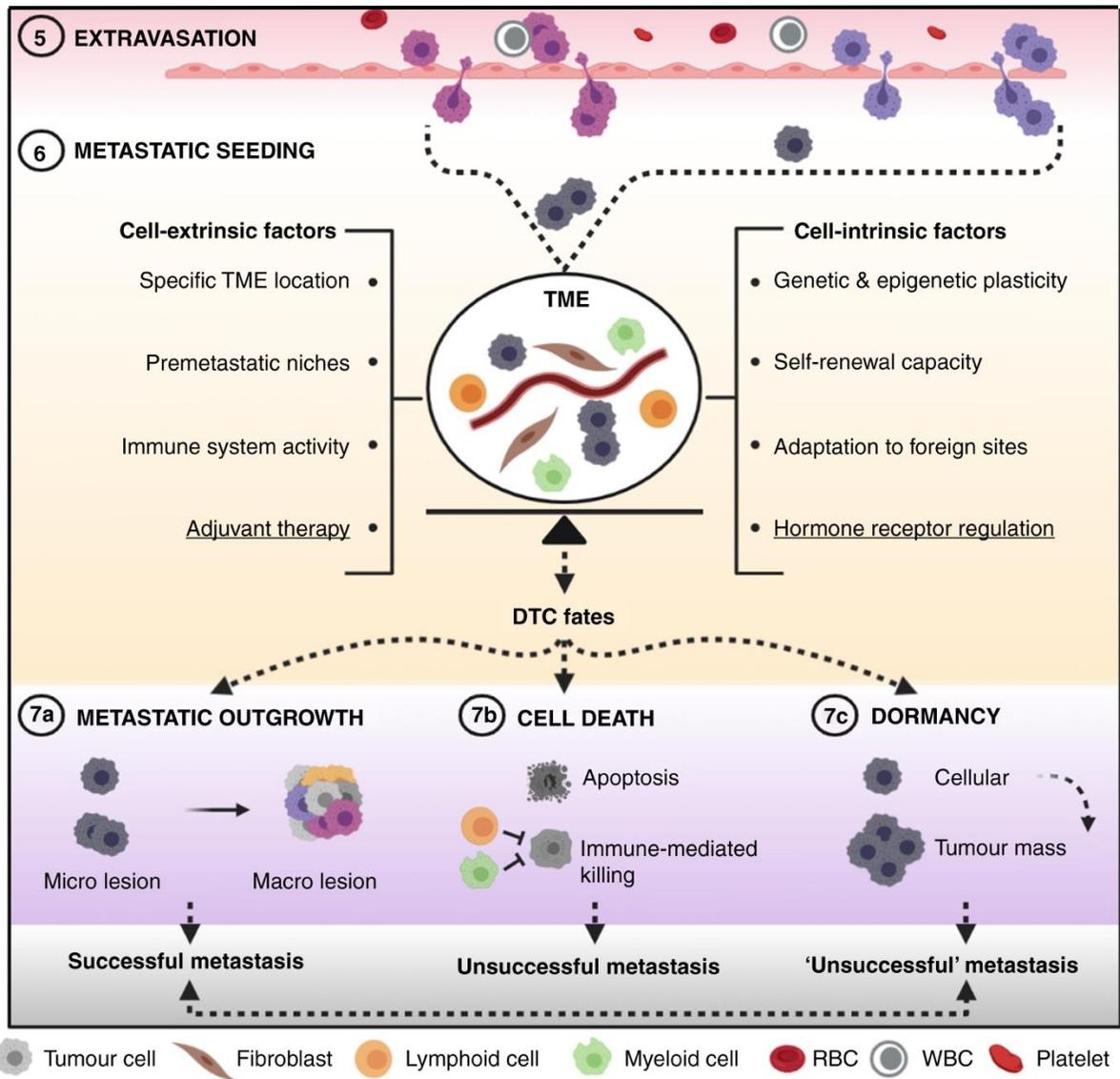


Figure 6. Cancer cell fate at the new metastatic loci

Following extravasation, the disseminated cancer cells will interact with the cells of the distant sites. Different cell-extrinsic and cell-intrinsic factors will determine the fate of DTCs. 7a. Cancer cells can form metastatic outgrowth, 7b. DTCs die via spontaneous or immune-mediated apoptosis, 7c. Tumor cells may remain dormant. While apoptosis of the cancer cells result is unsuccessful metastasis, dormant cancer cells might eventually resume dormancy and form metastasis. (TME: Tumor-microenvironment, RBC: Red blood cell, WBC: White blood cell)

Adapted from: (Riggio et al., 2021)

Dormancy

Metastasis is the primary cause of breast cancer-related deaths and the greatest difficulty challenging clinical oncologist is the inability to prevent and treat recurrent cancer. It is important to note that at the time of the first diagnosis, dissemination had already taken place in many patients whereas our understanding on the biology of disseminated cancer cells is extremely limited. Considering that disseminated dormant cells are possibly the sources of cancer recurrence, it is crucial to better comprehend the mechanisms of cancer cell dormancy (Klein, 2011).

The Australian pathologist Rubert A. Willis established the term "dormancy" in the first half of the 20th century (Willis, 1935). Cancer dormancy is a stage of tumor progression which is characterized by the presence of viable cancer cells without the expansion of the tumor mass. Clinically, it is defined by a prolonged period between the treatment of the primary tumor and the relapse, when the patient is considered as clinically free of cancer. Tumor dormancy can manifest as single dormant cells or as micrometastases. The term "single dormant cell" refers to cells that are at cell cycle arrest, while in the micrometastases, apoptosis and cell proliferation are in an equilibrium, preventing the increase of the tumor burden (Holmgren et al., 1995; Naumov et al., 2016).

Dormancy can last for extended period of time – even years, however, for reasons that are still unclear dormancy can resume and lead to recurrence. Numerous factors may contribute to the regrowth of cancer cells, thereby controlling the initiation and termination of dormancy: (1) genetic and epigenetic changes; (2) angiogenesis; (3) immune surveillance; and (4) the microenvironment (Figure 7).

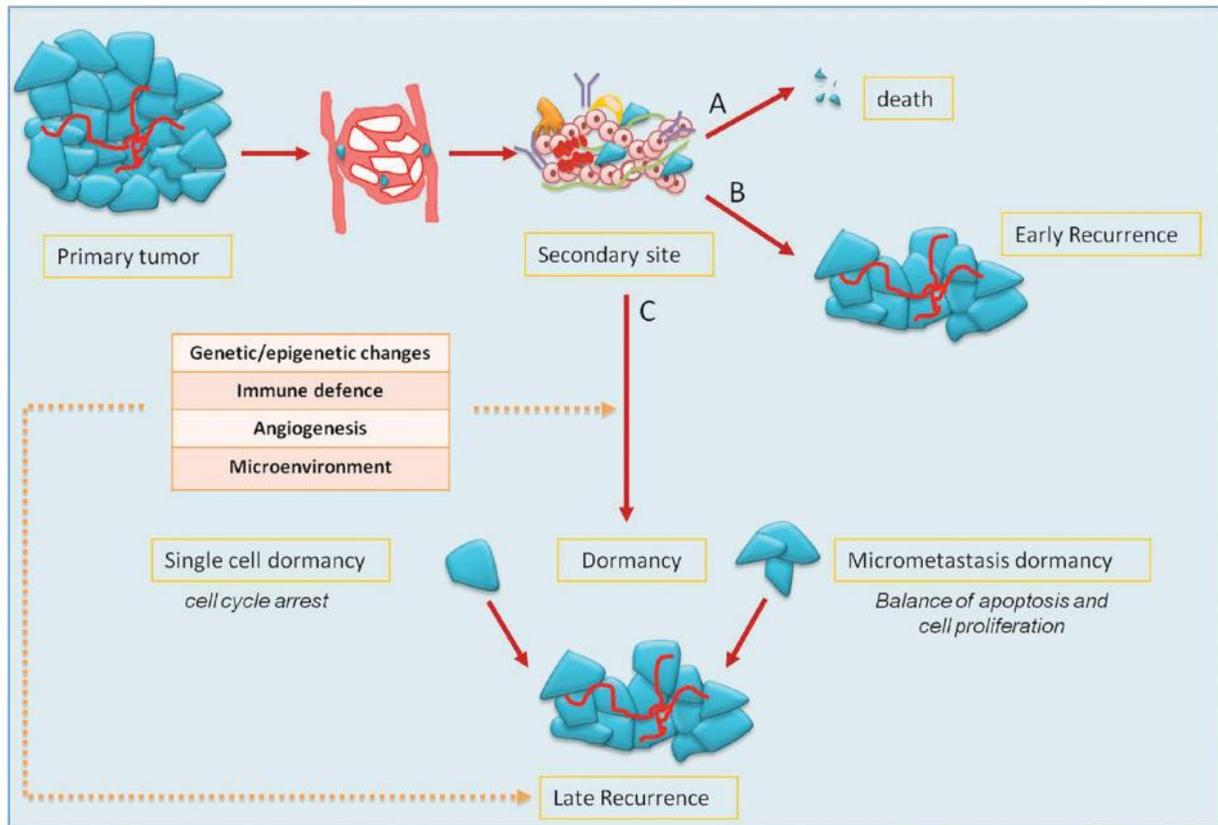


Figure 7. The possible fates of disseminated cancer cells

Disseminated cancer cells can face several fates at the secondary sites. If the microenvironment is not favouring the cell survival, the cells will die (A), or if the microenvironment is favourable, the arriving cancer cells can form metastatic lesions (B), or the cells can go into a dormancy (C). Dormancy can appear as single cell dormancy, where cells have the ability to initiate cell cycle arrest, or as micrometastasis (tumor mass) dormancy, where a balance in apoptosis and cell proliferation prevents tumor growth. Depending on several factors, such as genetic and epigenetic variations, the activity of the immune system, the blood vessel development (angiogenesis) and microenvironmental factors dormant cancer cells can quite dormancy and form late metastases.

Adapted from (Gelao et al., 2013)

Genetic and epigenetic differences influencing dormancy

Disseminated tumor cells might not only arise from the established primary tumors. Early DTCs are epithelial cells found in pre-invasive lesions that have the ability to conduct epithelial to mesenchymal transition and disseminate. Due to early-stage dissemination, such early DTCs that survive and may acquire the ability to proliferate, evolve differently from the primary tumor (Hüsemann et al., 2008). This could result in metastasis with features that differ from the primary tumor and could explain why treating metastasis with therapies solely based on the characteristics of the primary tumor are often unsuccessful. Furthermore, early DTCs can also shape or develop pre-metastatic niches for other disseminated tumor cells. Therefore, even if early DTCs themselves are dormant or senescent, they might still have an impact on the development of metastasis (Figure 8).

Almog and colleagues developed a tumor dormancy *in vivo* model for breast carcinoma, liposarcoma, glioblastoma and osteosarcoma (Almog et al., 2009). By using genome-wide transcriptional analysis, the authors compared the gene expression patterns of fast-growing tumors and quiescent tumors. Their findings revealed that fast-growing tumors displayed a downregulation of the expression of thrombospondin, an angiogenic inhibitor, as well as a decrease in sensitivity to angiostatin, another angiogenic inhibitor. Their findings indicate that the tumor vascular niche might be important in the dormancy escape.

MicroRNAs have also been associated to cancer dormancy. As an example, it was discovered that 16 microRNAs were overexpressed in dormant tumors and their downregulation was linked to the transformation of dormant to growing malignancies (Almog et al., 2012).

Another method of controlling gene expression in both dormant and proliferative cancer cells are epigenetic modifications. Using 20 breast cancer samples, Metge et al. showed that the promoter of the breast cancer metastasis suppressor 1 (BRMS1) gene was hypermethylated in 45% of the primary tumors and 60% of the matching lymph node metastases. This aberrant methylation resulted in the loss of its gene expression (Metge et al., 2008).

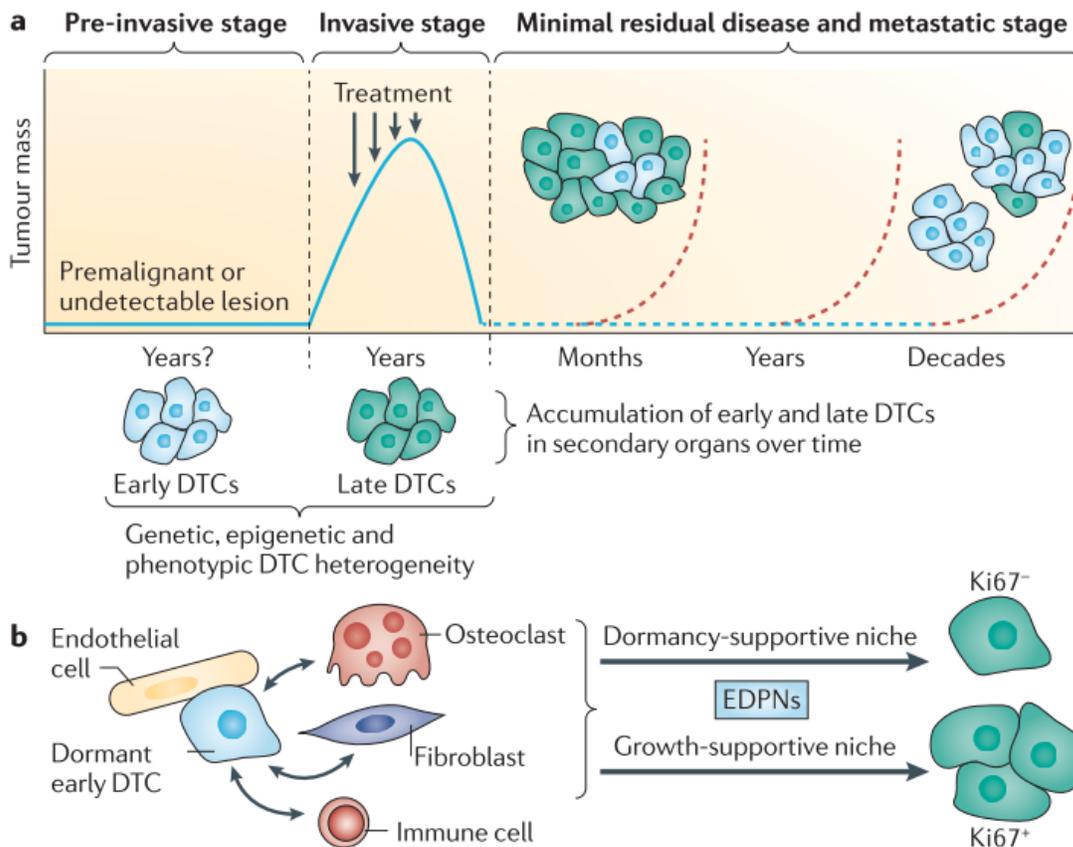


Figure 8. Heterogeneous DTC subpopulations form distinct metastases

a. Metastasis might be also initiated by cells (Early DTCs) from the pre-invasive lesions. The genetic and epigenetic phenotype of such early DTCs possibly differ from Late DTCs. As it is indicated by the blue line, the size of the tumor mass decreases as the primary tumor is diagnosed, treated and surgically removed. After months, years or even decades, the size of the tumor mass could increase as a metastasis is formed (dashed red lines). DTCs, that disseminate at different stages of tumor progression might form heterogeneous masses, resulting in metastases with varying phenotypic characteristics (Klein, 2009) **b.** Schematic representation of the hypothesis, about how Early DTCs could interact with different stromal cells (such as endothelial cells, osteoclasts, fibroblasts or different immune cells) to form Early DTC pre-metastatic niches (EDPNs), that could favour either the dormancy or the proliferation of the later arriving DTCs.

Adapted from (Sosa et al., 2014)

Micrometastasis dormancy

Angiogenic-dormancy

A vascular supply provided by angiogenesis is necessary for the growth of a tumor mass (Folkman, 1989). According to several studies, quiescent micrometastases are non-vascularised tumors with a balance between pro-angiogenic factors (vascular endothelial growth factor (VEGF), PDGR, fibroblast growth factor (FGF), Angiopoietin) and anti-angiogenic factors (endostatin, angiostatin, and TSP), with a minor dominance of the latter ones (Hanahan & Folkman, 1996) (Figure 9). Thus, the ability of micrometastasis to release angiogenic factors and downregulate angiogenic suppressors determines their capability to proliferate.

Using cell lines from breast cancer, osteosarcoma and glioblastoma, Naumov et al. established a model of human tumor dormancy that compares non-angiogenic and angiogenic malignancies (Naumov et al., 2006). When SCID (severe combined immunodeficient) mice were subcutaneously injected with non-angiogenic human cancer cell lines, the majority of the tumors that developed remained microscopic (1-mm diameter) for extended periods of time. Some of them became vascularized and were isolated and the acquired cells were used as angiogenic tumor cells. Surprisingly, these angiogenic cancer cells developed palpable tumors more frequently and earlier than the non-angiogenic cell lines. The authors came to the conclusion that the prolonged dormancy in the non-angiogenic cell lines was probably caused by decreased angiogenic capacity, rather than the mechanisms of quiescence or apoptosis, considering that there were no significant differences in the rates of proliferation and apoptosis between the two cell types. This conclusion was confirmed by a genome-wide transcriptional analysis that revealed significant alterations in the expression of genes linked to angiogenesis in various cancer cells (Almog et al., 2009).

The phrase "angiogenic switch" refers to the capacity of the tumor to switch from a non-angiogenic to an angiogenic phenotype, thus might be responsible for dormancy escape. This transition is triggered by decreased expression of angiogenesis inhibitors and increased expression of pro-angiogenic proteins by both cancer and stromal cells (Naumov et al., 2016). To better understand the angiogenic switch and consequently, find novel therapeutic targets to either induce or maintain tumor dormancy or alternatively, to trigger dormant cell death, a plethora of efforts are still required.

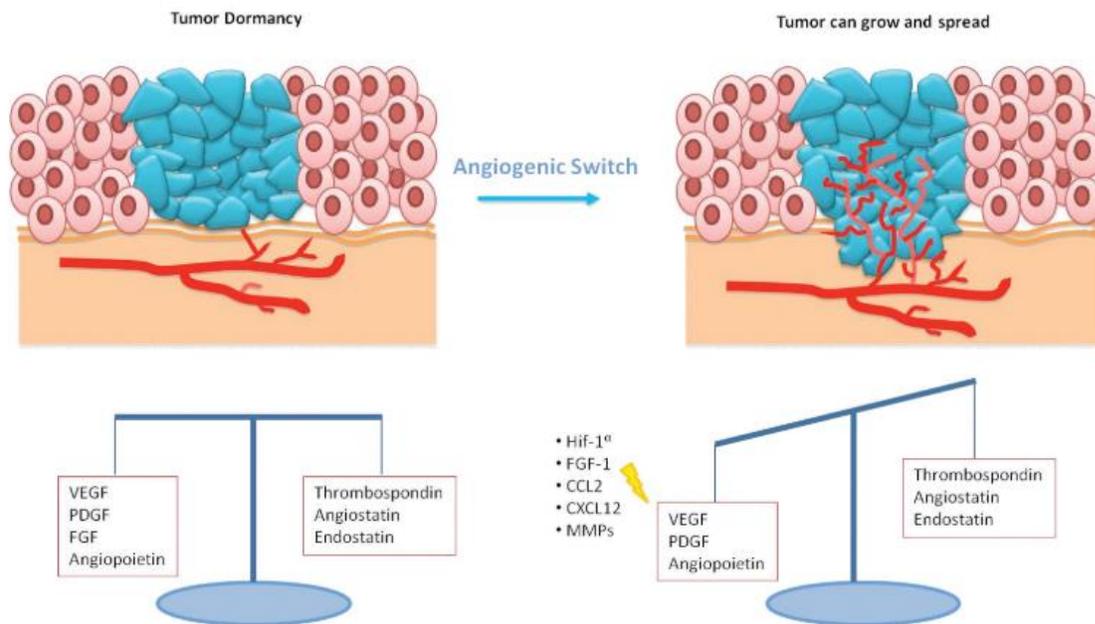


Figure 9. Schematic representation of angiogenic dormancy and the angiogenic switch
 In dormant micrometastases the poorly developed vascular system can be responsible for the constant tumor burden (lack of expansion). In angiogenic dormancy the pro-angiogenic (VEGF, PDGF, FGF, Angiopoietin) and anti-angiogenic (Thrombospondin, Angiostatin, Endostatin) factors are in equilibrium. The capability of micrometastasis to release angiogenic factors and downregulate angiogenic suppressors determines their ability to proliferate and to escape dormancy.

Adapted from (Gelao et al., 2013)

Immune-mediated dormancy

Immune surveillance has long been recognized as a crucial component in the prevention of tumor growth and development. Furthermore, tumor cells in established tumors have to acquire the ability for immune evasion (Dunn et al., 2004). In addition to its role in preventing early tumor development, the immune system also proved to be crucial for tumor mass dormancy.

The cancer immune-editing hypothesis suggests that the three stages of tumor development are elimination, equilibrium, and escape (Dunn et al., 2002) (Figure 10). In this model, the cancer surveillance phase is represented by the elimination phase, wherein the cells of the innate and adaptive immune system can eradicate the tumor and protect the host against the subsequent cancer development. If this process fails, the tumor cells might enter an equilibrium phase where they could be maintained or immunologically shaped by "immuno-editors" to create new populations of tumor variants. The equilibrium phase refers to dormancy. The chromosomal instability of the cancer cells is one of many variables that affect how long this equilibrium lasts. Chromosomal abnormalities in tumor cells accumulate over time resulting in a different phenotype, gives them the potential to elude the antitumor immune response and emerge from dormancy (Dunn et al., 2004).

Numerous studies show that the humoral and cellular immune systems both help to maintain dormancy. Interestingly, by immunizing against tumor cells, it is possible to induce tumor dormancy in immune-competent hosts. For instance, in the BCL1 lymphoma model, immunization with immunoglobulin (Ig) generated from BCL1 to elicit an anti-idiotypic immune response can induce tumour dormancy (Uhr, 1995).

Also, Eyles et al. demonstrated, using a spontaneous melanoma model in metallothionein (MT)-ret/AAD mice, that CD8⁺ T cells are crucial in the maintenance of tumor dormancy at the metastatic site. In this melanoma model, CD8⁺ antibody-induced immune system suppression significantly accelerated the spread of metastatic disease. Genome-wide single-nucleotide polymorphism analysis revealed a high degree of genetic similarity between the primary and metastatic sites, suggesting that metastases may have originated from tumor cells that disseminated from the primary tumor at an early stage. According to these findings, disseminated tumor cells would become dormant and avoid immune surveillance, and their escape from the immune system would lead to the establishment of metastatic lesions (Eyles et al., 2010).

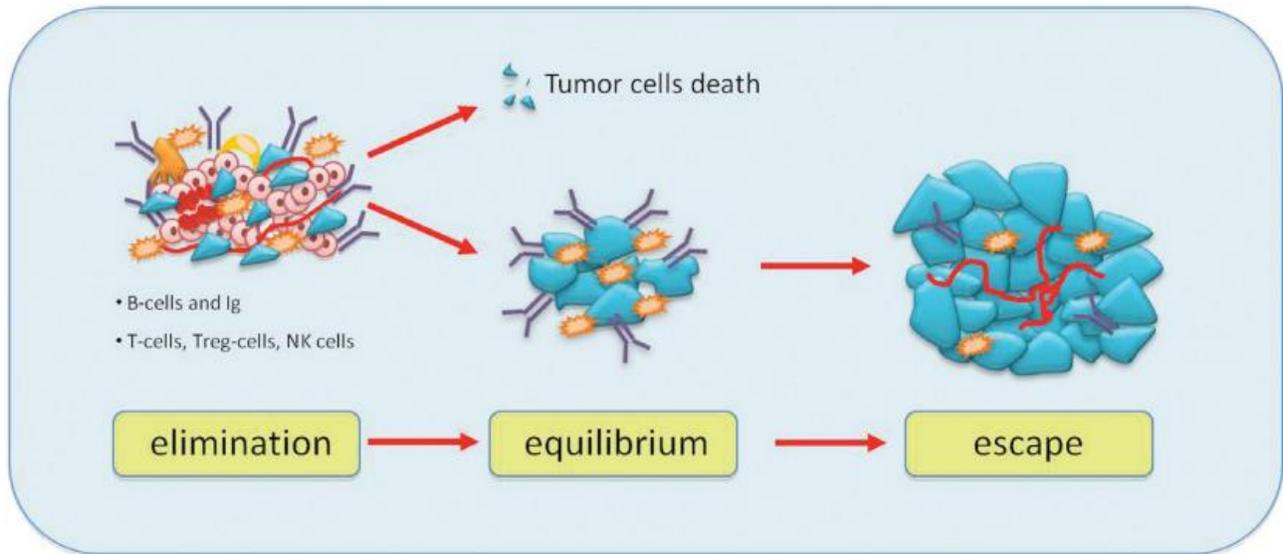


Figure 10. Immuno-editing hypothesis

*Proliferating tumor cells are maintained at sub-clinical numbers by the activity of the immune system. Several cell types, such as T-cells, B-cells, regulatory T-cells (Treg-cells), natural-killer cells are involved in the **elimination** of the cancer cells. Some of the cells might manage to evade the immune system and enter an **equilibrium**, which represent the immune-mediated tumor mass dormancy. After years or even decades later, if the tumor cells can **escape** immunosurveillance, cancer cells can progress into overt metastatic lesions.*

Adapted from (Gelao et al., 2013)

Cellular dormancy

Cellular dormancy is another type of cancer dormancy (Vera-ramirez & Hunter, 2017). Cellular dormancy is frequently identified as a reversible non-proliferative state of a cell. Currently, three distinct non-proliferative cellular phenotypes have been identified: cancer dormancy, senescence and quiescence. Senescent cells are metabolically active, but even in a favourable environment, their ability to proliferate is diminished (Takahashi et al., 2006). On the contrary, one of the main features of the quiescent and dormant cells is their ability to resume proliferation and re-enter cell-cycle, following an inactive period (Coller et al., 2006). Quiescence and dormancy are largely comparable cell statuses; however, they differ in how the cells re-enter the cell cycle. Quiescence, commonly known as G0, is a brief pause in proliferation that will be resumed when circumstances are favourable (Considine & Considine, 2016). The perception of dormancy as a more profoundly arrested condition suggests that it is more persistent than quiescence (Coller et al., 2006).

A growing body of research has shed light on the molecular mechanisms of cellular dormancy and reawakening. According to a plethora of studies, the extracellular signal-regulated kinase (ERK) activity is strongly implicated in the determination, whether cancer cells will enter, stay in dormant state or start to proliferate. Persistently proliferating cells possess constitutive ERK activity, which allows the transition between G0, G1 and S phases and therefore cell division (Chambard et al., 2007; Mebratu & Tesfaijzi, 2009). Upon ERK-induced proliferation, high p38 mitogen-activated protein kinase (p38) acts as an inhibitory regulator of ERK and limits cell proliferation, by initiating G0-G1 cell cycle arrest or promoting senescence and apoptosis (Aguirre-Ghiso et al., 2003; Dhillon et al., 2007). Indeed, a study, using a luciferase reporter system demonstrated direct evidence of p38/ERK activity as a marker of dormant cancer cells in various types of cancer, such as prostate cancer, melanoma, fibrosarcoma and breast cancer (Aguirre-Ghiso et al., 2003). Cancer cells with low p38 activity and high ERK activity were highly proliferative *in vivo*, whereas cancer cells with high p38 activity and low ERK activity were unable to proliferate, indicating that they were dormant *in vivo*. Meanwhile, numerous pharmacological and genetic therapeutic strategies that shift the balance of p38/ERK activity towards ERK were able to terminate dormancy and trigger cancer progression. Therefore, it appears that regulatory

factors that alter the balance of ERK and p38 activities have a significant impact on whether cancer cells proliferate or remain dormant (Sosa et al., 2011).

By regulating epidermal growth factor receptor (EGFR) signalling, the urokinase plasminogen activator (uPA) pathway has been linked to a transition from cancer dormancy to proliferation (D. Liu et al., 2002). uPA is a serine protease, produced and secreted by a wide variety of cells, including immune cells, fibroblasts and epithelial cells. The binding of uPA to its receptor (uPAR) triggers a proteolytic cascade, which result in the transformation of plasminogen to plasmin (Mahmood et al., 2018). Through its proteolytic activity, plasmin degrades a variety of extracellular components and by activating other enzymatic proteins like metalloproteases, it facilitates the invasion of cancer cells. The uPAR-uPA interaction activates integrin and EGFR signalling, independently of the catalytic activity (Figure 11). This, in turn, lowers p38 activity and concurrently activates ERK, therefore promoting the mitotic cascades (Aguirre-Ghiso et al., 2001; Aguirre-Ghiso et al., 2003). However, it has been found that dormant cancer cells express low levels of uPAR, which results in low levels of integrin and EGFR activation and therefore, high p38 and low ERK activity (Aguirre-Ghiso et al., 2003). Additionally, ERK^{low}/p38^{high} activity promotes G0-G1 arrest by downregulating G1 exit-promoting transcription factors (TFs) like FOXM1 and c-Jun and upregulating G0-G1 arrest-promoting TFs such basic helix-loop-helix protein 3 (BHLHB3) and nuclear receptor subfamily 2 group F member 1 (NR2F1) (Adam et al., 2009). Therefore, the quiescence program of dormant cancer cells is mediated by the combinational regulation of TFs by p38^{high}/ERK^{low} activity (Sosa et al., 2011).

Microenvironmental regulation of cellular dormancy

Extracellular matrix and dormancy

The fate of dormant cancer cells, whether they apoptose, remain dormant or emerge from dormancy within the metastatic milieu also depends on the composition of the extracellular matrix and its impact on adhesion signalling.

Integrin-mediated awakening

Transmembrane heterodimeric glycoproteins called integrins mediate communication cascades from cells to cells and from cells to the extracellular matrix (ECM). In both cancer cells and normal healthy cells, integrin signalling regulates cell proliferation, survival and motility by activating a number of intermediaries, such as cytosolic tyrosine kinases (Barkan & Green, 2011). Numerous studies have shown that the transition from cellular dormancy to metastatic expansion both *in vitro* and *in vivo* is strongly regulated by integrin signalling, particularly by that of β -1 integrin (Barkan et al., 2008, 2010; Barkan & Green, 2011; Shibue & Weinberg, 2009). Downregulation of the uPA-uPAR interaction results in a loss of β -1 integrin signalling, which may facilitate the transition of cancer cells from a proliferative to a dormant state (Aguirre-Ghiso, et al., 2003). Also, in a three-dimensional basement membrane assay, blocking β -1 integrin signalling using an antibody induced the growth arrest of breast cancer cells (Weaver et al., 1997). Interestingly, cell cycle arrest was restored, and cancer cell proliferation was restarted when the anti-integrin antibody was removed.

The mouse mammary tumor cell lines D2.OR and D2A1 both proliferate exponentially in two-dimensional cultures, but D2.OR is unable to grow in three-dimensional cultures and goes into dormancy; this is consistent with *in vivo* findings that D2A1 generates lung metastases rapidly, whereas D2.OR has a prolonged latency. Additionally, the ability of the highly metastatic D2A1 mammary carcinoma to grow in three-dimensional cell culture seems to be strongly dependent on the presence of fibronectin, β -1 integrin signalling and downstream phosphorylation of the myosin complex, suggesting that upregulating β -1 signalling allowed dormant cancer cells to re-enter the cell cycle (Barkan et al., 2008). The mouse mammary cancer cell lines D2.OR and D2A1 were

found to be dependent on β -1 integrin signalling for metastatic outgrowth in another *in vivo* study as well. Collagen binding to integrin receptors led to the activation of FAK/Src and subsequent ERK phosphorylation. Cell proliferation induced by ERK activation *via* integrins promoted the spread of metastatic disease (Boyerinas et al., 2013).

A downstream molecule of β -1 integrin called focal adhesion kinase (FAK) has also been associated to the control of cancer cellular dormancy. In a mouse mammary tumor virus (MMTV) transgenic breast cancer model, Cre-LoxP-mediated deletion of β -1 integrin inhibits tumor growth *in vivo* by decreasing FAK phosphorylation (White et al., 2004). These findings imply that the interaction between β -1 integrin/FAK and the MAPK pathway is crucial for the growth of cancer cells (Figure 11).

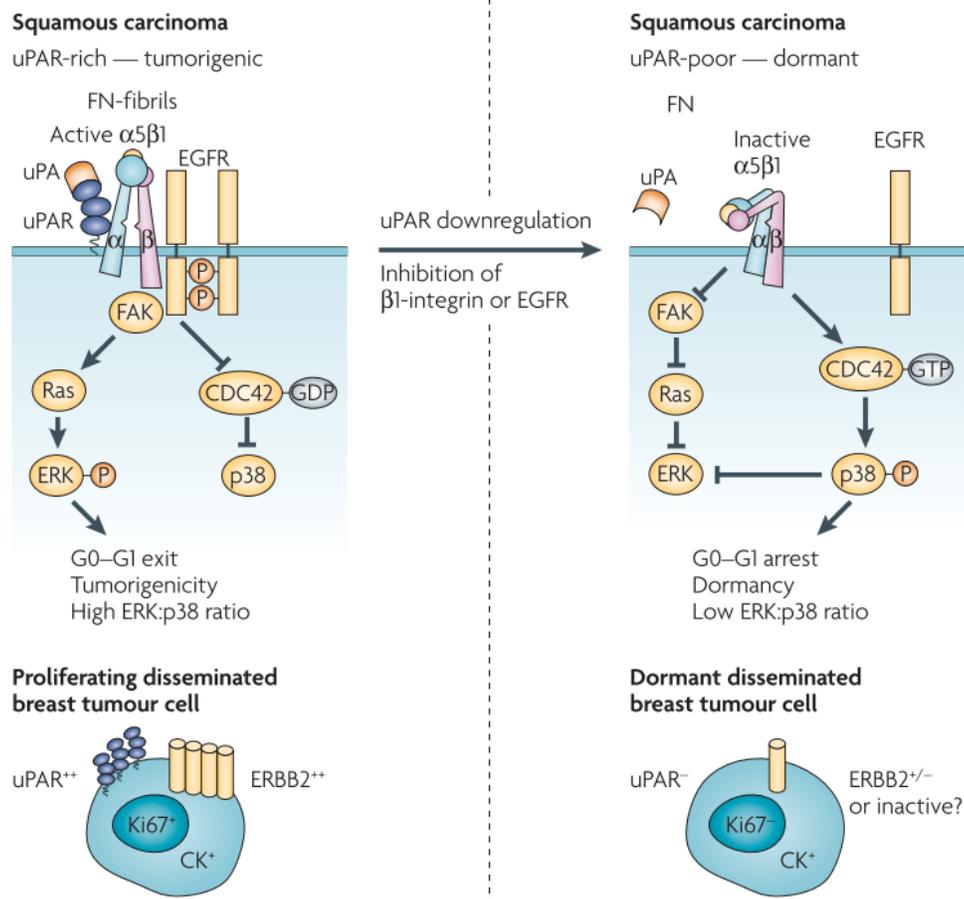


Figure 11. Integrins, uPAR and EGFR in cellular dormancy

Metastatic cells can modify and alter their microenvironment to introduce signals that promote growth when they have the proper microenvironment and receptors. In uPAR-rich squamous carcinoma, fibronectin-derived signals are transduced by uPAR- $\alpha5\beta1$ integrin-EGFR (or ERBB2) and FAK, generating ERK activation and p38 inactivation via cell division cycle 42 (CDC42), resulting in G0-G1 exit and cancer cell proliferation (Ki67+). Loss of any of these surface receptors (uPAR, $\alpha5\beta1$ integrin, EGFR), that transmit the growth signals (such as fibronectin) cause stress signalling (Low FAK-Ras-ERK and High CDC42 and p38 activity), which might trigger dormancy (Ki67-).

Adapted from (Aguirre-Ghiso, 2007a)

Discoidin domain receptor 1-mediated awakening

Collagen binding can activate the non-canonical discoidin domain receptor 1 (DDR1) signalling, which is known to promote cancer cell growth at metastatic regions. Mechanistically, the protein kinase C α (PKC α) is activated by the interaction of DDR1 and syntenin 2 *via* tetraspanin transmembrane 4 L six family member 1 (TM4SF1). Activated PKC α then phosphorylates Janus Kinase 2 (JAK2), which in turn phosphorylates Signal Transducer and Activator of Transcription 3 (STAT3). Constitutive activation of STAT3 in cancer enhances cancer cell proliferation by increasing the transcription of cell cycle regulators such c-Myc and cyclin D. Indeed, micrometastatic lesions are generally surrounded by collagen, according to histopathologic examination of metastatic murine breast cancer cells. The majority of tumor cells away from collagen in metastatic tissues are showing dormant phenotype, while those close to collagen are proliferative. These findings also demonstrate how the interplay between dormant cancer cells and the ECM affects cancer cell behaviour and reactivation (Gao et al., 2016) (Figure 12).

Wnt-mediated awakening

Wnt signalling has also been associated to the ECM-induced reactivation of dormant cancer cells. Wnt is a well-known inducer of proliferation and is known to regulate a wide range of biological processes. Wnt activation facilitates the G1-to-S transition by controlling cyclin D1, cyclin E1, and c-Myc both transcriptionally and non-transcriptionally (Lecarpentier et al., 2019). Consequently, one mechanism by which cancer cells enter quiescence is the suppression of Wnt signalling by the production of Dickkopf WNT signalling pathway inhibitor 1 (DKK1). Tenascin-C is known to promote the metastatic expansion of breast cancer cells by enhancing Wnt signalling. Tenascin-C is initially produced by metastasis-initiating cancer cells and later released from stromal fibroblasts. Tenascin-C promotes Wnt signalling activation and facilitates metastatic colonization by binding to syndecan-4, a coreceptor of the Wnt receptor Frizzled-7 (FZD). Additionally, it has been identified, that Periostin can attract Wnt ligands to the metastatic area. Periostin as well as Tenascin-C, mostly secreted by TGF- β activated stromal fibroblasts. Additionally, by co-assembling with fibronectin and altering its adhesiveness and stiffness, both Tenascin-C and Periostin can promote integrin signalling through an indirect pathway. These data

collectively imply that the ECM components from both stromal and cancer cells may create a permissive environment that facilitate the activation of signalling pathways that enable metastatic expansion (Malladi et al., 2016) (Figure 12).

Inflammation and dormancy

There is mounting evidence that chronic inflammation contributes to the development of cancer. For instance, patients with inflammatory bowel disease have an increased risk of developing colorectal cancer. Additionally, the incidence of liver cancer development is correlated with hepatitis and fatty liver disease and oesophageal cancer can result from acid reflux esophagitis.

Free radicals, such as reactive oxygen and nitrogen species (RONS) accumulate during inflammation and induce double-strand breaks in DNA, which are mutagenic if they are not rapidly and precisely repaired, and thus capable of triggering the transformation of normal cells into cancerous cells (Yang et al., 2018). Free radicals can also activate a variety of signalling pathways, such as MAPK/ERK, PI3K/Akt, and nuclear factor kappa-B (NFκB), which promote the malignancy of cancer cells (Liou & Storz, 2010).

However, not all individuals who have encountered a chronic inflammatory condition will acquire cancer throughout their lifetime. These phenomena made people wonder if there was a cause-and-effect connection between cancer and chronic inflammation. One explanation for this contradictory evidence is that reawakening dormant cancer cells might play a significant role in the emergence of cancer from chronic inflammation.

Chronic inflammation, for instance, promotes angiogenesis, which as explained above, can terminate dormancy by providing oxygen and nutrients and thus promotes cancer growth (Teng et al., 2008). Furthermore, recurrence of cancer, including oral (Okubo et al., 2016), endometrial (Machida et al., 2017) and particularly breast cancer and inflammation are strongly correlated (Bowers et al., 2014; Hughes et al., 2015). It has been reported, that the inflammatory cytokine interferon-gamma (IFN- γ) has the ability to trigger cancer break out from its dormant state (Kmieciak et al., 2013). Furthermore, this theory is also supported by the correlation between high blood inflammatory cytokine levels and cancer recurrence. High levels of circulating acute-phase proteins (APPs) were significantly correlated with distant recurrence in a cohort of 734 breast cancer patients (Cole, 2009). In addition, other inflammatory indicators in the blood like C-reactive protein (CRP) and interleukin 6 (IL-6) have demonstrated their potential as post-treatment prognostic monitoring factors for determining the likelihood of cancer recurrence and patient mortality (Duffy et al., 2008; Shrotriya et al., 2018; Toiyama et al., 2013). IL-6 regulates the

release of hepatocyte CRP and tumor necrosis factor (TNF) and interleukin-1 (IL-1) enhance the production of CRP. Increasing levels of CRP in the serum frequently indicate tissue injury.

According to a mouse model of pancreatic cancer, circulating cancer cells underwent epithelial to mesenchymal transition (EMT) and implanted metastatic colonies in the liver. The rate of EMT and invasive potential were the highest at inflammatory locations. However, treatment with an immunosuppressive drug, called dexamethasone eradicated EMT and cancer invasiveness. These findings suggest that by promoting the EMT process in cancer cells, inflammation can act as a driver in the development of cancer (Rhim et al., 2012).

The formation of macroscopic metastases can also be induced by localized inflammation in the lungs, which can promote cancer cell dormancy escape (Weaver et al., 1997). Within this process, the expression of Zeb1, a potent inducer of EMT was necessary for cancer cells to emerge from dormancy. On the other hand, cancer cell reactivation in the lung was prevented by neutrophil depletion, achieved by the injection of antibodies against the locus of the lymphocyte antigen 6 complex (Ly6G) (Albregues et al., 2018a). Collectively, these findings are consistent with the hypothesis, that inflammation function as a tumor promoting factor by triggering the awakening of dormant disseminated cancer cells.

Cellular factors in dormancy escape

Natural killer cells and dormancy

It has been discovered that DCCs are more resistant to the cytotoxicity of natural killer cells (NK cells), however, the role of NK cells in cancer dormancy and reactivation has not yet been established. DTCs were shown to activate p38 and self-renewal pathways through SRY-Box Transcription Factor 2 (Sox 2) and SRY-Box Transcription Factor 9 (Sox 9) in a “Latency-competent tumor model”, wherein dormant clones were collected from an *in vivo* metastasis assay. Additionally, it has been demonstrated that Sox2 promotes DKK1 production, which blocks Wnt signalling as well as associated proliferative pathways (Malladi et al., 2016). While DTCs with low DKK1 expression are nonetheless proliferative and vulnerable to NK cell cytotoxicity, DCCs that enter dormancy via DKK1 are able to prevent NK cell-mediated cell death (Figure 12).

CD4+ and CD8+ T-cells and dormancy

Recently, a rising amount of evidence has drawn attention to the potential contribution of CD4+ and CD8+ T cells to the maintenance of cancer dormancy (Linde et al., 2016; H. Wang et al., 2019). Dormant cancer cells exhibited minimal levels of cancer antigen expression and were significantly less sensitive to adaptive immunity. Furthermore, an *in vivo* xenograft model using clones of dormant murine breast cancer cells selected with constitutive doxorubicin treatment has shown that CD8+ and CD4+ T cells are also participating in chemotherapy-mediated dormancy (Lan et al., 2019). Through an autocrine and self-sustaining upregulation of interferon regulatory factor 7 (IRF7) chemotherapy treatment stimulated IFN signalling in cancer cells. CD4+ and CD8+ T cell expansion was enhanced by IRF7/IFN signalling, while inhibiting the deployment of CD11b+Gr1+ myeloid-derived suppressor cells. Together these effects promote immune cytotoxicity, which leads to immune-mediated cancer dormancy (Figure 12).

Pericytes and dormancy

It has been shown in mice brain, that disseminated cancer cells can cross the blood-brain barrier and attach to brain capillaries (Valiente et al., 2014). Their position and behaviour resembles to pericytes as they spread on the brain capillaries, moved along the vessels and remained dormant for extended periods of time (Er et al., 2018). Despite the fact that the mechanism of dormancy was not investigated in that study, it was discovered that the adhesion molecule L1CAM on DTCs mediates access to the perivascular niche and drives transcriptional programs that awaken dormant disseminated tumor cells, which in turn trigger metastasis. So far it is unclear whether the reactivation of the dormant cancer cells were driven by their pericyte-like behaviour or by the modifications of the perivascular niche.

Fibroblasts and dormancy

Fibroblasts are one of the most common cell types in tumor microenvironment and participate in a variety of biological and pathological processes, including inflammation, wound healing and metastasis (Malanchi et al., 2012). However, there is limited and frequently contradicting study on how fibroblasts contribute to cancer dormancy, probably due to their high heterogeneity. It has been demonstrated that normal fibroblasts repress and confine nearby aberrant hyperplasia to a dormant state (Zhou et al., 2016). The "neighbour suppression phenomenon" describes how reversibly activated fibroblasts in the early stages of tumor initiation prevent neighbouring transformed cells from growing by interfering *via* cell-to-cell contact (STOKER et al., 1966). Furthermore, it has been demonstrated that normal lung fibroblasts can prevent the expansion of human prostatic cancer cells without any physical contact as well (Kirk et al., 1981). Additionally, by lowering p16 and cyclin D1 levels *in vivo*, fibroblasts in mouse skin have been demonstrated to suppress the proliferation of melanoma cells (Zhou et al., 2016). These suggest that the neighbour suppression-dependent tumor dormancy by the normal fibroblast can prevent the cancer cells from expansion.

On the contrary, Fane et al. found that following an age-induced reprogramming, fibroblasts are able to awaken dormant melanoma cells within the lung. They identified WNT5A as an activator of dormancy in melanoma cells and interestingly, they showed that fibroblast

secreted sFRP-1 can inhibit WNT5A and thus inducing the metastatic outgrowth of disseminated dormant cancer cells (Fane et al., 2022).

Cancer-associated fibroblasts (CAFs) are irreversibly activated fibroblasts. In the tumor microenvironment, CAFs are the main producers of TGF- β , which has been shown to keep disseminated tumor cells dormant (Bhowmick et al., 2004). In head and neck squamous cell carcinoma (HNSCC) high levels of TGF- β have been found to support the maintenance of the dormant state of cancer cells (Bragado et al., 2013). However, TGF- β can also promote the proliferation of cancer cells. According to Qin et al., TGF- β stimulated the expression of a matrix-specific protein, called periostin, which facilitated the growth, migration, and invasion of HNSCCs (Qin et al., 2016).

During the desmoplastic process, CAFs have a strong capacity for ECM synthesis and remodelling. Cancer cell dormancy is correlated with the stiffness of the tumor stroma, which is primarily regulated by CAFs. When A549 lung cancer cells were cultured in an ECM-mimicking Matrigel system, the expression of uPA, uPAR, MMP2, MMP7, MMP9 significantly decreased, and the cells went into a drug-resistant dormant state (Keeratichamroen et al., 2018). The fate of the dormant cancer cells may also depend on the composition of the ECM. According to research by Barkan et al., type I collagen and fibronectin can activate PI3K/Akt and FAK to promote the proliferation of dormant D2.OR cells (Barkan et al., 2008, 2010). Tenascin-C, which is increasingly produced by activated fibroblasts in the metastatic niche, might prevent pro-dormancy cellular reprogramming by sequestering TGF- β in an inactive state and thus promote proliferative signalling (Oskarsson et al., 2011) (Figure 12).

Neutrophils and dormancy

Neutrophils have received a lot of interest lately due to their ability to reactivate dormant cancer cells. The evidence for the role of neutrophils in dormancy escape was provided by Albrengues et al. In these experiments, chronic lung inflammation was induced by cigarette smoke exposure or lipopolysaccharide inhalation, which also transformed dormant cancer cells into rapidly proliferating cells, leading to a rise in metastasis. Dormant cancer cell awakening was mediated through the formation of neutrophil extracellular traps (NETs), which are scaffolds of chromatin that release cytotoxic enzymes and proteases into the extracellular area. In more details,

NETs released two proteases, neutrophil elastase and matrix metalloproteinase 9 (MMP9), which cleaved and modified laminin one after the other. In turn, the modified laminin stimulated the proliferation of dormant cells via activating integrin $\alpha3\beta1$ signalling (Albregues et al., 2018b) (Figure 12).

Macrophages and dormancy

The contact between cancer cells and myeloid cells is also a factor contributing to the development of cancer. As an example, by secreting the chemokine C-C-motif ligand 2 (CCL2) and VEGF, inflammatory monocytes with Ly6C expression can promote the extravasation of cancer cells in the lung (Doak et al., 2018; B.-Z. Qian et al., 2011). Also, it has been reported that macrophages can attach to cancer cells to prolong their survival. During this process, the vascular cell adhesion protein-1 (VCAM-1) on cancer cells interacts with the β -1-integrin-positive macrophages, which results in the activation of Akt signalling and enable cancer cells to elude the TNF-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Q. Chen et al., 2011).

The differentiation of monocytes into macrophages has also been implicated in the metastatic outgrowth of cancer cells in an in-direct manner. Macrophages were found to promote metastatic outgrowth by forming a more fibrotic microenvironment. In a genetically engineered mouse model of pancreatic ductal adenocarcinoma (PDAC), macrophages produced granulin in the liver, which in turn stimulated the conversion of resident hepatic stellate cells into myofibroblasts. Myofibroblasts then released periostin, which enhanced the fibrotic microenvironment and promoted integrin signalling activation, leading to dormant cancer cell activation (Nielsen et al., 2016).

In a recent work, wounds developed in an immunogenic breast cancer model were used to illustrate the role of inflammation-associated myeloid cells in controlling dormancy. In this study, it was discovered that those breast cancer cells, which didn't have the potential to metastasize, began to spread and proliferate in distant tissues following surgery. Additionally, surgical injury has triggered a systemic inflammatory response and in line with this, treatment with the non-steroidal anti-inflammatory drug (NSAID), called meloxicam, had a suppressive effect on tumor growth. Increased production of inflammatory mediators, such as the main monocyte chemoattractant CCL2 and elevated levels of circulating myeloid cells (monocytes and

neutrophils) were indicating the inflammatory response. Accordingly, tumor growth has been partially decreased as a result of CCL2 down-regulation (Krall et al., 2018).

Also, it is important to point out that both primary tumors and bone metastases from breast cancer patients were found to be enriched with macrophages showing an M2-like differentiation that were situated close to blood arteries. According to an *in vivo* study, these macrophages expressed vascular endothelial growth factor A (VEGFA). Following chemotherapy, malignancies lacking VEGFA+ macrophages recurred more slowly than tumors with VEGFA+ macrophages (Hughes et al., 2015).

Another connection between dormancy and macrophages was found by demonstrating how chemokines like CCL2 and CCL5 that recruit monocytes are involved in dormancy escape. As previously indicated, the function of CCL2 in promoting monocyte recruitment and subsequent dormancy escape were studied in the context of wound healing (Krall et al., 2018). In a research setup where HER2 down-regulation has resulted in the development of residual tumors and subsequent tumor recurrence, CCL5-induced enhanced macrophage presence, that facilitated emergence from dormancy. This effect was driven by a rise in CCR5+ macrophages that deposit collagen and the overexpression of CCL5 in tumor cells has accelerated the rate of recurrence (Walens, DiMarco, et al., 2019). Additionally, this study has discovered an intriguing link between TNF α activity and dormancy via the nuclear factor-kappa B (NF- κ B) pathway. It has been demonstrated that HER2 down-regulation triggered a pro-inflammatory program that includes TNF α , which led to the expression of several chemokines, through the activation of the IKK-NF- κ B pathway. CCL5 can be found between these chemokines, that was responsible for the increased presence of CCR5+ macrophages that promoted dormancy escape.

Another work done by Borriello et al. in 2022 provided evidence about the importance of macrophages in the metastatic process. They confirmed the hypothesis, that the primary tumor microenvironment is able to prime the disseminating tumor cells for a dormant phenotype. They discovered that spontaneously disseminated tumor cells have increased speed of extravasation and better survival following extravasation compared to experimentally metastasized cancer cells. Furthermore, they also found that spontaneously metastasizing tumor cells display dormancy or stem-like markers more frequently. However, the depletion of macrophages at the primary tumor sites reduces the level of dormant disseminating tumor cells, suggesting a fundamental role of macrophages in priming DTCs for dormancy (Borriello et al., 2021).

The involvement of macrophages has been described in many mechanisms that influence the success rate of the metastatic process. However, whether direct interaction between macrophages and dormant cancer cells could lead to emergence from dormancy, has not yet been sufficiently demonstrated.

The functions of macrophages in the metastatic cascade

The importance of macrophage populations within the primary tumor to promote metastatic progression is supported by a vast amount of research. These tumor-associated macrophages (TAMs) influence the development of the tumors by a variety of mechanisms, including stimulation of angiogenesis, inhibition of tumor-specific immune responses and matrix remodelling (Condeelis & Pollard, 2006; Lamagna et al., 2006; Pollard, 2004; B.-Z. Qian & Pollard, 2010; Ruffell et al., 2012; Sica et al., 2006). Understanding how macrophages at the metastatic site contribute to the late stages of metastasis, such as secondary site arrest, extravasation, early colonization or dormancy escape, is a less explored but expanding area of research. According to recent research, these metastasis-associated macrophages (MAMs), which arise from both tissue-resident macrophages and bone marrow-derived macrophages (BMDMs), promote metastasis in the secondary site. The metastatic potential of disseminated cancer cells is decreased upon the depletion of macrophages at any stage of the metastatic process, indicating that interactions between MAMs and cancer cells have an impact on such important procedures as cancer cell adhesion to the vasculature, transmigration through the endothelium and colonization of secondary tumors (Headley et al., 2016a; B. Qian et al., 2009; B.-Z. Qian et al., 2011).

Different macrophage phenotypes

Understanding precisely how macrophages are recruited and activated during an immune response can shed light also on how these cells might affect the tumor microenvironment as well. Macrophages represent a heterogeneous population of immune cells which are commonly classified as tissue resident macrophages or bone marrow-derived macrophages. Tissue resident macrophages are differentiated from foetal liver or yolk sac progenitors to help the embryonic development. Later, these cells acquire auto-proliferation functions, specialize into a number of subtypes to conduct tissue-specific tasks such as waste and foreign object phagocytosis and immune response initiation (Davies et al., 2013). In contrast, bone marrow-derived macrophages are recruited to the different tissues as myeloid-derived cells, where they differentiate into macrophages and in response to local signals they polarize into different subtypes.

Macrophage colony-stimulating factor (M-CSF) and granulocyte macrophage colony-stimulating factor (GM-CSF) are two crucial cytokines that control macrophage number and activity. Among these, M-CSF, also known as colony-stimulating factor 1 (CSF-1), is essential in the maintenance of multiple myeloid lineage populations, whereas alveolar macrophage maturation and the effector differentiation of invariant natural killer T cells (iNKT) depend on GM-CSF (Bezbradica et al., 2006; Hamilton, 2008; Shibata et al., 2001). In general, M-CSF polarizes macrophages toward an anti-inflammatory phenotype, whereas GM-CSF triggers a pro-inflammatory phenotype. Moreover, M-CSF is frequently used *in vitro* to drive the activation of bone marrow-derived monocytes into macrophages. M-CSF acts through its receptor, called colony-stimulating factor 1 receptor (CSF-1R). M-CSF-activated macrophages show high phagocytic activity, as well as low antigen-presenting ability (Ushach & Zlotnik, 2016).

Traditionally, macrophages can be categorized as either classically-activated (M1) or alternatively-activated (M2) macrophages. In general, it is believed that M1 polarization promotes pro-inflammatory, tissue-destructive actions, while M2 macrophages promote rather anti-inflammatory and tissue-repair functions. Despite the fact that these definitions imply that M1 and M2 macrophages are separate entities with distinct roles, nowadays, this classification system is considered as an oversimplified view. Instead, distinct M1 and M2 phenotypes are distributed along a continuum which allows a seamless phenotypic transition between pro-inflammatory, anti-inflammatory/recovery and homeostatic statuses (Biswas & Mantovani, 2010; F. O. Martinez & Gordon, 2015; Murray et al., 2014). However, the balance of polarization in many pathological conditions is tilted towards chronic inflammation (M1-like) or tissue repair (M2-like). Since M2-like macrophages facilitate immune escape, angiogenesis, local tissue remodelling and secrete pro-tumorigenic growth factors, in malignant tissues, cancer cells mainly promote the polarization of this macrophage subtype. However, depending on the type of the given tumor and its environment, a number of M1-associated cytokines, including tumor necrosis factor (TNF)- α and interleukin (IL)-6, may either be pro- or anti-tumorigenic (Bertazza & Mocellin, 2010; Sethi, 2008). Numerous macrophage subpopulations may help in the development and spread of tumors. Even though bone marrow-derived macrophages are believed to make up the majority of tumor-associated macrophages, sometimes it can be challenging to discriminate tumor associated macrophages from tissue resident macrophages (Ruffell et al., 2012). Interestingly, a recent mass cytometry-based analysis identified up to 17 different macrophage populations in a renal cell

carcinoma sample. This finding emphasises the need to further comprehend how the phenotypic variety of macrophages affects the development of cancer (Chevrier et al., 2017).

Macrophage functions in cancer cell invasion and intravasation

One of the hallmarks of malignancy in primary tumors is invasion, which is fuelled by tumor- intrinsic and extrinsic factors that depend on the type of cancer and its microenvironment. During the first stages of invasion, cells within the primary tumor resolve their cell-cell junctions and migrate as single or multiple cells along the fibrillar matrix. These migratory activities are controlled by factors like chemokines, cytokines and growth factors that stimulate the reprogramming of cytoskeletal and adhesion proteins for migration (Friedl & Alexander, 2011). Cellular contributors to the induction of tumor-invasive factors include neutrophils, TAMs, and their progenitors. Importantly, these cell types are often controlled by the cancer cell *via* paracrine loops. The interaction between breast cancer cells and TAMs is one of the most well-described processes regulating the intravasation of cancer cells. It has been observed in a mice transgenic cancer model, that epidermal growth factor (EGF), a chemoattractant and migration-promoting factor for tumor cells, is expressed by TAMs in response to the expression of CSF-1, also known as M-CSF by breast cancer cells (Condeelis & Pollard, 2006; Wyckoff et al., 2004) (Figure 13). TAMs also release cathepsins that promote tumor cell invasion which is regulated by IL-4 released by CD4+ T cells (Akkari et al., 2014). Furthermore, breast cancer secreted neuregulin can induce notch signalling in TAMs, which triggers the release of jagged-1 (Jag-1) by macrophages, which also leads to an increase in tumor invasion (Cabrera et al., 2018). Additionally, macrophages secrete platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF) and WNTs which are soluble factors which might be implicated in tumor cell invasion and migration (Lewis & Pollard, 2006).

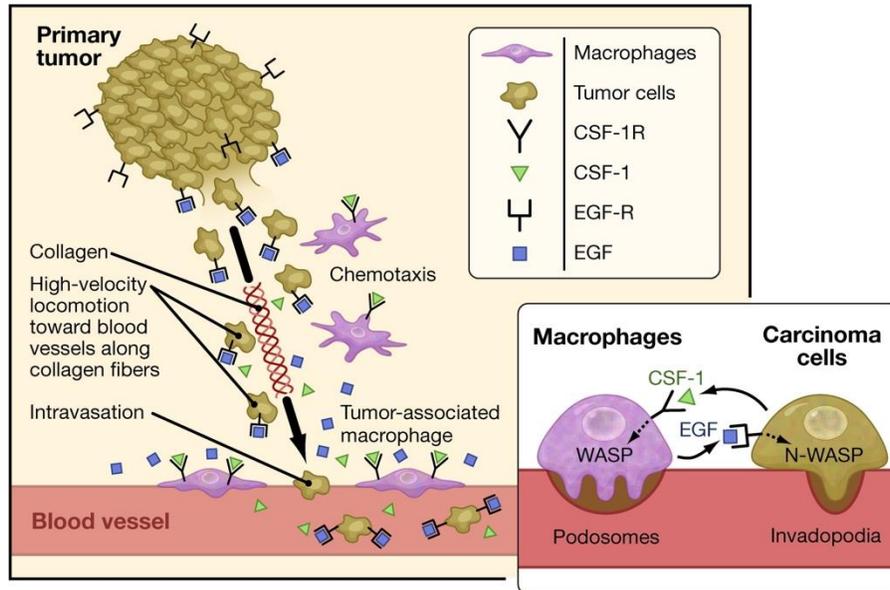


Figure 13. Macrophage-secreted EGF facilitates cancer cell migration toward blood vessels

At the initial stage of metastasis, in response to tumor cell-secreted CSF-1, macrophages secrete EGF, which serves as a chemoattractant for carcinoma cells. Within this chemotaxis, EGF receptor (EGFR)-expressing cancer cells migrate along collagen fibers toward blood vessels and the site of intravasation. These interactions between cancer cells and macrophages affect the activity of actin regulators (such as Wiskott-Aldrich syndrome protein (WASP)) resulting in the development of invadopodia in tumor cells and podosomes in macrophages to facilitate tumor cell intravasation.

Adapted from: (Condeelis & Pollard, 2006)

The majority of the pre-invasive forms of solid tumors are enclosed in a basement membrane matrix, that is composed of collagen IV, laminin and proteoglycans (Bissell & Hines, 2011). This barrier can be destroyed if the tumor possesses high proteolytic activity, thus enabling the cancer cells to penetrate the nearby tissue (Wan et al., 2013). The main groups of proteases released by both tumor cells and stromal cells, such as TAMs or neutrophils are Matrix Metalloproteases (MMPs), serine proteases, cysteine cathepsins as well as A Disintegrin And Metalloproteinases (ADAMs) (Joyce & Pollard, 2009; Mason & Joyce, 2011). In TAMs, different cytokines control the release of these proteases. As an example, IL-4, IL-10 and IL-6 can activate STAT-6 and STAT-3, which in turn regulates cathepsin secretion. Therefore, the inhibition of these pathways could prevent the macrophage-mediated tumor invasion (Yan et al., 2016).

The permeability of the blood vessels increases when pro-angiogenic factors, such as VEGF-A or TGF- β bind to the VEGF receptor 2 (VEGFR2), VE-cadherin- β -catenin complex in endothelial cells. Following these interactions, endothelial cells lose their adherens junctions and thus allow a greater trans-endothelial permeability (Reymond et al., 2013). Interestingly, when these factors are overexpressed, it results in immature angiogenic vessels with deficient basement membrane and pericyte coverage. Intravital imaging has demonstrated that VEGF-A-expressing TIE2⁺ macrophages generate localized areas of vascular leakiness. Importantly, it has been demonstrated that cancer cells can utilise those leaky regions for intravasation in mice breast cancer model (Harney et al., 2015).

Macrophages in epithelial to mesenchymal transition of cancer cells

The process known as epithelial-mesenchymal transition (EMT) enables epithelial cancer cells to lose their cell-cell junctions and shift into a migratory and invasive phenotype (Lambert et al., 2017). Growth factors including EGF, HGF, and FGF as well as inflammatory factors like TGF- β , TNF- α , IL-1 β and CCL18 have been shown to induce EMT in cancer cells (Figure 14). These stimuli promote the expression of transcription factors such as Zeb1 and 2, Snail, Slug, and Twist in tumor cells, which downregulate the gene expression of E-cadherin and Epithelial Cell Adhesion Molecule (EpCAM), thus ultimately leading to the loss of cell-cell junctions (Bonde et al., 2012; Friedl & Alexander, 2011; Lambert et al., 2017; Su et al., 2014; Suarez-Carmona et al., 2017). Pastushenko and his colleagues used a breast cancer model that expresses yellow fluorescent protein as a reporter to analyse the EMT states of cancer cells. They observed that tumor cells with high degrees of EMT were found in CD68+ macrophage rich and well vascularized areas within the tumors. Following the inhibition of CSF1 and CCL2, both the rate of EMT and the number of circulating tumor cells (CTCs) decreased, indicating that TAMs are involved in the control of EMT in breast cancer (Pastushenko et al., 2018).

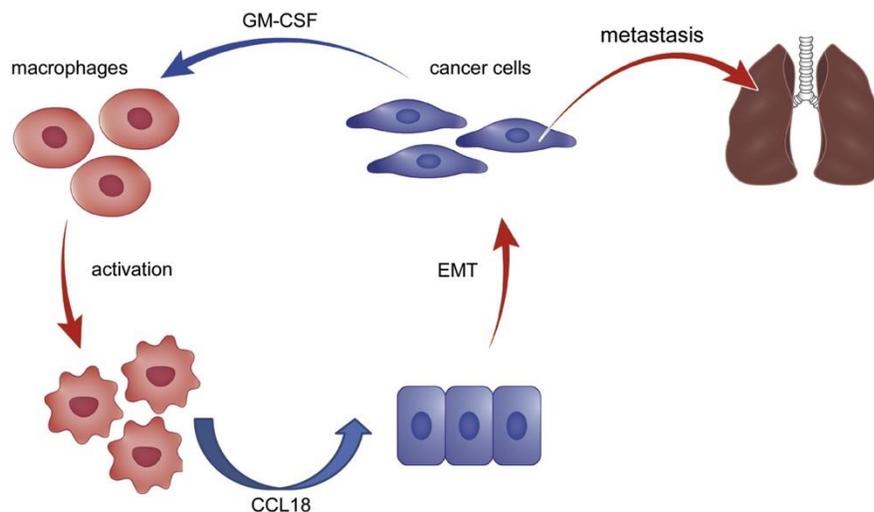


Figure 14. Schematic representation of macrophage-secreted CCL18-induced cancer cell EMT
Su et al. demonstrated that cancer cell-derived GM-CSF dedifferentiates macrophages toward tumor-associated macrophage phenotype. In turn, these activated TAMs release CCL18, which trigger EMT in the breast cancer cells, thus facilitating the metastatic process.

Adapted from: (Su et al., 2014)

Macrophages at the pre-metastatic site

Prior to the dissemination of the cancer cells into the blood circulation, primary tumors can already prime the formation of a favourable location in a distant organ, known as pre-metastatic niche. Generally, monocytes, myeloid-derived suppressor cells (MDSCs) and neutrophils are recruited to these pre-metastatic niches, where they start the remodelling of the extracellular matrix (ECM) and foster an immunosuppressive environment that favours the survival and seeding of disseminating cancer cells.

It has been demonstrated that the production of inflammatory mediators by the tumor cells from the primary tumor promotes the recruitment of myeloid cells to the pre-metastatic lung. For instance, TNF- α and TGF- β can stimulate the production of small calcium-binding proteins, like S100A8 and S100A9, which serve as chemo-attractants for myeloid cells (Hiratsuka et al., 2006). Myeloid cells were first implicated in pre-metastatic lung, where they were recruited as monocyte-like VEGFR1+ CD11b+ BM-derived cells that adhere to fibronectin areas by very late antigen 4 (VLA-4) (Kaplan et al., 2005).

Primary tumor cells, such as many other cell types, are also known to release extracellular vesicles (EVs) into the bloodstream. These EVs are generally composed of proteins, lipids, DNA, mRNA and microRNAs. However, it has been shown that those EVs that are secreted by tumor cells, can facilitate the development of an immunosuppressive pre-metastatic niche (Wortzel et al., 2019). It has been demonstrated that EVs generated by pancreatic ductal carcinoma contain macrophage migration inhibitory factor 1 (MIF-1), which is known to be involved in liver metastasis formation. Extracellular vesicle-derived MIF-1 was shown to stimulate Kupffer cells to secrete TGF- β and was involved in macrophage infiltration and subsequent fibronectin remodelling (Costa-Silva et al., 2015).

Clinical research demonstrates that neoadjuvant chemotherapy recipients are more likely to develop metastases. An explanation for this phenomenon was offered by a recent study which showed that the administration of neoadjuvant chemotherapy in a breast cancer model increases EV production in comparison to untreated breast cancer. They found that this effect accelerated the rate of metastasis. Mechanistically, they showed that cancer cell-secreted EVs contain Annexin A6, which activate the NF- κ B pathway in endothelial cells, which, in return, secrete CCL2. This

endothelial-derived CCL2 attracts Ly6C+CCR2+ monocytes to the lung and facilitates the metastatic seeding (Keklikoglou et al., 2019).

Cox et al. observed an increased lysyl oxidase (LOX) expression in primary, ER- breast cancer cells upon bone metastasis. They found that, LOX stimulates osteoclastogenesis and by cross-linking collagen 4 facilitates the adhesion of bone marrow-derived macrophages and the subsequent cancer cell extravasation into the bone (Cox et al., 2015). The secretion of TGF- β by the primary tumor cells has also been implicated in bone metastasis by triggering osteoclastogenesis and the release of MMP9, which also promote the formation of the pre-metastatic niche (Luis-Ravelo et al., 2011). Additionally, LOX produced by hypoxic ER-mammary cancer cells localizes to areas of the lung that are rich in fibronectin, collagen IV cross-links and supports BMDM recruitment (Erler et al., 2009).

Macrophages in extravasation and early colonization of cancer cells

Interactions between arriving cancer cells and macrophages at the metastatic sites accelerate the metastatic behaviour. Frequently, cancer cells are recruited to the metastatic site using the same mechanisms as macrophages employ to attract leukocytes during inflammation. These mechanisms include for example increasing adhesion and transmigration. Also, the same processes that macrophages use to promote tissue repair can be applied to assist secondary tumor formation. These include suppressing immune responses, fostering cellular proliferation, activating angiogenesis and remodeling the matrix. Furthermore, to maintain the pro-tumorigenic immunological milieu, these MAMs continuously recruit bone marrow-derived macrophages.

There is strong evidence from numerous studies that the development of a metastatic lesion depends on the presence of metastasis-associated macrophages during the metastatic extravasation process (Headley et al., 2016b; Kitamura et al., 2015; B. Qian et al., 2009). Following macrophage depletion with L-clodronate, Qian et al. injected mice with breast cancer cells into the tail veins. They discovered that the number of cancer cells in the lung of macrophage-depleted mice was significantly lower 36 hours after injection, indicating that macrophages help the extravasation of disseminated tumor cells into the lung. Additionally, the tumor growth rate in the animals with macrophages was twice as fast as in mice without macrophages, emphasising the significance of

macrophages in maintaining a pro-tumoral microenvironment. Furthermore, CD11b+ BMDMs were interacting directly with extravascular cancer cells, demonstrating that the adhesion of tumor cells to macrophages might also help to transmigrate the endothelium (B. Qian et al., 2009).

Direct evidence links CCR2 expression by macrophages to the development of the metastatic disease (Kitamura et al., 2015). The activation of CCL2/CCR2 stimulates macrophages to produce CCL3, which, in a CCR1-dependent manner, assists to the retention of macrophages at the metastatic site. In addition, the CCL3/CCR1 axis was shown to be crucial for the direct interaction between macrophages and cancer cells *via* an $\alpha 4$ -integrin mediated processes (Kitamura et al., 2015; B. Qian et al., 2009).

Macrophage-expressed $\alpha 4$ -integrin is known to bind to VCAM-1, which is generally present on leukocytes and endothelial cells, however it also can be overexpressed by tumor cells (Holzmann et al., 1998; Osborn et al., 1989). According to Chen et al., upon mammary fat pad injection, VCAM-1-silenced MDA231-LM2-4175 cancer cells proliferated at the site of injection but generated significantly less metastasis in the lung compared to the non-silenced cells, due to their lower binding affinity to macrophages. Although VCAM-1-silencing did not affect the accumulation of tumor cells within the lung following tail vein injection, significantly reduced metastatic outgrowth was observed compared to non-silenced counterparts. Thus, the macrophage-cancer cell interaction *via* VCAM-1 and $\alpha 4$ -integrin does not necessary for the colonization of the lung by the metastatic cells but regulates the expansion of the metastatic lesions. The molecular background of this phenomenon was also revealed. It has been demonstrated that the interaction between the macrophage $\alpha 4$ -integrin and the VCAM-1 activates the PI3K-AKT pathway in cancer cells which leads to increased migration, survival and reduced apoptosis of the cancer cells and thus promote secondary cancer formation (Q. Chen et al., 2011; Vara et al., 2004).

Other macrophage-derived factors, such as VEGF and endothelin-1 (ET-1) have also been demonstrated to enhance the metastatic colonization. It has been shown in mammary tumor models that macrophage-derived VEGF helps the retention of MET-1 mammary tumor cells in the lung. Additionally, *in vitro* studies demonstrated that VEGF drives the migration of MET-1 cells across the endothelium by increasing the endothelial permeability (B.-Z. Qian et al., 2011). In addition to endothelial cells, VEGF may also act on VEGFR1-expressing macrophages to trigger the secretion of MMP9 and C-X-C Motif Chemokine Ligand 12 (CXCL-12) (Hiratsuka et al., 2002). MMP9 drives the development of tumors by increasing the permeability of the vessels, degrading the

extracellular matrix, cleaving several growth factors that subsequently promote tumor growth and also by promoting angiogenesis to fuel the high nutrient requirements of proliferating cancer cells (Lamagna et al., 2006). Additionally, the cycle of bone marrow-derived macrophage recruitment is also supported by macrophage VEGF-VEGFR1 signalling, by establishing gradients of the CXCL12 chemokine, a potent chemoattractant for monocytes and CXCR4+ cancer cells (Cardones et al., 2003; Kaplan et al., 2005; B.-Z. Qian et al., 2011).

The previously mentioned ET-1 is a soluble vasoconstrictor, that has been also implicated in the promotion of the metastatic colonization. Tail-vein injection of muscle-invasive bladder cancer cells that has been knocked down for the vasoconstrictor ET-1 results in a decreased recruitment of bone marrow-derived macrophages and less expanded metastatic burden, compared to the wild type cells. Furthermore, inhibiting the ET-1 receptor on macrophages limits the release of downstream pro-tumoral cytokines, like CCL2, MMPs and IL-6 and thus reducing macrophage recruitment, angiogenesis and secondary tumor formation (Said et al., 2011; Titus et al., 2005).

In addition to influencing extravasation through direct contacts with cancer cells, macrophages recruited to secondary sites can also interact with other stromal cells in the pre-metastatic microenvironment to facilitate metastasis. As mentioned in earlier sections, interactions between macrophages and endothelial cells can increase the ability of cancer cells to metastasize by enhancing endothelial affinity and permeability, which promotes circulating tumor cell adherence and transmigration into new the metastatic loci (Akkari et al., 2014; Hiratsuka et al., 2002; B.-Z. Qian et al., 2011; Wyckoff et al., 2004). Additionally, it is well described that TAMs can also be polarized by CAFs in order to conduct pro-tumorigenic actions at the primary site (Chiarugi, 2013). The collaboration of CAFs and metastasis-associated macrophages has also been emphasised at the secondary site. It has been described in both liver and lung metastasis that the primary tumor stimulated the release of fibronectin by fibroblasts, which attracts BMDMs to the secondary location (Erler et al., 2009). Also, as mentioned previously, macrophages recruited to the liver promote the metastasis formation of pancreatic ductal adenocarcinoma by secreting granulins, thus activating hepatic stellate cells into myofibroblasts. These myofibroblasts subsequently promote the progression of metastatic tumors *via* the secretion of periostin, which stimulates the Wnt and $\alpha_v\beta_3$ -Akt/PKB signalling pathways in PDAC cells. Granulin depletion hindered the formation of the MAM-dependent fibrotic environment and the following expansion

of PDAC metastatic cells, while inhibition of periostin secretion by CAFs substantially reduced the metastatic outgrowth of PDAC cells (Nielsen et al., 2016).

Even though these studies emphasise the significance of metastasis-associated macrophages in mechanisms supporting tumor cell extravasation and expansion at the metastatic site, given the variety of macrophage functions at the primary sites, there might be additional MAM-related contributory factors to metastasis. Additional studies are needed to evaluate the implications of macrophages at the metastatic site, as well as their role in determining the cell fate of the disseminated tumor cells.

Interactions between macrophages and the extracellular matrix

Mechanisms of ECM remodelling

The cellular phenotypes and their molecular determinants are not only dependent on cell-cell interactions, but also on the bidirectional communication between the cells and the extracellular matrix (Winkler et al., 2020). About 300 different matrix macromolecules has been described within the core matrixome. These macromolecules can be categorized as collagens, glycoproteins (such as laminin, fibronectin, tenascin, elastin) and proteoglycans (like heparan sulphate proteoglycans or hyaluronan) (Naba et al., 2016). Furthermore, a variety of remodelling enzymes, including oxidases and proteases have the ability to modify these ECM components post-translationally. Additionally, the ECM also binds different soluble factors, including growth factors and several other ECM-associated proteins. Notably, these ECM-bound components and factors can interact with cell surface receptors, thus mediate a number of different cellular processes (Hastings et al., 2019; Humphrey et al., 2014; Winkler et al., 2020).

There are two main types of ECM, one is the interstitial matrix, which interconnects cells within the stroma and to the second type of ECM, which is referred to as basement membrane. The interstitial matrix not only controls processes like cell migration and differentiation but also ensures the structural integrity of tissues and organs. The interstitial matrix is mostly composed of fibronectin, elastin, and collagens I, III and V. The composition differs between tissue types and even microenvironments within the same tissue. Furthermore, it also can change in response to mechanical stress or damage, for example during wound healing or tissue regeneration. Remodelling of the interstitial ECM in cancer results in a wide spectrum of biophysical and biochemical alterations that have an impact on ECM stiffness, cell signalling, cell migration and cancer development (Egeblad et al., 2010). The basement membrane, in contrast, is a more substantial, thick layer that covers the basal surface of several cell types, including epithelial and endothelial cells, encloses muscle cells, surrounds adipocytes and divides tissues into various, orderly compartments. The major components of the basement membrane are collagen IV and laminins, which are linked by a variety of network-bridging proteins as nidogen and heparan sulphate proteoglycans (HSPGs) (Pozzi et al., 2017). For the establishment of epithelial cell polarity, as well as for many developmental events and the maintenance of tissue homeostasis, cell binding to the basement membrane is crucial. Also, cancer cells must remodel the basement

membrane in order to invade the stromal tissue and develop into a malignant tumor (Jayadev & Sherwood, 2017; Winkler et al., 2020).

We differentiate 4 different ECM remodelling mechanisms: (1) Deposition of ECM, which affects the biochemical and mechanical characteristics of the ECM through altering the abundance and composition of ECM components; (2) Chemical modification, that impacts the biochemical and structural features of the ECM at post-translational level; (3) Proteolytic degradation which liberates bioactive ECM fragments and ECM-bound components; (4) Force-mediated ECM remodelling, which changes the organization of the extracellular matrix by aligning ECM fibers and creating channels for cell migration (Figure 15).

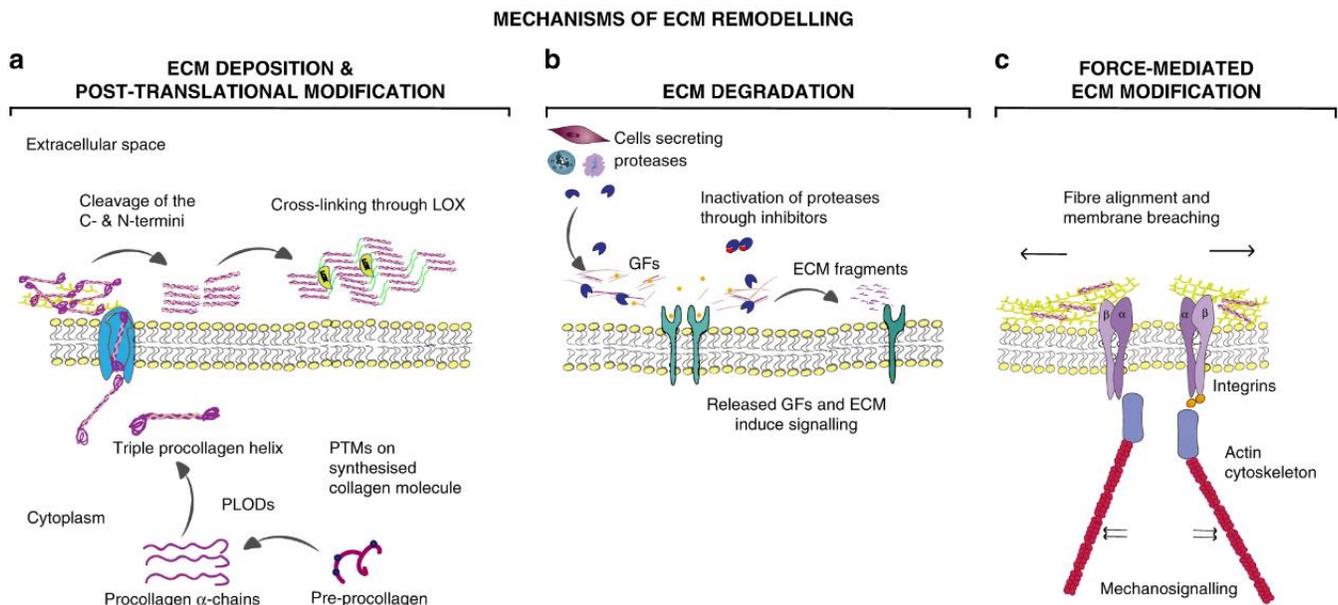


Figure 15: The different mechanisms of ECM remodelling

- a. ECM deposition and post-translational modification:** Synthesized pre-procollagen is transferred to the Golgi where it is converted into a procollagen α -chain. Then, it undergoes various post-translational modifications (PTMs). These PTMs include the hydroxylation of lysine residues via procollagen-lysine 2-oxoglutarate 5-dioxygenase (PLOD), which enables the triple helix formation and thus the secretion into the extracellular space. Here, proteases cleave the C- and N-terminal of the pro-peptides to form collagen fibrils. These collagen fibrils can be cross-linked by lysyl oxidases (LOX).
- b. ECM degradation:** Proteases, such as MMPs, cathepsins and serine proteases degrade the extracellular matrix and thus releasing matrix-bound growth factors (GFs), cytokines and ECM fragments, such as matrikines, but also removing the barriers of cell migration.
- c. Force-mediated ECM modification:** Integrin binding to the ECM exerts forces on the ECM molecules, which may alter the conformation of the ECM molecule and expose binding sites, that could facilitate fibril production and fiber alignment.

Adapted from (Winkler et al., 2020)

ECM-mediated macrophage infiltration in the tumor

Generally, immune infiltration determined by immune cell density in malignancies serves as a prognostic factor (Galon et al., 2012). The TAM infiltration varies in density across different cancer types, but TAMs are especially prevalent in breast tumors, where they constitute as much as 50% of the cancerous mass (Pollard, 2008). Correlational results imply a relationship between the nature of the tumor-specific ECM and the infiltration of TAMs. In a cohort of 278 women with breast cancer, increased hyaluronan deposition, an ECM glycosaminoglycan, was associated with higher macrophage numbers and a poor prognosis regardless of the tumor subtype (Tiainen et al., 2015). Furthermore, in 20 breast cancer patients, the quantity of macrophages at the invasive front was positively correlated with ECM stiffness and activation of TGF- β signalling, both of which are typically linked to fibrosis (Acerbi et al., 2015).

In mouse breast tumorigenesis models, such associations have also been observed. For example, in a spontaneous mammary carcinogenesis model, higher presence of TAMs within the tumor was associated with increased collagen deposition (Esbona et al., 2016). Similar to this, constitutive synthesis of CCL2 in the mammary epithelium, which promotes macrophage infiltration, was linked to elevated stromal deposition of collagen, which may increase the risk of tumor development (Sun et al., 2017). Additionally, CCL2 overexpressing stromal cells in the mammary glands increased the infiltration of macrophages within the breast and subsequently lead to elevated collagen deposition. However, when CD11b-expressing cells were depleted, both of these effects were abolished (Kuziel et al., 2020). These findings imply that tumors can alter the CCL2/CCR2 pathway to promote the influx of collagen producing macrophages.

Using an orthotopic breast cancer model, where the grafted cancer cells were modified to express either high or low levels of Tenascin-C, Deligne et al. found that the accumulation of macrophages within the Tenascin-C high tumors were significantly higher compared to Tenascin-C low tumors. Additionally, they observed, that the macrophage infiltration followed exclusively those “tracks”, that were generated by the deposition of Tenascin-C. Moreover, treating mice with anti-Tenascin-C antibodies lead to the accumulation of macrophages only at the edge of the tumor, while in non-treated mice the majority of tumor infiltrating macrophages invaded the entire tumor (Deligne et al., 2020)(Figure 16). These findings demonstrate the ability of ECM molecules to stimulate TAM infiltration during carcinogenesis and indicate that the tumor-specific matrix can

regulate the spatial emplacement of TAMs once they entered the TME (Deligne & Midwood, 2021).

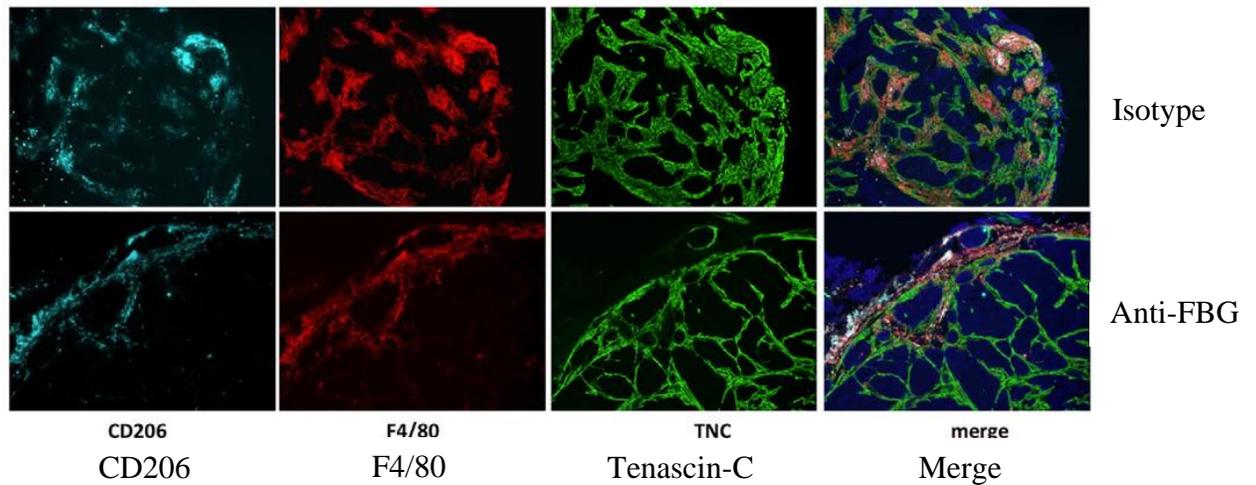


Figure 16: Tenascin-C-dependent macrophage accumulation within the TME

Blocking the FBG domain of Tenascin-C decreases the rate of macrophage accumulation within the TME and confines their presence to the tumor edge. Mammary tumors were treated with anti-FBG antibody (lower panels) or its isotype control (upper panels) and were collected 21 days following engraftment and stained (CD206-light blue, F4/80-red, anti-Tenascin-C-green).

Adapted from: (Deligne & Midwood, 2021)

Macrophages as shapers of the extracellular matrix

The tumor-associated matrix has the ability to induce the infiltration of macrophages into the tumor microenvironment and evidence suggest that it can even alter the phenotype of the arriving macrophages (Deligne & Midwood, 2021). However, not only the matrix can affect the macrophages, but also macrophages are capable to directly alter the organization and the composition of the extracellular matrix. Interestingly, Gallerand and his collaborators demonstrated, that monocytes and macrophages are essential for the expansion of the brown adipose tissue and showed that monocyte depletion led to altered tissue architecture, thus highlighting the importance of monocyte recruitment in tissue remodelling (Gallerand et al., 2021).

Matrix metalloproteases

Macrophages secrete multiple types of matrix metalloproteases (MMPs). MMPs are enzymes that are capable of degrading the ECM proteins and thus are essential for promoting cell migration (Paolillo & Schinelli, 2019). In addition to promoting migration, in breast cancer, MMP-2, 9, 13 and 14, were shown to be associated to a wide range of other cancer-related processes, such as neo-angiogenesis and metastasis (Jena & Janjanam, 2018).

In CSF-KO mice – where macrophage differentiation is impaired – both the number of mammary gland resident macrophages and the circulating macrophages were significantly decreased. Although the level of collagen within the mammary tissue did not change, collagen fibrillogenesis into long fibers was impaired in CSF-KO mice (Ingman et al., 2006). Furthermore, in an orthotopic breast cancer model, in the absence of TAMs, the microstructure of the fibrillar collagen within the tumor was also altered and these alterations were associated to a decrease in the number of lung metastases (Burke et al., 2013). This phenomenon could be explained by the fact, that macrophage-derived MMPs contribute to the degradation of the ECM.

In a CCL4-induced murine liver fibrosis model, treatment with anti-lysyl oxidase like 2 (LOXL2) was shown to improve the resolution of fibrosis *via* facilitating the accumulation of MMP-14 secreting monocyte-derived macrophages. Both the increased accumulation of macrophages and the elevated collagenolytic activity were reversed by the inducible and selective suppression of infiltrating macrophages (Klepfish et al., 2020).

Lysyl oxidases

Lysyl oxidases and Lysyl oxidase like 1-4 (LOXL1-4) are amine oxidases and are known to initiate the formation of covalent crosslinks between the collagen fibers of the extracellular matrix *via* oxidizing the lysine residues, thus stiffening the extracellular matrix (Rucker et al., 1998). Importantly, it has been shown that the development of metastases and cell migration are promoted by this action. Therefore, inhibition of these enzymes—specifically LOXL2—has been proposed as a therapeutic approach to prevent the spread of breast cancer (Ramos et al., 2022).

There is evidence showing that macrophages can modify extracellular matrix *via* LOX enzymes. Huang and collaborators found that LOX expression was significantly upregulated in the adipose tissue of obese mice (high fat diet receiving or ob/ob), compared to lean mice. Moreover, they observed higher LOX protein level, enzymatic activity and increased matrix stiffness in this tissue. Interestingly, histological analysis revealed that the LOX signal was predominantly co-localised with F4/80 macrophage signals. Furthermore, the LOX mRNA level, protein level and enzymatic activity was all found to be significantly elevated in macrophages isolated from obese mice compared to macrophages isolated from lean mice. Also, these obesity-associated macrophages showed capability to remodel and stiffen a type I collagen gel *in vitro* (Huang et al., 2021).

Additionally, macrophages can also trigger LOX-mediated matrix modifications via in-direct mechanisms. It was shown in a PDAC model, that macrophage-secreted oncostatin M (OSM) can act as an inducer of LOXL2 expression in cancer cells, which then promoted both primary and metastatic cancer expansion and lead to decreased overall survival (Alonso-Nocelo et al., 2022).

Serine proteases

Macrophages also secrete a variety of serine proteases, such as thrombin, plasminogen activators (PA), plasmin, trypsin and neuropsin. Among these, the urokinase plasminogen activator (uPA) was found to be implicated in the degradation of collagen IV, laminin, fibronectin and fibrin. uPa is secreted in an inactive form, called pro-uPA and various proteases, like cathepsin B and L, trypsin and kallikrein can transform it into active uPA by limited proteolysis. Once uPA is activated, it can bind to its receptor, referred as urokinase plasminogen activator receptor (uPAR),

which is a glycosyl-phosphatidylinositol (GPI)-anchored cell membrane receptor. The majority of the solid tumors, including breast, prostate, head and neck and brain cancer express high levels uPAR. The uPA-uPAR interaction results in the conversion of plasminogen into plasmin, which in turn get released from the cell surface to degrade the extracellular matrix both directly and *via* the activation of other proteases, like pro-MMPs. uPA is mainly expressed by tumor-associated macrophages and fibroblasts. The role of uPA has been described in several cancer-related mechanisms. As an example, the interaction between uPAR and distinct ECM elements can control cell adhesion, as well as the migration of cancer cells (Mahmood et al., 2018). Moreover, uPa promotes cancer cell proliferation by the proteolytic activation of growth factors, such as EGF, fibroblast growth factor-2 (FGF-2) or hepatocyte growth factor (HGF) (Naldini et al., 1995; TANIGUCHI et al., 1998). Furthermore, as it was described previously, the uPA-uPAR system has also been implicated in dormancy escape *via* a ligand-independent activation of the EGFR and subsequent ERK activation (Aguirre-Ghiso et al., 2001).

Cathepsins

As a part of the interstitial collagen turnover in physiologic conditions, collagen is fragmented by pericellular collagenases, endocytosed *via* collagen receptors, then transported to the lysosomes, where cathepsins further degrade it. Malignant tumors are generally defined by high extracellular matrix turnover rates. Madsen et al. demonstrated that tumor-associated macrophages are the main cell type involved in the endolytic collagen degradation within the tumor. They found that CCR2+ monocyte-derived TAMs degrade collagen through cellular uptake in a mannose receptor-dependent manner, followed by lysosomal degradation *via* cathepsins. Accordingly, they showed that mannose receptor-deficient mice exhibit elevated levels of intratumoral collagen (Madsen et al., 2017).

These findings indicate that macrophages are involved in the matrix organization and re-organization, while additional research suggests that macrophages may also contribute to the *de novo* synthesis of the tumor-specific ECM.

Secretion of matrix components

Extracellular matrix elements can be directly secreted by macrophages. This was confirmed by an RNA expression analysis of primary human cells, which revealed that the majority of activated myeloid cells are capable of secreting Tenascin-C (Giblin et al., 2020). Similar to this, it was shown, that TLR4-dependent stimulation of macrophages enables them to synthesize distinct forms of collagen (Piccinini et al., 2016). In another mammary tumor engraftment, CCL5 secretion by dormant tumor cells lead to the accumulation of CCR5+ macrophages within the tumor microenvironment. These infiltrated macrophages were shown to facilitate the emergence of cancer cells from dormancy *via* increased collagen deposition (Walens, Dimarco, et al., 2019). Although little is known about the importance of macrophages in the synthesis of matrix elements within the breast, some information are available from other tumor types. As an example, using an orthotopic colorectal cancer model, Afik et al. observed that the composition of the extracellular matrix in tumors with high or low levels of TAM differs significantly. They also demonstrated that monocytes upregulate programs related to matrix remodelling upon differentiation into TAMs. These include genes associated with the synthesis and assembly of collagen types I, VI and XIV. Moreover, they show by imaging that TAMs also regulate the deposition, cross-linking and linearization of the collagen fibers within the tumor (Afik et al., 2016).

Results

Objectives

Worldwide, breast cancer is the most frequently diagnosed malignant tumor in women. In addition, breast cancer not only leads the list of incidences but also represents 15% of all cancer-related deaths in women (Bray et al., 2018). Metastasis is the main cause of breast cancer-related mortality and the biggest challenge facing clinical oncologists is their inability to prevent and cure recurrent cancer.

The term "disseminated cancer cells" (DTCs) refers to tumor cells that have physically separated from the primary tumor and spread to a distant location. These DTCs may remain in a dormant, non-proliferative state, thereby escaping the various therapeutic approaches. However, for reasons that are not fully understood, dormant cancer cells can resume their proliferation, thus leading to the formation of metastatic outgrowth and recurrence (Klein, 2011). Therefore, it is crucial to better comprehend those mechanisms which regulate dormancy escape.

Evidence suggests that chronic inflammation is associated with an increased risk for breast cancer recurrence (Bowers et al., 2014; Hughes et al., 2015). Macrophages represent the major cell type that regulates the initiation, maintenance, and resolution of inflammation (Nagatoshi et al., 2005) and their participation has been already described in numerous mechanisms that influence the success rate of the metastatic process. However, their involvement in the regulation of the dormancy state of cancer cells is still poorly understood.

Thus, the main objectives of my thesis were to:

1. Investigate whether direct interactions between M-CSF-activated primary macrophages and dormant cancer cells could lead to the awakening of dormant cancer cells
2. Identify the macrophage-derived mechanisms which mediate dormancy escape
3. Uncover which molecular pathways are activated in the dormant cancer cells upon macrophage-induced awakening.

Summary of the results

Despite the significant efforts focused on the understanding of the mechanisms that regulate dormancy, our knowledge on cancer dormancy regulation is still very limited. Therefore, during my doctoral studies I aimed to investigate the direct interactions of dormant cancer cells with one of the main cellular component of the stroma, macrophages.

In order to study these interactions between dormant cancer cells and macrophages, we used the D2.OR cell line, which was generated from spontaneous malignant tumors of Balb/C mouse mammary gland. These cancer cells show dormant phenotype when maintained on basement membrane matrices (Matrigel) in low serum containing media (Barkan et al. J. Vis. Exp. 2011). The dormant phenotype of D2.OR can be characterized as circular, solitary cells that do not proliferate (Ki67-). However, alterations in the microenvironment might trigger the awakening of these dormant cells and induce their proliferation.

Using this model, we established an *in vitro* co-culture system of bone marrow-derived, M-CSF-activated macrophages and dormant cancer cells to investigate whether macrophages themselves could support in the awakening of dormant cancer cells. Interestingly, we found that in such co-cultures, M-CSF-activated primary macrophages induced the awakening of dormant D2.OR cells as these cells formed macroscopic outgrowths and expressed the proliferation marker Ki67. However, in co-cultures with non-activated bone marrow-derived cells, neither cancer expansion nor proliferation was detected, demonstrating that non-differentiated bone marrow-derived cells cannot induce awakening. **Thus, we demonstrated for the first time that dormancy escape can be initiated by functional interactions between macrophages and dormant cancer cells.**

Then, we aimed to identify the macrophage-derived mechanisms which mediate dormancy escape. We tested whether macrophages trigger cancer cell awakening *via* direct cell-cell interactions or by secreted molecules. We found that treatment with conditioned media, collected from M-CSF-activated macrophages is sufficient to induce dormancy escape, indicating that soluble factors mediate this process.

Our next aim was to identify which macrophage-secreted molecules are involved in the macrophage-mediated cancer cell awakening. In order to compare the secretome of macrophages and bone marrow-derived cells we probed a proteome profiler screen with conditioned media from

both cell types. We discovered that eight molecules were upregulated upon M-CSF-activation of bone marrow-derived cells. We sought to confirm, whether among the identified molecules we could determine those that trigger the macrophage-mediated cancer cell awakening. For this, we used recombinant proteins, blocking antibodies and inhibitor treatments. We found that blocking MMP9 activity with an inhibitor treatment significantly decreased the awakening potency of the macrophage conditioned media, indicating that **macrophage-derived MMP9 is involved in the macrophage-mediated cancer cell awakening**. However, recombinant pro- or active MMP9 treatments were insufficient to restore the growth of dormant cancer cells, suggesting that MMP9 works in concert with other factors to enable dormancy escape.

MMP9 is associated with increased matrix remodelling, therefore we hypothesised that macrophages might mediate cancer cell awakening by matrix modifications. To test this hypothesis, we generated conditioned media-primed matrices. We found that these primed matrices could trigger the awakening of dormant cancer cells, suggesting that **macrophages mediate dormancy escape via matrix modifications**. Therefore, we performed a mini screen to identify which macrophage-mediated matrix modifications are involved in this process. We found that inhibition of lysyl oxidase activity within the macrophage conditioned media with a specific inhibitor (BAPN) decreased the awakening of D2.OR cells. Additionally, dual inhibition of MMP9 and LOX activity hampered D2.OR awakening, indicating that **MMP9 and ECM stiffening by LOX might cooperate to promote the macrophage-induced awakening of breast cancer cells**.

We also aimed to uncover the molecular pathways in the dormant cancer cells which are activated upon macrophage-mediated awakening. Our results suggest that **macrophage-derived matrix modifications activate an integrin- β 1/FAK/Src axis that triggers Her2 receptor tyrosine kinase activation in a ligand-independent manner**. This leads to the activation of ERK/MAP Kinases, which facilitate the switch from a dormant to a proliferative state.

In conclusion, we identified macrophages as a new cell type that is able to trigger the dormancy escape of cancer cells. We demonstrated that macrophage derived MMP9 and LOX activities are involved in the formation of an extracellular matrix that enables the growth of dormant cancer cells. Furthermore, we showed that D2.OR cells sense these matrix modifications *via* integrin- β 1, which triggers a ligand-independent Her2 activation and result in the awakening of dormant cancer cells through ERK activation.

Manuscript

M-CSF-activated macrophages prime the extracellular matrix to promote awakening of dormant breast cancer cells.

Kitti Jenovai¹, Pascal Lopez¹, Enora Lecorgne¹, Isabelle Bourget¹, Jean Albregues¹ and Cedric Gaggioli¹.

1. University Cote d'Azur, INSERM U1081, CNRS UMR7284, Institute for Research on Cancer and Aging, Nice (IRCAN), Medical School, 28 Avenue Valombrose, F-06107, Nice, France.

*Corresponding author

Cedric Gaggioli, PhD

Université Cote d'Azur

Institute for Research on Cancer and Aging, Nice

INSERM, U1081, CNRS, UMR7284

28. Av de Valombrose 06107 Nice Cedex 02, France

Tel: + 33 (0) 4 93 37 77 53

Fax: +33 (0) 4 93 37 76 76

Email : gaggioli@unice.fr

Running title: macrophages awaken dormant cancer cells.

Key words: macrophages, MMP9, extracellular matrix, dormancy and recurrence.

The authors declare no conflict of interest.

Abstract

Breast cancer relapse and metastatic recurrence are frequent and are responsible for the death of patients. Tumor recurrence at metastatic or primary lesion sites arise from disseminated dormant cells, that can stay quiescent for years, and are insensitive to therapies. It is therefore essential to determine how dormant cancer cells awake to identify new therapeutic perspectives and therefore, prevent recurrence. While inflammation has been widely linked to tumor initiation and metastatic development, yet the role of macrophages in dormant breast cancer cell awakening remains poorly understood. Three-dimensional (3D) co-culture of dormant breast cancer cells and primary macrophages identified that macrophages prime the extracellular matrix (ECM) to promote the awakening of the dormant cancer cells. We demonstrate that macrophages secrete both MMP9 and LOX to induce ECM remodelling and subsequent awakening of quiescent tumor cells through active proliferation. Mechanistically, we identify Her2 activation in the dormant cancer cells as the key factor of the macrophage-dependent awakening. Collectively, our findings link inflammation and awakening of metastatic dormant cells and thereby highlight a new cellular and molecular crosstalk between macrophages and breast cancer cells that may initiate breast cancer metastatic relapse.

Introduction

Tumor recurrence is a common phenomenon in solid cancer patients, especially in breast and prostate tumors (Retsky & Demicheli, 2014; Weckermann et al., 2001). Tumoral relapse could occur decades after initial remission, either at the initial primary lesion or at metastatic sites, and these latest are responsible for the vast majority of patient death. In breast cancer, metastatic dissemination is an early event and might occur even before the primary tumor is detectable (Harper et al., 2016). At molecular level, this early dissemination is driven by a Her2+ signalling that triggers local invasion of early disseminated cancer cells and secondary target organ lodging (Harper et al., 2016). These long-term surviving early disseminated metastatic cancer cells maintained in a state of dormancy are insensitive to adjuvant therapy. So far, little is known about the biological processes that favour the survival of a presumably small fraction of disseminated tumor cells that have been quiescent for a considerable amount of time. Similarly, it is uncertain which crucial factors control latent tumor cells to enter a proliferative state that results in clinical recurrence. Understanding the molecular mechanisms of metastatic cellular dormancy escape is an important step toward the development of anti-relapse therapies.

Increased extracellular matrix (ECM) remodelling and stiffening are key features of solid tumors (Larsen et al., 2006; Pickup et al., 2014). Matrix metalloproteases (MMP) are drivers of ECM deconstruction and tumoral expansion leading to metastasis development (Kessenbrock et al., 2010; Liotta et al., 1980; Niland et al., 2021). Among many, MMP9 is a well characterized proteolytic enzyme that degrades collagen IV, which favours the dissemination and metastatic outgrowth of tumor cells, therefore its increased expression correlates with a poor clinical outcome in patients with breast cancer (Liotta et al., 1980; Ren et al., 2015). Moreover, ECM collagens are crosslinked by the LOX family enzymes, triggering an increase in ECM stiffness which ultimately

results in elevated cancer cell proliferation, therapeutic resistance and metastatic dissemination (C.-H. Lin et al., 2015; Samuel et al., 2011). Cancer cells can sense the ECM stiffening, notably through ECM transmembrane receptors, called integrins (Levental et al., 2009). ECM interactions with early disseminating cancer cells are crucial environmental factors that control both dormancy entry and exit at the metastatic sites (Aguirre Ghiso et al., 1999). At a molecular level, the ratio of ERK and p38 MAPK activities, controlled by integrin-mediated intracellular signalling activity, promotes the ECM-dependent molecular switch of dormancy escape (D. Liu et al., 2002). Moreover, increased fibrosis and LOX-dependent collagen crosslinking is involved in creating a growth permissive fibrotic microenvironment that favours metastatic growth (Cox et al., 2013). Crosstalk between myeloid cells and cancer cells triggers tumoral expansion and metastatic development. Secretion of proteases, cytokines, chemokines and growth factors by inflammatory monocytes with Ly6C expression promotes extravasation of cancer cells in the lung (Doak et al., 2018; Qian et al., 2011) and prolongs their survival in the metastatic niche (Chen et al., 2011). The differentiation of monocytes into macrophages has been also implied in the metastatic outgrowth of cancer cells in an indirect manner. Macrophages promote metastatic outgrowth by forming a more fibrotic microenvironment through myofibroblasts. This fibrotic environment promotes integrin signalling activation, leading to dormant cancer cell activation (Nielsen et al., 2016). Macrophages can be categorized as either classically-activated (M1) or alternatively-activated (M2) macrophages. In general, it is believed that M1 polarisation promotes pro-inflammatory, tissue-destructive actions, while M2 macrophages promote rather anti-inflammatory and tissue-repair functions. Despite the fact that these definitions imply that M1 and M2 macrophages are separate entities with distinct roles, nowadays, this classification system is considered as an oversimplified view. Instead, distinct M1 and M2 phenotypes are distributed along a continuum

which allows a seamless phenotypic transition between pro-inflammatory, anti-inflammatory and homeostatic statuses (Biswas & Mantovani, 2010; Martinez & Gordon, 2015; Murray et al., 2014). The involvement of macrophages has been described in many mechanisms that influence the success rate of the metastatic process. However, whether direct interactions between macrophages and dormant cancer cells could lead to dormancy escape, has not yet been demonstrated. Consequently, in this study, we investigated the role of M-CSF polarised macrophages in the awakening of D2.OR cancer cells. Using a 3D Matrigel model of cancer dormancy, we established that macrophages trigger D2.OR dormant cell awakening and proliferative state entry. We found that secretion of MMP9, together with a LOX-dependent ECM remodelling, primed the ECM to induce awakening. At a mechanistic level, we identified EGFR signalling pathway to be crucial for D2.OR dormancy escape. Interestingly, we found that integrin β -1 and Src/FAK activation was responsible for a Her2-mediated MEK/ERK awakening, in a ligand independent manner. Altogether, this work established that macrophages promote ECM remodelling that induces a permissive microenvironmental niche that favours the emergence of metastatic recurrence.

Results

M-CSF-activated macrophages trigger the awakening of dormant D2.OR cells.

To investigate the role of M-CSF-polarised macrophages (M0) in the awakening of dormant breast cancer cells, we exploited the D2.OR murine breast tumoral cell model of dormancy (Barkan & Green, 2011). We used a 3-dimensional (3D) basement membrane matrix (Matrigel) culture system, where D2.OR cells enter in a low-cycling dormant state and developed co-culture assays with bone marrow-derived cells (BMD) or with M-CSF-activated macrophages (M0) (**Fig. 1A**). Luciferase-expressing D2.OR cells stayed as single cells with round-shape morphology, reminiscent of dormancy, when cultured on a 3D Matrigel (**Fig. 1B and 1C**). The dormancy status of the cells is demonstrated by a low basal luciferase activity (**Fig. 1D**) and an absence of Ki67 expression, as shown by immunofluorescence (**Fig. 1E and 1F**). A similar phenotype of D2.OR dormancy is observed when these tumoral cells are co-cultured with BMD cells (**Fig. 1B**). Microscopy image analysis and luciferase quantifications (**Fig. 1C and 1D**) of D2.OR awakening showed that BMD cells did not affect the dormancy status of the tumoral cells. Moreover, quantification of Ki67+ D2.OR cells also confirmed that a 4-day co-culture period with BMD cells did not trigger the shift of dormant cells to proliferation (**Fig. 1E and 1F**). However, we observed a striking macroscopic elongation of D2.OR cell clusters when co-cultured in presence of M0 cells (**Fig. 1B and 1C**) which was accompanied by a 6-fold induction of the luciferase activity, demonstrating an increase of D2.OR cell number (**Fig. 1D**). This awakening was further demonstrated by the presence of a Ki67+ staining specifically in the D2.OR cells (**Fig. 1E and 1F**).

The mode of action of the macrophage-dependent awakening of the D2.OR breast cancer cells was further studied. Macrophages are known to promote an inflammatory response through the

secretion of growth factors and cytokines, which prone tumoral development (Y. Lin et al., 2019). Thus, we investigated whether the secretome of M-CSF-activated macrophages was sufficient to promote D2.OR awakening *in vitro*. Conditioned media of BMD (BMD_CM) and M0 (M0_CM) cells were assessed for their capacity to awaken D2.OR dormant cells cultured on 3D Matrigel for a period of 14 days. We showed that only M0_CM promoted D2.OR awakening, whereas BMD_CM did not (**Fig. 1G-1I**). M0-derived secretome induced elongated D2.OR cellular shape, luciferase transcriptomic activation and importantly, the appearance of Ki67+ nuclei in D2.OR cells, indicating that tumoral cells exit from dormancy (**Fig. 1J and 1K**). Moreover, we demonstrated that the D2.OR awakening was strongly promoted by the M0 secretome and slightly by M2 (IL-4 + IL-13) polarised macrophages, whereas M1 (LPS + IFN γ) polarised macrophages were not able to promote D2.OR awakening *in vitro* (**Supp. Fig 1A-1E**). Altogether, our data indicate that M0 cells are capable of promoting a proliferative state of dormant cancer cells by a paracrine mode of action.

MMP9 is required but not sufficient for macrophage-dependent breast cancer cell awakening.

As conditioned media obtained from M-CSF-polarised macrophages was sufficient to promote tumor cell awakening, we hypothesised that the paracrine crosstalk was transduced by tertiary structured molecules. Accordingly, M0_CM lost the capacity to awake dormant D2.OR cells when subjected to heating (100°C for 15 min; **Fig. 2A and 2B**), which validated our hypothesis. Therefore, we next aimed to identify candidate molecules that may trigger dormancy exit of the cancer cells. We quantified the molecular composition of both M0 and BMD cell-conditioned media, using a membrane-based antibody array (R&D Systems; #ARY028) that allows the relative

quantification of 111 growth factors, cytokines and chemokines. We found 9 molecules (CD14, LDLR, CCL6, M-CSF, CXCL10, CXCL16, endostatin, HGF and MMP9) to be significantly upregulated in M0_CM, when compared to BMD_CM (**Fig. 2C and 2D**). Next, we tested each of the 9 identified molecules for their capacity to induce D2.OR cell awakening when seeded on the 3D Matrigel. We found that inhibition of MMP9, by specific MMP9 inhibitor, hampered D2.OR awakening, whereas none of the 8 other identified molecules impacted the awakening of dormant D2.OR cells (**Fig. 2E and Supp. Fig. 2A-2C**). Accordingly, zymography analysis of M0_CM showed both the pro- and active form of MMP9 to be secreted by M0 but not by BMD cells (**Supp. Fig. 2D**). We further tested the ability of MMP9 to promote breast cancer cell awakening by culturing D2.OR cells on the 3D Matrigel in presence of either the pro-MMP9 and an active-MMP9. In these experimental conditions, we observed that D2.OR cells stayed dormant (**Fig. 2F**). Taken together, our data demonstrate that active MMP9, secreted by M-CSF-polarized macrophages is responsible for breast cancer awakening, however, MMP9 alone was not sufficient.

MMP9 and Lysyl oxidase activities are required to promote breast cancer cell awakening by macrophages.

MMP9 plays an important role in reactive processes including inflammation and is expressed constitutively by macrophages (Newby, 2016). MMP9 degrades type IV collagen (Newby, 2016) to exert physiological and pathological ECM remodelling. We developed a 3D Matrigel matrix priming assay to assess the implication of M0-dependent matrix remodelling during D2.OR cancer cell awakening. The 3D Matrigel matrices were subjected to remodelling by M0_CM or by BMD_CM for a period of 7 day. Next, the D2.OR cells were seeded on the top of the remodelled

3D Matrigel matrices for a period of 14 day to assess their dormancy exit (**Fig. 3A**). Macroscopic analysis and Ki67 staining showed that D2.OR cells awakened when plated on primed matrix remodelled by M0_CM (PM_M0_CM) but not on BMD_CM-primed matrix (PM_BMD_CM) or control (PM_Veh.) (**Fig. 3B-D**), demonstrating that ECM remodelling is a fundamental step for macrophage-dependent dormancy exit. Next, we investigated whether MMP9 activity is required in this process. Quantification of luciferase activity from D2.OR cells demonstrated that inhibition of MMP9 activity during matrix priming by M0_CM was sufficient to significantly, but not completely, revert D2.OR awakening (**Fig. 3E and 3F**). This partial inhibition of D2.OR awakening observed in the presence of MMP9 inhibitor led us to hypothesise that MMP9 acts in concert with other proteases. Indeed, macrophages can remodel ECM by the secretion of proteases, such as tissue plasminogen activator or cathepsins that have been linked to lung metastasis development (Xiao et al., 2021) and to the recruitment of neutrophils, which are major actors of breast cancer awakening (Albregues et al., 2018). Therefore, using a pharmacological inhibition of cathepsins, tissue plasminogen activator and a blocking antibody of a neutrophil-dependent cryptic cleavage of laminin, we showed that M0-dependent awakening of D2.OR cells is not dependent on such molecular mechanisms (**Supp. Fig. 3A-3H**).

Mechanical signals from the ECM drive tumoral expansion and metastasis development (Levental et al., 2009). Lysyl oxidase like 2 (LOXL2) is secreted by macrophages to crosslink collagen and elastin to favour the metastatic dissemination of pancreatic cells (Alonso-Nocelo et al., 2022). In breast cancer, LOXL2 expression promotes dormant cancer cell awakening (Weidenfeld et al., 2016). Therefore, we investigated a potential cooperative effect of lysyl oxidase activity and MMP9 during M0-dependent D2.OR awakening. We first showed that M0 macrophages possess LOX activity, whereas BMD cells does not (**Fig. 3G**) and demonstrated that

addition of BAPN, a pan-LOX inhibitor, significantly decreased D2.OR awakening in response to M0_CM (**Fig. 3H**). Furthermore, a dual inhibition of MMP9 together with LOX activity hampered D2.OR awakening suggesting that MMP9 and ECM stiffening act in concert to promote breast cancer cell awakening by macrophages (**Fig. 3H**). Similar observation was also shown during matrix priming by M0_CM (**Fig. 3I**). Interestingly, we demonstrated that collagen IV degradation induced by M0_CM *in vitro* is transduced by MMP9 activity. Additionally, MMP9 blocking inhibited collagenase activity of the M0_CM by approximately 50% and BAPN or combinational treatments both resulted in a comparable levels of collagenase activity inhibition (**Fig. 3J and 3K**). This suggests that LOX might be an upstream regulator of MMP9 activity. Altogether, these data demonstrate that macrophages promote the awakening of breast cancer cells through the secretion of MMP9 and LOX-dependent ECM remodelling.

Macrophage-mediated matrix modifications promote ligand-independent EGFR activation to induce the awakening of dormant D2.OR cells.

EGFR activation initiates signals which can promote tumor growth. Importantly, this can occur independently of EGFR ligand binding (D. Liu et al., 2002). Instead, EGFR activation can be mediated by an integrin-dependent adhesion to ECM (Aguirre Ghiso et al., 1999). Both EGFR and its family member receptor Her2 sense matrix rigidity to be activated through a Src-dependent kinase phosphorylation (Saxena et al., 2017). In cancer cells, increased stiffening of the surrounded ECM promotes cancer expansion through EGFR activation in a ligand independent mechanism (Grasset et al., 2018). Subsequently to the demonstration that matrix modifications are involved in the M0-dependent awakening of the D2.OR cells, we investigated whether EGFR activation might be involved. We showed that indeed, EGF stimulation triggered the awakening of dormant D2.OR

cells *in vitro* (**Fig. 4A and 4B**). Additionally, pharmacological EGFR kinase inhibition by Ag1478 and Gefitinib (**Fig. 4C**) impeded the D2.OR awakening in co-cultures (**Fig. 4D**) and the awakening of the D2.OR cells by M0 conditioned media (M0_CM) (**Fig. 4E**). Interestingly, either using the membrane-based antibody array (**Supp. Fig. 4A and 4B**) or a direct ELISA assay to quantify EGF within the M0_CM we found that EGF is not secreted by the M0 cells (**Supp. Fig. 4C**). Accordingly, addition of a monoclonal blocking antibody, called Cetuximab that inhibits ligand binding to EGFR, did not impact the awakening of the D2.OR cells in co-cultures (**Fig. 4D**) and in response to M0_CM (**Fig. 4E**), while it blocked the awakening induced by EGF (**Supp. Fig. 4D**). We found that macrophage secretome promotes an EGFR-dependent awakening of dormant breast cancer cells independently of ligand activation, suggesting that EGFR activation may be triggered by a MMP9 and LOX dependent matrix remodelling.

To determine whether the bio-mechanical regulation of ECM by macrophages promotes EGFR activation in D2.OR cells, we performed a western blot analysis of Her2 receptor activation. We observed an increased phosphorylation of Her2 in response to M0_CM primed matrix, by contrast, primed matrix by BMD_CM did not promote Her2 activation (**Fig. 4F-4G**). In addition, we showed an increased phosphorylation of the FAK and ERK kinases, consistent with D2.OR awakening (**Fig. 4F-J**) (D. Liu et al., 2002). Finally, 3D Matrigel dormancy assays strongly suggested that an integrin β -1/Src/FAK/ERK signalling pathway triggered the awakening of D2.OR cells in response to M0-dependent matrix modifications, since inhibition of this signalling cascade promoted cancer cell dormancy *in vitro* (**Fig. 4K-4M**). Taken together, these results directly implicate MMP9 and LOX as mediators of macrophages-dependent ECM modifications to prompt dormant cancer cells into fast growing tumoral cells.

Discussion

Macro-metastatic growth and on the contrary, long-term dormancy of the disseminated cancer cells are dictated not only by intrinsic genetic alterations (Y. Liu et al., 2021; Nobre et al., 2022; Trieu et al., 2022), but also, by varieties of external cues (Bragado et al., 2012). Therefore, the metastatic initiation depends on interactions between disseminating cancer cells and its surrounding microenvironment within the secondary target organ (Kaplan et al., 2006).

While several stromal cell types have been implicated in the metastatic fate of disseminating cancer cells (Bragado et al., 2012), our study identifies macrophages as initiators of solitary dormant to proliferative cell switch. We demonstrate that macrophages primed the ECM to reactivate the proliferation of dormant cells. ECM priming occurs through the secretion of MMP9 within the extracellular milieu and works in concert with lysyl oxidases, leading to a ligand-independent activation of the Her2 tyrosine kinase receptor. We found that Her2 activity triggers cancer cell awakening upon macrophage-dependent ECM remodelling.

Ligand-independent activation of tyrosine receptor kinases have been demonstrated in previous studies (Grasset et al., 2018). Dormancy entry can occur following a decreased expression of the urokinase plasminogen activator receptor (uPAR) in human carcinoma cells. uPAR downregulation leads to a reduced MAPK ERK activity due to reduced avidity of the $\alpha 5 \beta 1$ integrin to fibronectin (Aguirre Ghiso et al., 1999). In our model, β -1 integrin blockage also hampered the macrophage-dependent awakening of D2.OR dormant cancer cells (**Fig. 4K and 4L**), however, uPA inhibition does not affect their quiescent phenotype when cultured on 3D Matrigel matrix (**Supp. Fig. 3**), indicating, that macrophages do not restore the uPAR/ $\alpha 5 \beta 1$ complex to resume proliferation. However, macrophage-dependent matrix priming reactivate the MAPK ERK signalling to promote awakening.

Macrophages have been long recognized as active components of the tumor microenvironment and their involvement in the progression of the primary tumor has been well characterized (Condeelis & Pollard, 2006; Lamagna et al., 2006; Pollard, 2004; Qian & Pollard, 2010; Ruffell et al., 2012; Sica et al., 2006). However, our knowledge on their contribution to dormancy and dormancy escape is very limited. Macrophages within the primary tumor microenvironment can prime disseminating tumor cells for a dormant phenotype (Borriello et al., 2021). Macrophages were also implicated in dormancy escape in an indirect manner. In a pancreatic ductal adenocarcinoma model, macrophages favour the formation of a fibrotic microenvironment, through conversion of resident stellate cells into myofibroblasts, which triggers the awakening of dormant cancer cells *via* activation of integrin signalling (Nielsen et al., 2016). Here, we demonstrate a novel mechanism of breast cancer cells awakening, linking tissue inflammation and ECM remodelling by protease secretion from macrophages. Accordingly, accumulation of myeloid cells, as a part of the inflammatory response following surgery, correlates with increased metastatic recurrence (Krall et al., 2018). Our study demonstrates that direct matrix remodelling by macrophages favour dormancy escape, therefore, providing evidence that in patients with increased risk of recurrence, inflammation should be monitored and controlled to avoid metastatic relapse.

MMPs shape the tumor microenvironment in cancer (Niland et al., 2021), their expression correlates with poor clinical outcome (Z. Liu et al., 2010) and favour the dissemination of cancer cells (Gonzalez-Avila et al., 2019). Cleavage of the ECM by MMPs is a frequent process, and their implication in cancer cell awakening has been previously documented (Albregues et al., 2018). Moreover, in breast metastasis to the brain, astrocyte-dependent accumulation of laminin-211

favours dormancy (Dai et al., 2022). A proteolytic cleavage of the laminin-211 by MMPs, following brain inflammation by microglia macrophages might initiate metastatic outgrowth. Interestingly, our work suggests a cooperative effect of MMP9 and LOX activities toward the pro-awakening ECM modifications by macrophages. Several evidence have demonstrated a link between LOXL2 and MMP9. In hepatocellular carcinoma, LOXL2 promotes MMP9 expression, thus promoting the pre-metastatic niche formation (Wu et al., 2018). In breast cancer, LOXL2 level is associated with invasive property (Kirschmann et al., 2002) by regulating the expression and activity of MMP9 (Barker et al., 2011). LOXL2 in pancreatic ductal adenocarcinoma increases both the primary and metastatic growth of cancer cells. LOXL2 expression is upregulated by Oncostatin secretion by macrophages (Alonso-Nocelo et al., 2022). In our model, we found that macrophages secrete active LOX and MMP9, and cooperation between these two matrix modifier enzymes may also exist. Therapeutic approaches to block tumoral development by targeting MMPs have failed in clinic, and clinical trials focusing on LOX inhibition in breast cancer are so far inexistent (Augoff et al., 2022; Ferreira et al., 2021). This study suggests that patients with high risk of relapse may benefit from therapeutic approaches targeting MMPs and LOX upon tissue inflammation of such organs (lung, liver or brain) where dormant breast cancer cells preferably disseminate and stay quiescent for years before awakening.

Materials and Methods

Cell culture

D2.OR cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (#21969035, Gibco) supplemented with 10% fetal bovine serum (FBS) (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122), 2mM Glutamine (#25030-024, Gibco). To generate mCherry- and luciferase-expressing cells, a pGIPz vector containing cDNA for mCherry and luciferase (Open Biosystems) was used. D2.OR cells were infected with the viral supernatants overnight in the presence of 10 µg/mL polybrene (#TR-1003-G, Sigma) and selected with 1 µg/ml puromycin (#sc-108071, Santa Cruz Biotechnology).

Bone marrow-derived cell isolation and macrophage activation

For bone marrow-derived cell isolation, Balb/C mice were sacrificed via cervical dislocation. After covering the hind legs with 70% ethanol, the skin was peeled from the top of each hind leg down over the foot. After, the hind legs were removed from the hip joint by a scissor. Both femur and tibiae were cleaned and collected from each hind leg and placed in a plastic dish containing cold RPMI (#31870025, Gibco) supplemented with 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco). Under sterile conditions, one end of each bone has been cut off using a sterile scalpel. Then, a 1ml syringe, with a 27-G sterile needle was filled with sterile RPMI (#31870025, Gibco) supplemented with 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco). Using this syringe, the bone marrow cavity of both the femur and tibiae were flushed twice or until the bone cavity appears to be white. The cells were centrifuged for 10 minutes at 1500 rpm, then the cell pellet was resuspended in bone marrow-derived cell media (RPMI (#31870025, Gibco) supplemented with 20% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco), 1mM Pyruvate (#11360070, Gibco) and β-mercaptoethanol (#M3148, Sigma-Aldrich)). 1×10^7 cells were diluted in 30 ml Bone marrow-derived cell media (BMD media) and seeded on 150 mm non-culture treated dish (#351058, Falcon) and placed in a 5% CO₂ incubator at 37°C (Day 0). For macrophage activation, the BMD media was supplemented with 10 ng/ml Macrophage-colony stimulation factor (M-CSF #416-ML-010, R&D Systems). On Day 3, additional 20 ml of fresh BMD media was added for the non-activated bone marrow-derived

cells and M-CSF-supplemented BMD media for the macrophages. On Day 5 both non-activated bone marrow-derived cells and M-CSF-activated macrophages were ready to be harvested for co-culture experiments or conditioned media preparation. For M1 and M2 macrophage differentiation M0 macrophages were further stimulated. Using Lipopolysaccharide (LPS) (#L4391, Sigma) at 100 ng/ml and murine recombinant Interferon- γ (IFN- γ) (#315-05, Peprotech) at 100U/mL for 48h for M1 phenotype or with 20ng/mL recombinant murine IL-4 (#214-14, Peprotech) and 20ng/mL recombinant murine IL-13 (#210-13, Peprotech) for 48h for M2 phenotype.

Proliferation assay in co-cultures

96-well culture-plates were coated with 50 μ L of Matrigel (#356234, Corning) and incubated at 37 °C for 20 minutes. Activated macrophages were rinsed twice with cold PBS (#14200-067, Gibco) and detached using StemPro Accutase (#A1110501, Thermo Fisher Scientific). Macrophages were centrifuged for 5 minutes at 1500 rpm, then 1×10^4 cells were resuspended in 100 μ L DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 μ g/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco) and 10ng/ml M-CSF (#416-ML-010, R&D Systems). Non-activated bone marrow-derived cells were centrifuged for 5 minutes at 1500 rpm, then 1×10^4 cells were resuspended in 100 μ L DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 μ g/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco). Both M-CSF-activated macrophages and non-activated bone marrow-derived cells were seeded on the Matrigel (#356234, Corning) coated plates. 24 h later, D2.OR mCherry-luciferase cells (2×10^3) were seeded in co-culture with either M-CSF-activated macrophages or non-activated bone marrow-derived cells or alone on Matrigel (#356234, Corning) coated plates in DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 μ g/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco). The media was changed every second day. After 4 days, 100 μ l of medium containing 5 μ g/ml of luciferin (#12507, AAT Bioquest) were added to the wells and proliferation was measured by bioluminescence imaging (BLI) using a plate reader (FLUOstar Omega Microplate Reader - BMG Labtech).

Conditioned media preparation

M-CSF-activated, M1, M2 macrophages and non-activated bone marrow-derived cells were acquired as described above. As activated macrophages are adhesive cells even on non-cell culture treated dishes (#351058, Falcon), BMD media was removed and cells were washed twice with PBS (#14200-067, Gibco), then PBS (#14200-067, Gibco) was replaced with 15ml DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco). The non-activated bone marrow-derived cells are non-adhesive cells on non-cell culture treated dishes (#351058, Falcon), therefore these cells were collected in a falcon tube and centrifuged for 5 minutes at 1500 rpm, and resuspended first in 10 ml PBS (#14200-067, Gibco), then centrifuged again for 5 minutes at 1500 rpm, then resuspended in 15ml DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco). Both macrophage and non-activated bone marrow-derived cell conditioned media was collected 24h later. The collected conditioned media was centrifuged for 5 minutes at 1500 rpm in order to eliminate any possible cell debris and used in conditioned media experiments right away or kept in -80°C until next experiments.

Proliferation assay with conditioned media

96-well culture-plates were coated with 50 µL of Matrigel (#356234, Corning) and incubated at 37 °C for 20 minutes. mCherry-luciferase cells (2×10^3) were resuspended in 100 µL of DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco), then grown on the coated wells. The next day, media were replaced with 100 µL of the indicated conditioned media (CM), supplemented with the indicated inhibitors. The CM was changed every second days. After 14 days, treatments were replaced with 100 µL CellTiter-Glo® 3D assay reagent (#G9681, Promega). Following a 5-minute incubation on an orbital shaker, proliferation was quantified by luminescence measurements using a plate reader (FLUOstar Omega Microplate Reader - BMG Labtech).

Proteome Profiler Antibody Array: membrane-based assay

Both BMD_CM and M0_CM were prepared as previously described. The assay was performed following the manufacturer's instructions (#ARY028, R&D Systems).

Gelatin zymography

Conditioned media from M0 macrophages or BMD cells were collected as described in previous sections in serum-free DMEM (#21969035, Gibco). Conditioned media was first concentrated, using 3kDa centrifugal filters (#UFC900308), then mixed at a 1:1 ratio with loading buffer (0.25 M Tris, pH 6.8, 2% SDS, 4% sucrose, bromophenol blue). Samples were then loaded on 10% SDS-PAGE gels containing 1 mg/ml gelatin (#G1890, Sigma). Following electrophoresis, the gels were rinsed twice in water and incubated in 2.5% Triton X-100 for 90 min at room temperature. The gels were then washed twice in substrate buffer (50 mM Tris pH 7.4, 200 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂) and incubated in substrate buffer at 37 °C for 20 h. Gels were stained with Imperial Protein Stain for 2 h (#24615, Thermo Scientific) and destained in ultrapure water overnight. Enzymatic activities appear as clear bands in a blue background.

Extracellular matrix remodelling assay

In this assay, 96-well culture-plates were coated with 50 µL of Matrigel (#356234, Corning) and incubated at 37 °C for 20 minutes. Matrigel (#356234, Corning) coated plates were then incubated with DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco) or BMD_CM or M0_CM (with or without inhibitors, as indicated in figure legends) for a period of 7 days at 37 °C. The primed matrices were then washed twice with phosphate buffer saline (PBS #14200-067, Gibco) before adding cancer cells (1x10³) to the culture. After 14 days, treatments were replaced with 100 µL CellTiter-Glo® 3D assay reagent (#G9681, Promega). Following a 5-minute incubation on an orbital shaker, proliferation was quantified by luminescence measurements using a plate reader (FLUOstar Omega Microplate Reader - BMG Labtech).

Lysyl Oxidase Activity Assay Kit (Fluorometric)

BMD_CM and M0_CM were prepared as previously described, then concentrated, using 3kDa centrifugal filters (#UFC900308). The assay was performed following the manufacturer's instructions (#ab112139, Abcam). Recombinant Mouse Lysyl Oxidase Homolog 2 /LOXL2 (#9259-AO-020, , R&D Systems) was used as positive control.

Immunofluorescence of cell cultures

For immunofluorescent analysis we used Matrigel-coated (50µg/ml in PBS overnight, 37°C) 0.2 kPa hydrogels, which were bound to a glass bottom in 96-well plate (#SW6-EC-0.2, Cell guidance systems). For the immunofluorescent analysis of co-cultures, BMD cells and M0 macrophages were labelled with Vibrant cell-labeling solution (#V22886, Invitrogen). D2.OR cells were then seeded in co-cultures with BMD cells or M0 macrophages (for a period of 4 day) or seeded alone and treated with the indicated conditioned media as previously described. For the immunofluorescent analysis of primed-matrices, we incubated these Matrigel-coated hydrogels for 7 days with DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco) or BMD_CM or M0_CM. Then, primed-matrices were washed twice with PBS (#14200-067, Gibco) before adding cancer cells (1×10^3) to the culture. After 14 days, cells were fixed with 4% PFA for 20 min at room temperature, rinsed twice in PBS (#14200-067, Gibco), incubated in 50 mM of NH₄Cl for 10 min, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were then incubated in blocking buffer (PBS containing 1% BSA) for 30 min and incubated with antibody against Ki67 (#NB110-89717, Novus Biologicals) . After two washes in PBS (#14200-067, Gibco), cells were incubated in the presence of Phalloidin for 40 min, rinsed twice in PBS (#14200-067, Gibco), stained with DAPI for 5 min, rinsed in water, and placed in PBS (#14200-067, Gibco) for subsequent analysis.

Enzyme-linked immunosorbent assay (ELISA) for EGF

BMD_CM, M0_CM, M1_CM, M2_CM were prepared as previously described. The assay was performed following the manufacturer's instructions (#MEG00, R&D Systems).

DQ Collagen IV experiments

DQ collagen IV (#D12052, Invitrogen) was mixed into Matrigel (#356234, Corning) at a final 25µg/mL concentration. Glass bottom, 96-well black plates were coated with 50µL Matrigel-DQ collagen IV mix and incubated at 37 °C for 20 minutes. D2.OR cells (2×10^3) were resuspended in 100 µL of DMEM (#21969035, Gibco) supplemented with 0,5% FBS (#SH30066.03, HyClone), 500 units/mL penicillin and 500 µg/mL streptomycin (#15140-122, Gibco), 2mM Glutamine (#25030-024, Gibco), then grown on the coated wells. The next day, media were replaced with 100 µL of the indicated conditioned media (CM), supplemented with the indicated inhibitors. Treatments were changed every second days. Fluorescence intensity was measured in a fluorescence microplate reader (FLUOstar Omega Microplate Reader - BMG Labtech). Cells were fixed with 4% PFA for 20 min at room temperature, rinsed twice in PBS (#14200-067, Gibco), incubated in 50 mM of NH₄Cl for 10 min, and permeabilized with 0.5% Triton X-100 for 5 min. Cells were then incubated in blocking buffer (PBS containing 1% BSA) for 30 min and cells were incubated in the presence of Phalloidin for 40 min, rinsed twice in PBS (#14200-067, Gibco), stained with DAPI for 5 min, rinsed in water, and placed in PBS (#14200-067, Gibco) for subsequent analysis.

Western blot

Cells grown on CM-primed Matrigel-coated 0.2 kPa hydrogel were lysed on ice in lysis buffer (25 mM Tris [pH 6.8], 2% sodium dodecyl sulfate (SDS), 5% glycerol, 1% β-mercaptoethanol, 0.01% bromophenol blue) and the samples were sonicated. Equal amounts of protein from each sample were loaded on SDS-polyacrylamide gel electrophoresis, separated, and transferred onto Immunobilon-P PVDF membrane (#IPVH00010). The immunoblots were incubated in blocking buffer (5% BSA, 10 mM Tris-HCl [pH 7.5], 500 mM NaCl) for 30 min at room temperature and probed with specific antibodies overnight at 4 °C. Then, the immunoblots were washed three times for 10 min in Tris-buffered saline Tween 20 (TBST, 10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% Tween 20), incubated with secondary antibodies for one hour at room temperature in blocking buffer, and washed three times in TBST again. Immunodetection was performed using chemiluminescent horseradish peroxidase (HRP) substrate (#WBKLS0500, Sigma-Aldrich).

Statistical analysis

All experiments were repeated at least three times and representative data/images are shown. Numerical quantifications for *in vitro* experiments represent mean \pm standard deviation (SD). Immunoblot images are representative of experiments that have been repeated at least three times. Quantification of immunoblot corresponds to the mean \pm standard deviation (SD) of the different replicate. For comparisons among groups, one-way ANOVA and post-hoc Tukey testing was performed. A P-value less than 0.05 was considered significant. All statistical analyses have been performed with GraphPad Prism software.

Cell culture reagents and antibodies

Recombinant Mouse M-CSF (#416-ML-050, R&D Systems) was used at 10ng/mL to activate macrophages. Interferon- γ (IFN- γ) (#315-05, Peprotech) was used at 100U/mL, Lipopolysaccharide (LPS) (#L4391, Sigma) at 100 ng/ml, Recombinant Murine IL-4 (#214-14, Peprotech) and Recombinant Murine IL-13 (#210-13, Peprotech) were used at 20ng/mL to polarize macrophages. MMP9 protein (#50560-MNAH, Sino Biological) was used at 2 μ g/ml and 4-Aminophenylmercuric acetate: APMA (#A9563, Sigma Aldrich) was used at 1mM to activate pro-MMP9 and MMP9 inhibitor 1 (#44278, Calbiochem) was used at 10 μ M to inactivate MMP9. 3-Aminopropionitrile fumarate salt: BAPN (#A3134, Merck) was used at 10 μ M to inhibit LOX activity. Ag1478 (#1276, Tocris Bioscience) and Gefitinib (#TB3000-RMU, Tocris Bioscience) was used at 1 μ M, Cetuximab (#L01XC06) was used at 10 μ g/mL to inhibit EGFR. PF-573228 (#S2013, Selleck) was used at 10 μ M to inhibit FAK activation, SU6656 (#572635, Calbiochem) was used at 10 μ M to inhibit Src, Anti-Integrin B1 antibody clone Ha2/5: CD29 (#5555003, BD Biosciences) was used at 10 μ g/ml to inhibit integrin-B1 activity, UO126 (#U120, Merck) was used at 10 μ M to inhibit ERK activation. Recombinant Mouse CD14 (#771902, BioLegend) was used at 1, 10, 100 ng/mL, Recombinant Mouse Endostatin (#570-ES-050, R&D Systems) was used at 10, 50, 100 ng/ml, Recombinant Mouse CXCL10 (#466-CR-010, R&D Systems) was used at 1, 5, 25, 50, 100 ng/ml, Recombinant Murine HGF (#315-23, Peprotech) was used at 1, 5, 10, 25, 50 ng/ml, Recombinant Mouse CCL6 (#487-C-010, R&D Systems) was used at 1, 5, 25 ng/ml, Recombinant Mouse LDLR (#2255-LD-025, R&D Systems) was used at 0,5, 1, 5 μ g/ml, Recombinant Mouse CXCL16 (#503-CX-025, R&D Systems) was used at 1 μ g/ml and 5ng/ml.

Recombinant Mouse Serpin E2/PN1 (#2175-PI-010, R&D Systems) was used 20nM and Cathepsin G inhibitor 1 (#219372, EMD Millipore) was used at 10 μ M.

For Western blot analysis, antibodies against ERK1/2 (#4695), pThr202/Tyr204-ERK1/2 (#4370), FAK (#3585), pTyr397-FAK (#8556), HER2/ErbB2 (#2165), pTyr1248-HER2/ErbB2 (#2247) were purchased from Cell Signaling and monoclonal anti- β tubulin (#T4026, Sigma-Aldrich) was purchased from Merck. For immunofluorescence staining of *in vitro* cultures, antibodies against Ki67 (#NB110-89717, Novus Biologicals), Alexa Fluor 568 phalloidin (#A12380, Thermo Fisher Scientific) was used to stain F-actin and Dapi (#D9S42, Sigma Aldrich) was used as DNA stain.

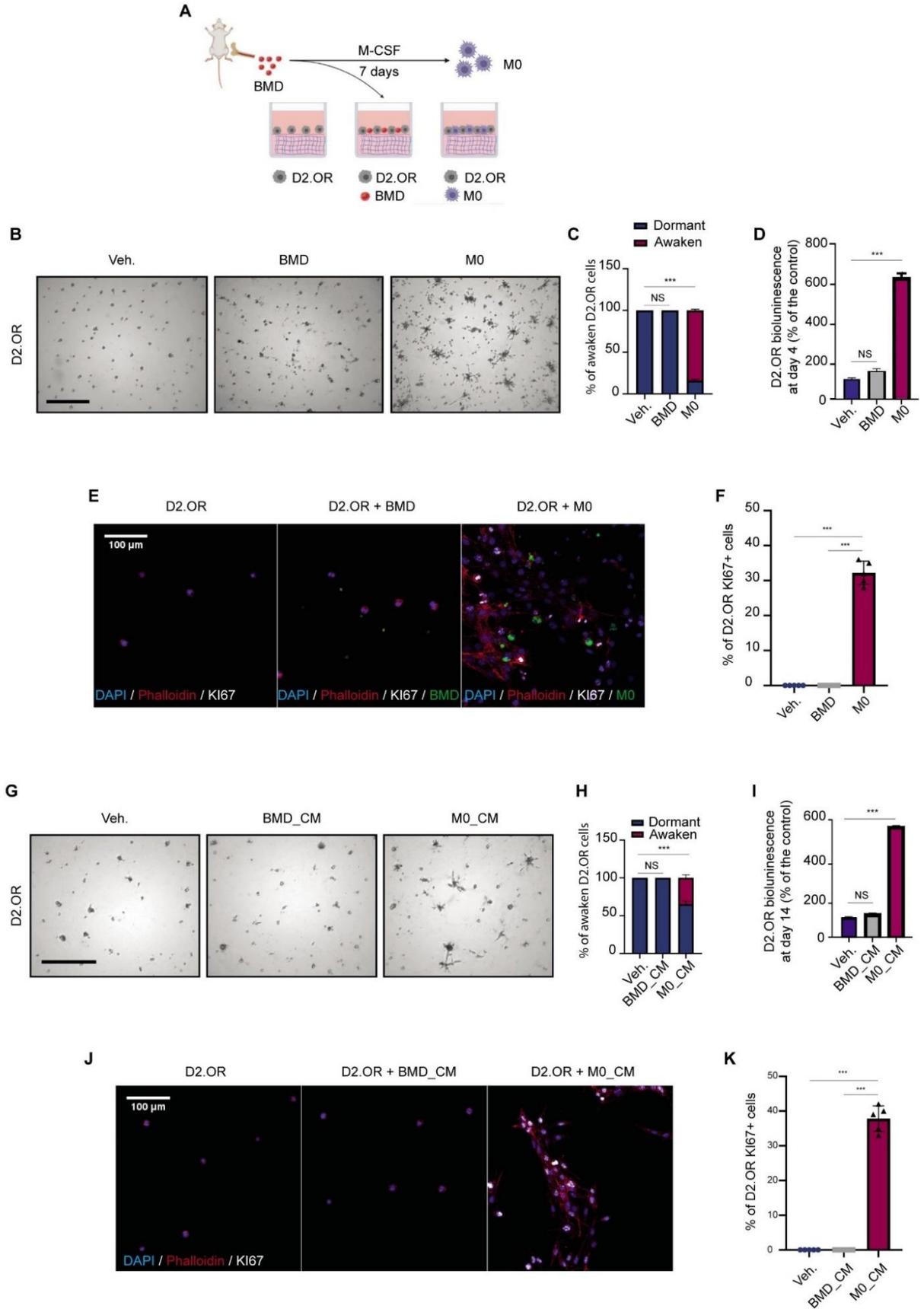


Figure 1 : MCSF-activated macrophages trigger the awakening of dormant D2.OR cells

Figure 1: M-CSF-activated macrophages trigger the awakening of dormant D2.OR cells

A) Schematic representation of the different co-culture experimental setups of D2.OR cells and bone marrow-derived cells (BMD) or Macrophage colony-stimulating factor (M-CSF)-activated macrophages (M0). **B)** Representative images of D2.OR cells grown on Matrigel in 0,5% DMEM alone (Veh.) or in co-culture with BMD cells (D2.OR+BMD) or with M0 macrophages (D2.OR+M0). Scale bar, 100 μ m. **C)** Quantitative image analysis of awakening D2.OR cells grown on Matrigel in 0,5% DMEM alone (Veh.) or when seeded in co-culture with BMD cells (BMD) or with M0 macrophages (M0). **D)** Quantification of D2.OR bioluminescence grown on Matrigel in 0,5% DMEM (Veh.) at day 4 or upon co-culturing with BMD cells (BMD) or with M0 macrophages (M0). **E)** Representative images of DAPI- (blue), Phalloidin- (red) and Ki67-specific antibody (white)-labelled D2.OR cells . BMD and M0 cells are labelled with a green fluorescent dye. Scale bar, 100 μ m. **F)** Quantification of the percentage of Ki67+ D2.OR cells in monocultures (Veh.) or upon co-culturing with BMD cells (BMD) or with M0 macrophages (M0). **G)** Representative images of D2.OR cells grown on Matrigel in 0,5% DMEM (Veh.) or treated with conditioned media (CM) collected from BMD cells (BMD_CM) or from M0 macrophages (M0_CM). Scale bar, 100 μ m. **H)** Quantitative image analysis of awakening D2.OR cells grown on Matrigel in 0,5% DMEM (Veh.) or when treated with BMD_CM or with M0_CM. **I)** Quantification of D2.OR bioluminescence at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with BMD_CM or with M0_CM. **J)** Representative images of D2.OR cells labelled with DAPI (blue), Phalloidin (red) and with a Ki67-specific antibody (white). Scale bar, 100 μ m. **K)** Quantification of the percentage of Ki67+ D2.OR cells upon treatment with 0,5% DMEM (Veh.) or treated with BMD_CM or with M0_CM.

(n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.

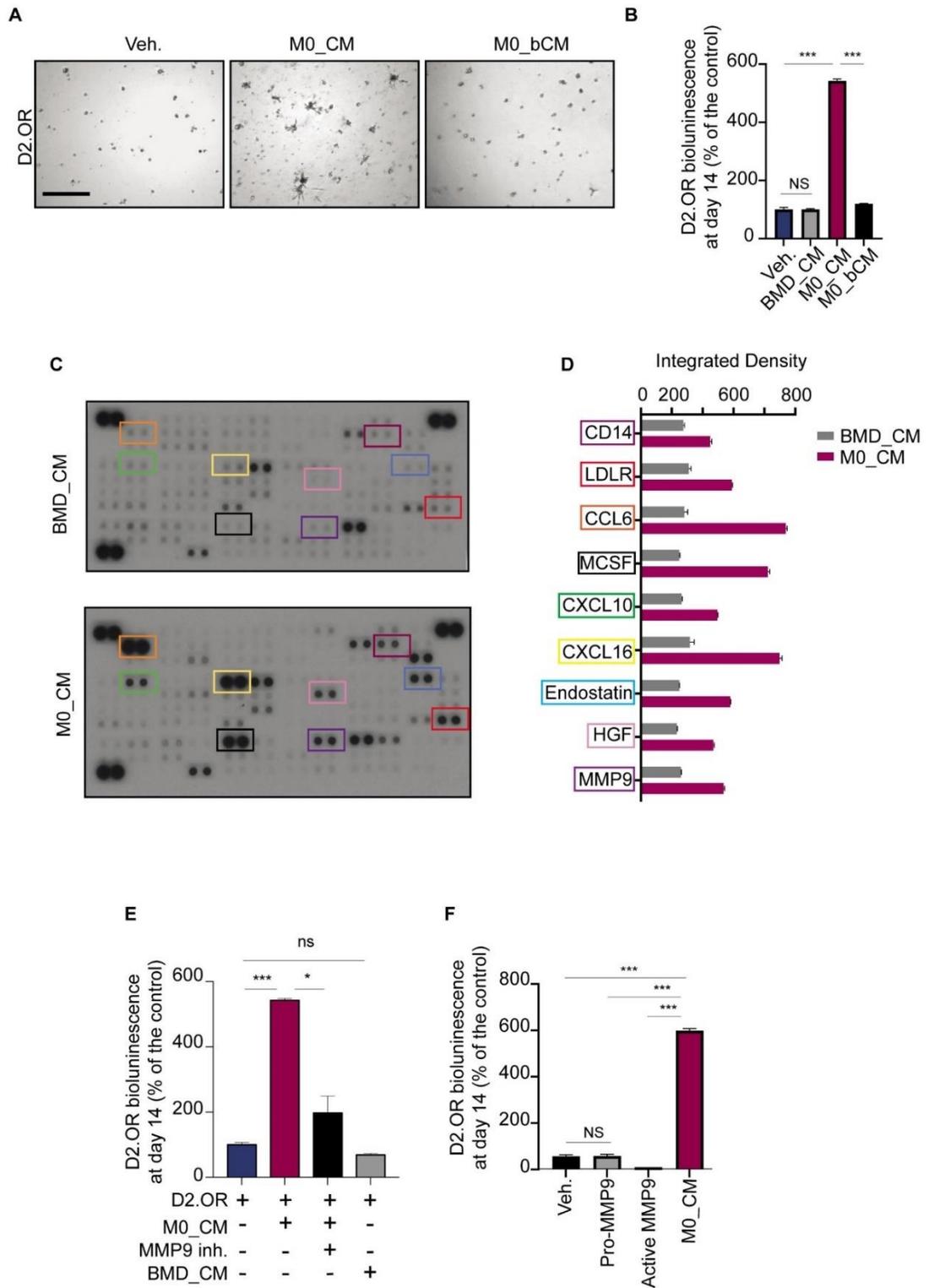


Figure 2 : MMP9 is required but not sufficient for macrophage-dependent breast cancer cell awakening

Figure 2: MMP9 is required, but not sufficient for macrophage-dependent breast cancer cell awakening

A) Representative images of D2.OR cells grown on Matrigel in 0,5% DMEM (Veh.), treated with M0_CM or boiled macrophage conditioned media (M0_bCM). Scale bar, 100 μ m. **B)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with BMD_CM, M0_CM or M0_bCM. **C)** Proteome-profiler cytokine arrays were probed with BMD_CM and M0_CM. The 8 identified molecules that showed upregulation in M0_CM are marked with squares in different colours. **D)** Quantification of the integrated density levels corresponding to each identified molecules on both membranes. **E)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon stimulation with 0,5% DMEM (first bar), M0_CM, M0_CM in the presence of MMP9 inhibitor or with BMD_CM **F)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon stimulation with 0,5% DMEM (Veh.), pro-MMP9, Active MMP9 or M0_CM.

(n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.

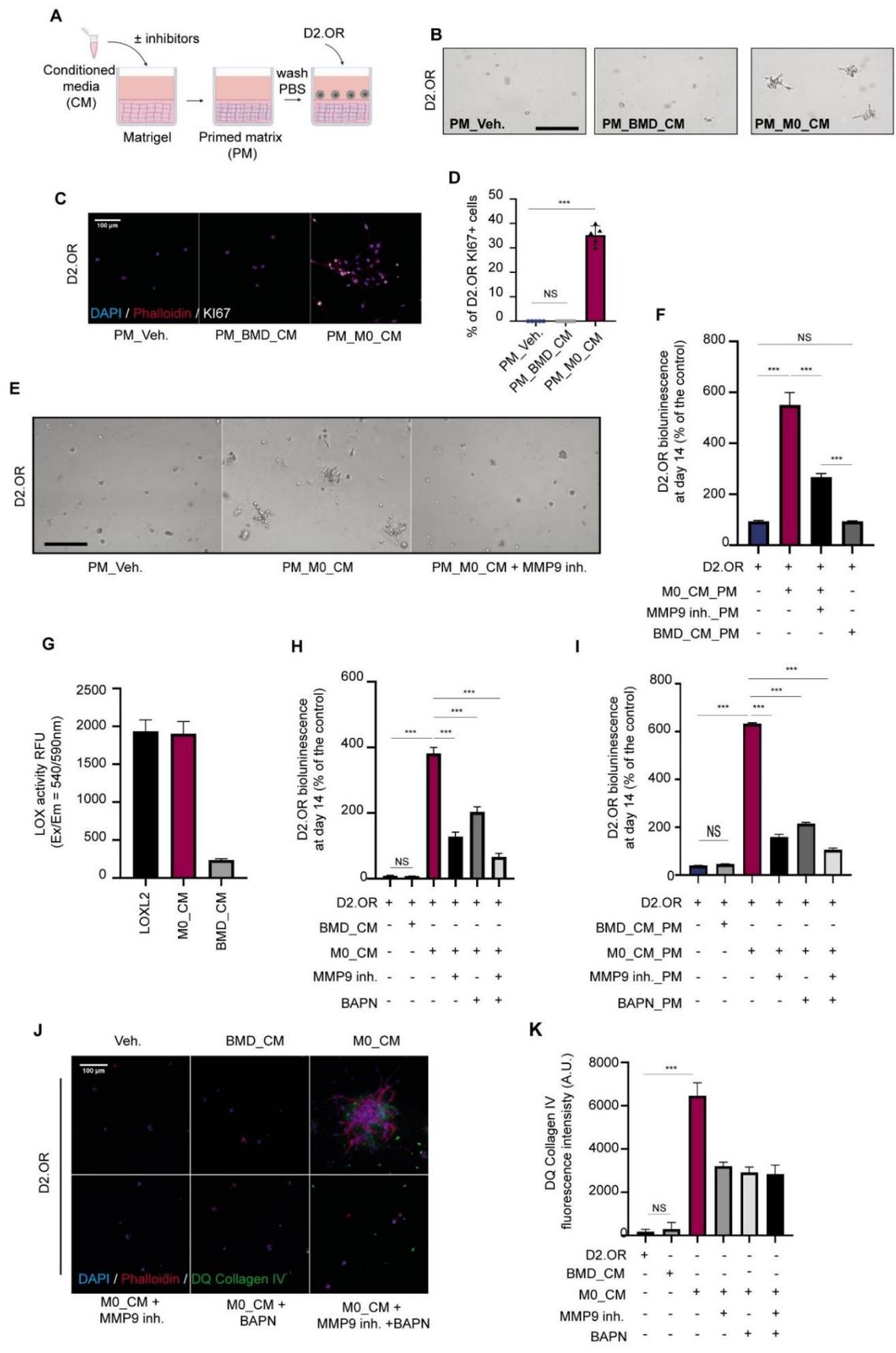


Figure 3 : MMP9 and lysil oxidase activities are required to promote breast cancer cell awakening by macrophages.

Figure 3: MMP9 and Lysyl oxidase activities by macrophages are required to promote breast cancer cell awakening

A) Schematic representation of the experimental design for matrix priming experiments. **B)** Representative images of D2.OR cells seeded on Matrigel matrices that were primed with the indicated conditioned media (PM_BMD_CM or PM_M0_CM) or 0,5% DMEM (PM_Veh.). **C)** Representative images of immunostaining of D2.OR cells on primed Matrigel matrices with Ki67 specific antibody (white) and labelled with DAPI (blue) and Phalloidin (red). Scale bar, 100 μ m. **D)** Quantification of the percentage of Ki67+ D2.OR cells seeded on matrices that were primed with the indicated conditioned media (PM_BMD_CM or PM_M0_CM) or 0,5% DMEM (PM_Veh.). **E)** Representative images of D2.OR cells seeded on Matrigel matrices that were primed with 0,5% DMEM (PM_Veh.), M0_CM (PM_M0_CM) or with M0_CM in the presence of MMP9 inhibitor (PM_M0_CM+MMP9 inh.) Scale bar, 100 μ m. **F)** Quantification of D2.OR bioluminescence 14 days after seeding them on the differentially primed Matrigel matrices with 0,5% DMEM (PM_Veh.), M0_CM (PM_M0_CM) or with M0_CM in the presence of MMP9 inhibitor (PM_M0_CM+MMP9 inh.). **G)** Quantification of LOX activity in M0_CM and BMD_CM. Recombinant mouse LOXL2 was used as positive control. **H)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon stimulation with 0,5% DMEM, BMD_CM, M0_CM, M0_CM in the presence of MMP9 inhibitor, M0_CM in the presence of BAPN (LOX inhibitor) or M0_CM in the presence of both MMP9 inhibitor and BAPN. **I)** Quantification of D2.OR bioluminescence 14 after seeding on the differentially primed Matrigel matrices with 0,5% DMEM, BMD_CM, M0_CM, or with M0_CM in the presence of MMP9 inhibitor; BAPN; or both MMP9 inhibitor and BAPN. **J)** Representative images of Ki67 immunostaining of D2.OR cells on primed matrices. Cells were further labelled with DAPI (blue), Phalloidin (red) and green signal indicates DQ Collagen IV degradation. Scale bar, 100 μ m. **K)** Quantification of the fluorescence intensity corresponding to DQ Collagen IV degradation upon stimulation with the indicated CM in the presence of the indicated inhibitors.

(n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.

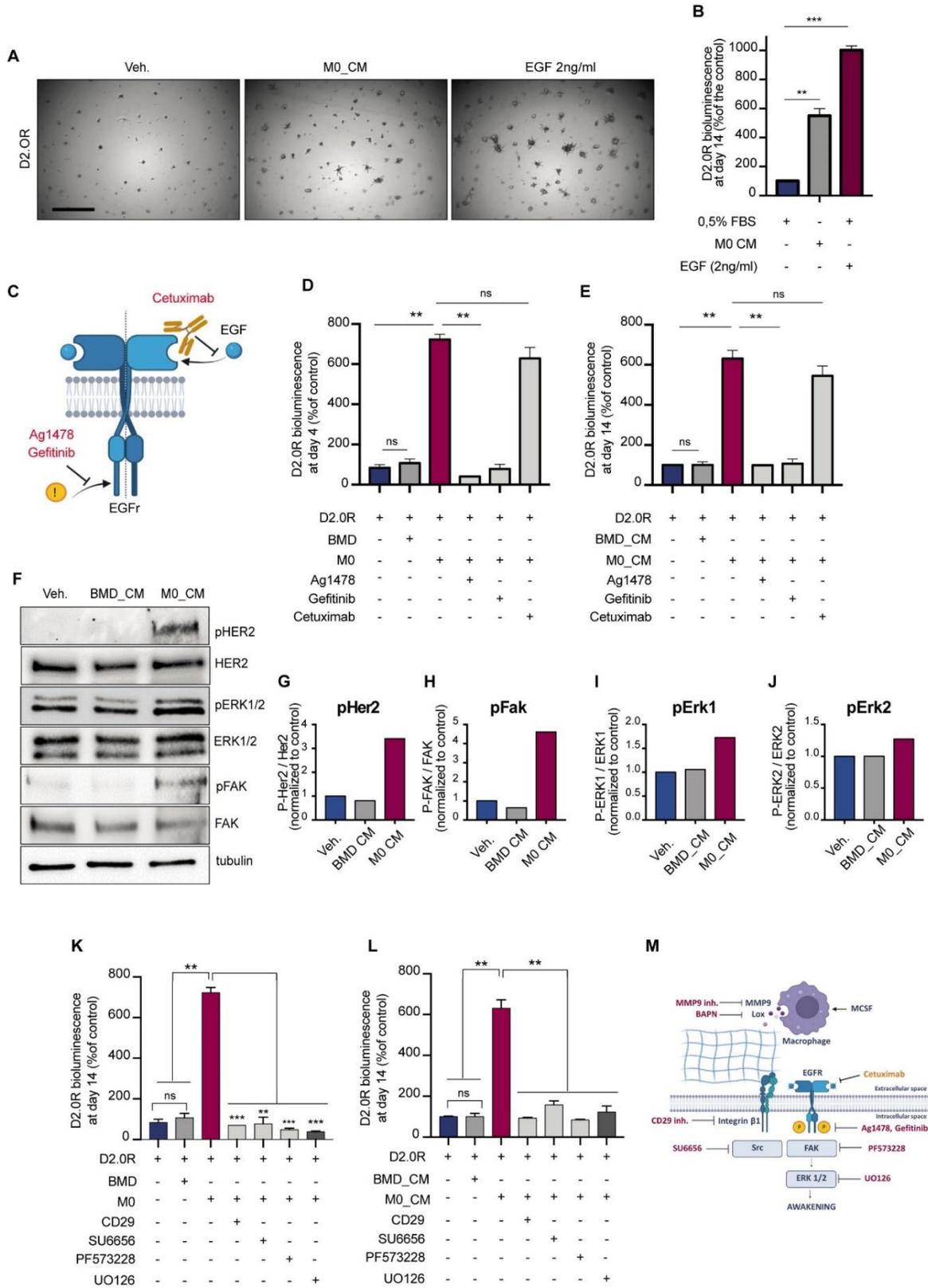
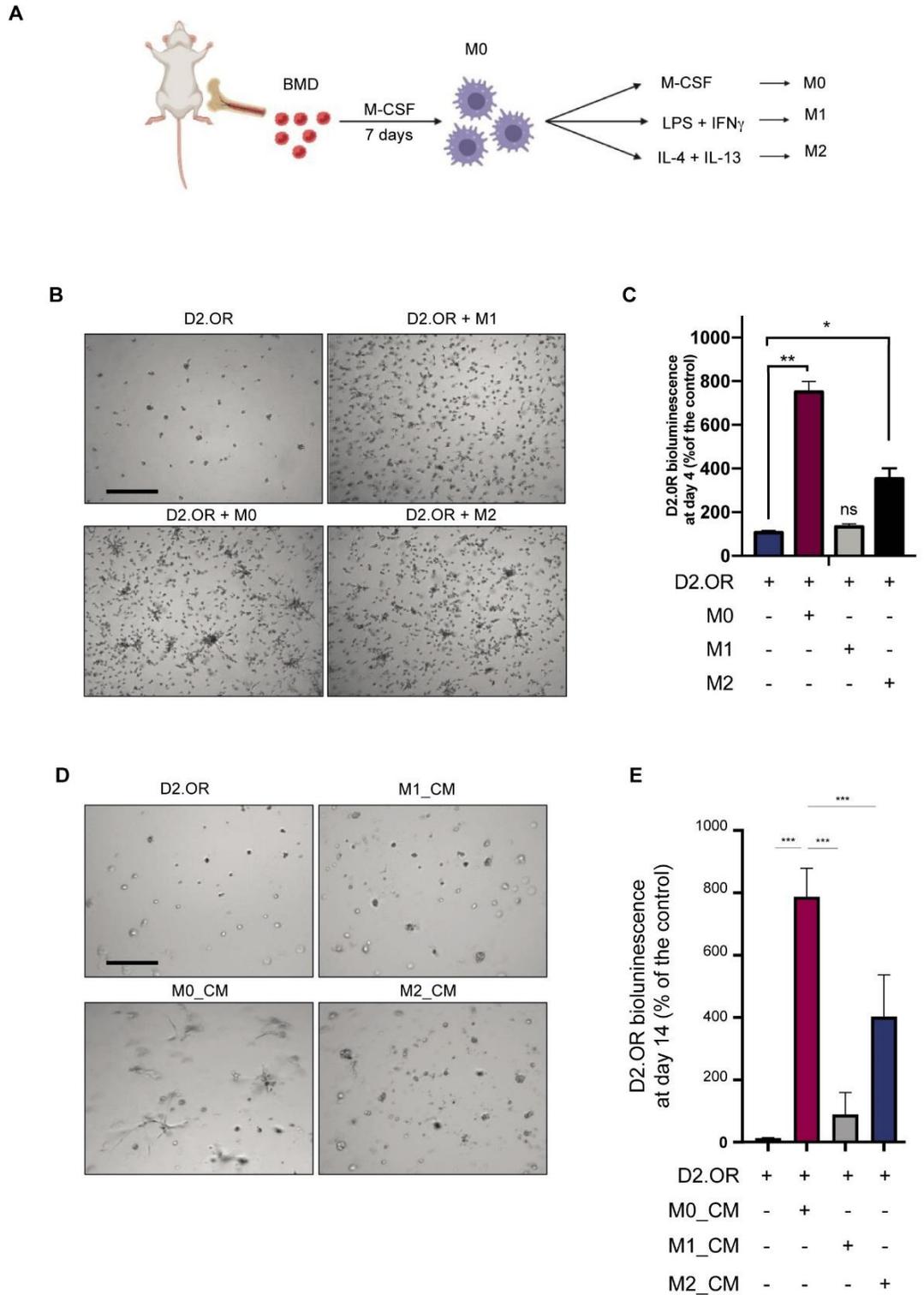


Figure 4 : Macrophage-mediated matrix modifications promote ligand-independent EGFR activation in dormant D2.0R cells

Figure 4: Macrophage-mediated matrix modifications promote ligand-independent EGFR activation in dormant D2.OR cells

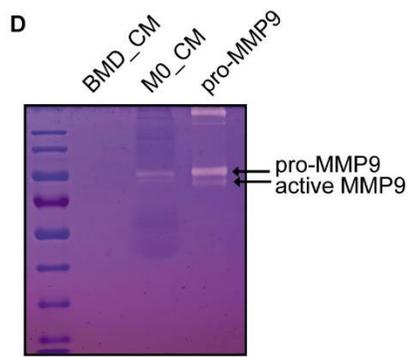
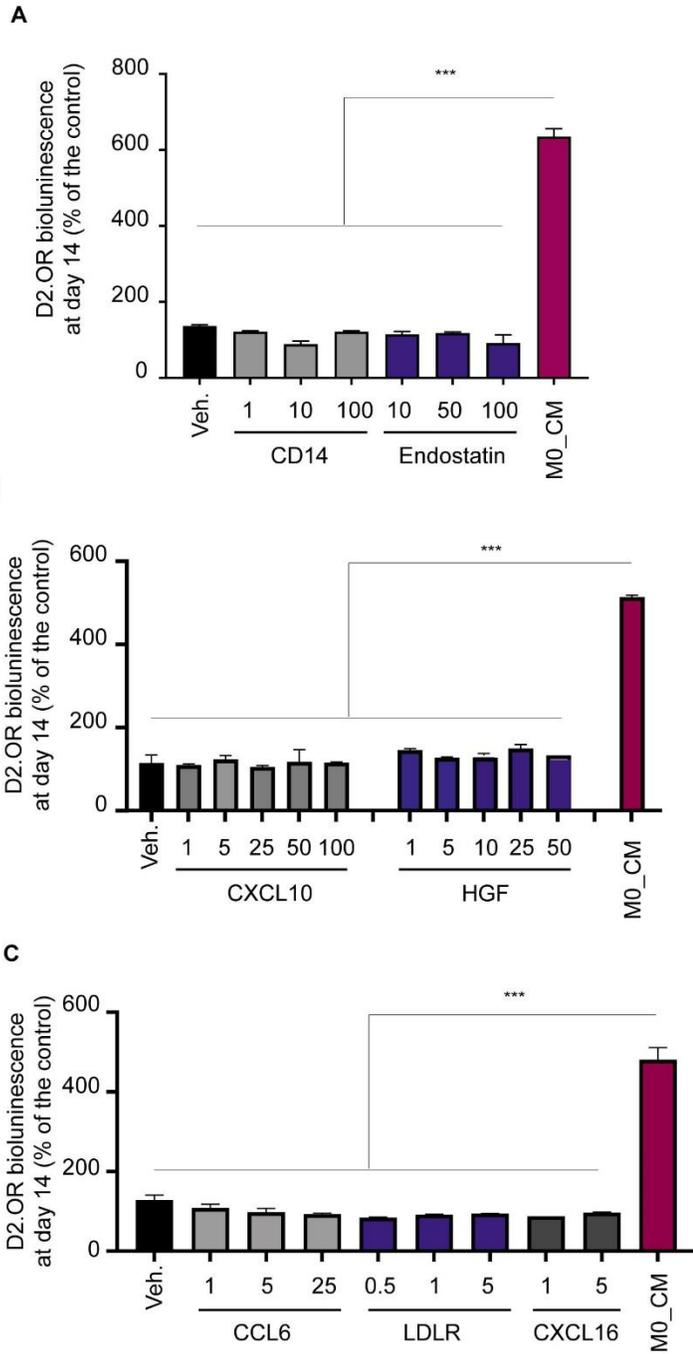
A) Representative images of D2.OR cells grown on Matrigel in 0,5% DMEM (Veh.), treated with M0_CM or EGF (2ng/ml). Scale bar, 100 μ m. **B)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.), treated with M0_CM or EGF (2ng/ml). **C)** Schematic representation of the mechanism of action of the different EGFR inhibitors. **D)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.), BMD_CM or treated with M0_CM in the presence of the indicated inhibitors. **E)** Quantification of D2.OR bioluminescence 14 after seeding them on the differentially primed Matrigel matrices with 0,5% DMEM, BMD_CM, M0_CM, or with M0_CM in the presence of the indicated inhibitors. **F)** Immunoblots corresponding to pHER2, pERK1/2, pFAK in D2.OR cells seeded on primed matrices with 0,5% DMEM, BMD_CM or M0_CM. HER2, ERK1/2, FAK and tubulin-specific antibodies were used as loading controls. **G-J)** Quantification of HER2, ERK1/2 and FAK phosphorylation levels were calculated by normalizing the phosphorylation-specific band intensities to those of their non-phosphorylated counterparts. **K)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with BMD_CM or M0_CM in the presence of the indicated inhibitors. **L)** Quantification of D2.OR bioluminescence 14 after seeding them on the differentially primed Matrigel matrices with 0,5% DMEM, BMD_CM, M0_CM, or with M0_CM in the presence of the indicated inhibitors. **M)** Graphical abstract of the suggested mechanisms driving the macrophage-mediated cancer cell awakening.

(n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.



Supplementary Figure 1:

A) Schematic representation of BMD cell activation into M0 macrophages, then trans-differentiation into M1 (stimulation with Lipopolysaccharide (LPS) and Interferon- γ (IFN- γ) for 48h) or M2 macrophages (stimulation with IL-4 and IL-13 for 48h). **B)** Representative images of D2.OR cells on Matrigel alone (D2.OR) or in co-culture with M0 (D2.OR+M0), M1 (D2.OR+M1), M2 (D2.OR+M2) macrophages. Scale bar, 100 μ m. **C)** Quantification of D2.OR bioluminescence on Matrigel at day 4 in monocultures or upon co-culturing with M0, M1 or M2 macrophages. **D)** Representative images of D2.OR cells grown on Matrigel in 0,5% DMEM (D2.OR) or treated with conditioned media (CM) collected from M0 (M0_CM), M1 (M1_CM), M2 (M2_CM) macrophages. Scale bar, 100 μ m. **E)**) Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM or treated with M0_CM, M1_CM or M2_CM. (n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.

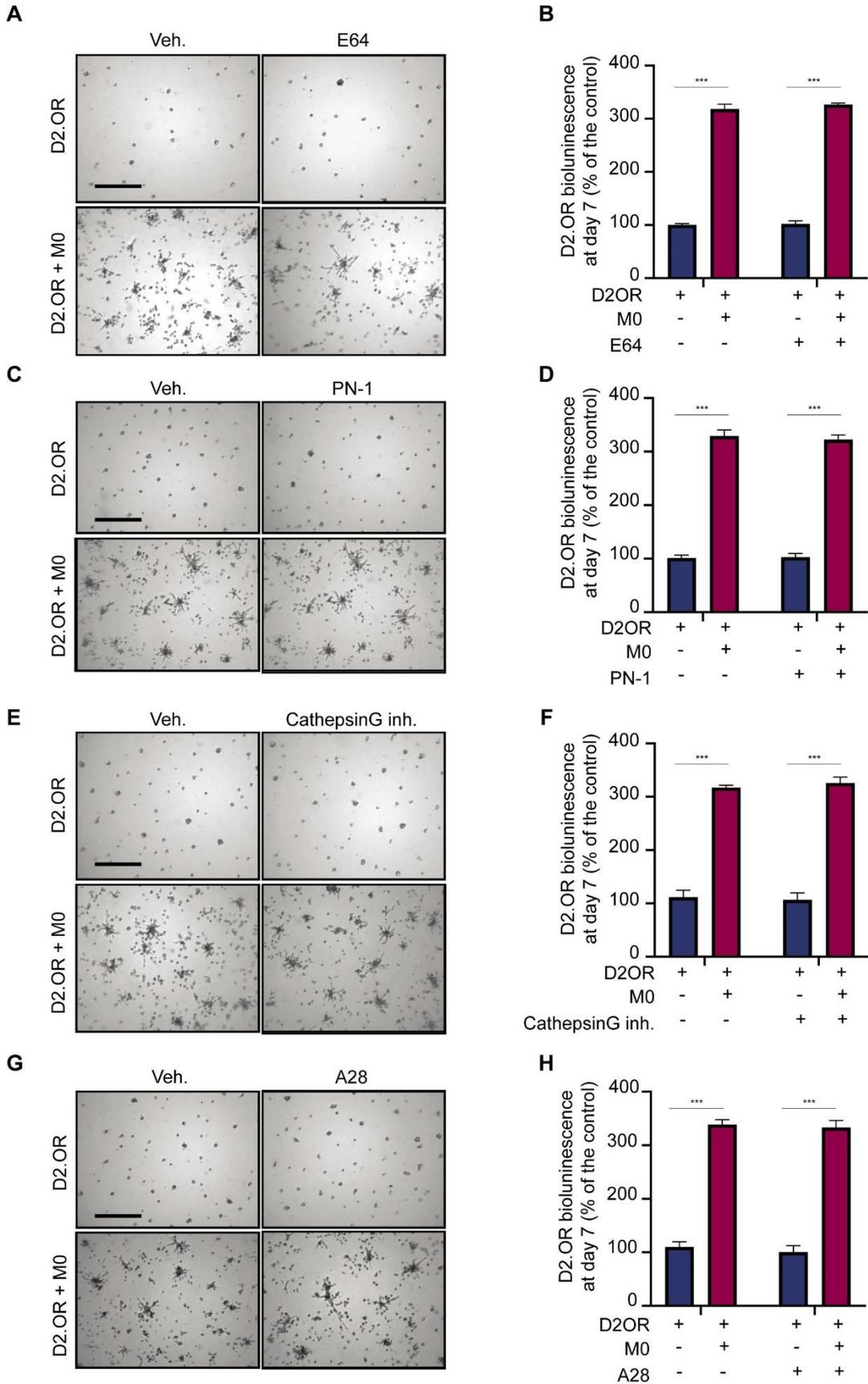


Supplementary Figure 2

Supplementary Figure 2:

A) Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with 1, 10 or 100 ng/ml recombinant mouse CD14, or 10, 50 or 100 ng/ml recombinant mouse Endostatin or with M0_CM. **B)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with 1, 5, 25, 50 or 100 ng/ml recombinant mouse CXCL10 or 1, 5, 10, 25, or 50 ng/ml recombinant mouse HGF or with M0_CM. **C)** Quantification of D2.OR bioluminescence on Matrigel at day 14 upon treatment with 0,5% DMEM (Veh.) or treated with 1, 5, 25 ng/ml recombinant mouse CCL6 or 0.5, 1 or 5 µg/ml recombinant mouse LDLR, or 1 µg/ml or 5ng/ml recombinant mouse CXCL16 or M0_CM. **D)** Representative image of Zymography test performed with BMD_CM, M0_CM and MMP9.

(n = 3; means ± SD). ***P < 0.001; **P <0.01; *P < 0.05; NS: not significant.

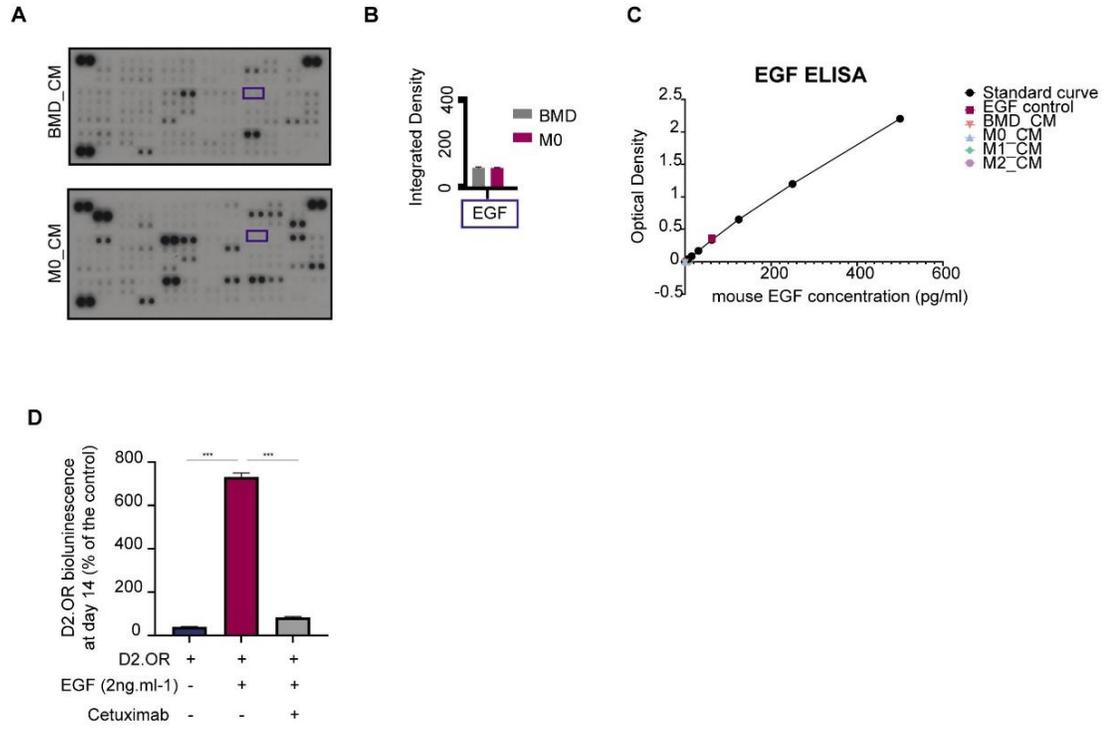


Supplementary Figure 3

Supplementary Figure 3:

A) Representative images of D2.OR cells grown on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with E64, a cysteine protease inhibitor (D2.OR E64); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with E64 (D2.OR+M0 E64). **B)** Quantification of D2.OR bioluminescence on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with E64 (D2.OR E64); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with E64 (D2.OR+M0 E64). **C)** Representative images of D2.OR cells grown on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with protease nexin-1 (PN-1), a serine protease inhibitor (D2.OR PN-1); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with PN-1 (D2.OR+M0 PN-1). **D)** Quantification of D2.OR bioluminescence on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with protease nexin-1 (PN-1), a serine protease inhibitor (D2.OR PN-1); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with PN-1 (D2.OR+M0 PN-1). **E)** Representative images of D2.OR cells grown on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with CathepsinG inhibitor (D2.OR CathepsinG inh.); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with CathepsinG inhibitor (D2.OR+M0 CathepsinG inh.). **F)** Quantification of D2.OR bioluminescence grown on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with CathepsinG inhibitor (D2.OR CathepsinG inh.); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with CathepsinG inhibitor (D2.OR+M0 CathepsinG inh.). **G)** Representative images of D2.OR cells grown on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with Antibody 28 (D2.OR A28); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with Antibody 28(D2.OR+M0 A28). **H)** Quantification of D2.OR bioluminescence on Matrigel alone in 0,5% DMEM (D2.OR Veh.) or upon treatment with Antibody 28 (D2.OR A28); or grown in co-cultures with M0 macrophages in 0,5% DMEM (D2.OR+M0 Veh.) or upon treatment with Antibody 28(D2.OR+M0 A28).

Scale bar, 100 μ m; (n = 3; means \pm SD). ***P < 0.001; **P < 0.01; *P < 0.05; NS: not significant.



Supplementary Figure 4

Supplementary Figure 4:

A) Representation of the proteome-profiler cytokine arrays that were probed with BMD_CM and M0_CM. EGF is indicated with blue squares. **B)** Quantification of EGF integrated density on the membrane arrays. **C)** Quantification of mouse EGF within BMD_CM, M0_CM, M1_CM, M2_CM using EGF-specific ELISA. **D)** Quantification of D2.OR bioluminescence on Matrigel at day 14 in 0,5%DMEM or upon stimulation with EGF in the presence of absence of Cetuximab. ***P < 0.001; **P <0.01; *P < 0.05; NS: not significant.

References

- Aguirre Ghiso, J. A., Kovalski, K., & Ossowski, L. (1999). Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *The Journal of Cell Biology*, *147*(1), 89–104. <https://doi.org/10.1083/jcb.147.1.89>
- Albregues, J., Shields, M. A., Ng, D., Park, C. G., Ambrico, A., Poindexter, M. E., Upadhyay, P., Uyeminami, D. L., Pommier, A., Küttner, V., Bružas, E., Maiorino, L., Bautista, C., Carmona, E. M., Gimotty, P. A., Fearon, D. T., Chang, K., Lyons, S. K., Pinkerton, K. E., ... Egeblad, M. (2018). Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science (New York, N.Y.)*, *361*(6409). <https://doi.org/10.1126/science.aao4227>
- Alonso-Nocelo, M., Ruiz-Cañas, L., Sancho, P., Görgülü, K., Alcalá, S., Pedrero, C., Vallespinos, M., López-Gil, J. C., Ochando, M., García-García, E., David Trabulo, S. M., Martinelli, P., Sánchez-Tomero, P., Sánchez-Palomo, C., Gonzalez-Santamaría, P., Yuste, L., Wörmann, S. M., Kabacaoğlu, D., Earl, J., ... Sainz, B. (2022). Macrophages direct cancer cells through a LOXL2-mediated metastatic cascade in pancreatic ductal adenocarcinoma. *Gut*. <https://doi.org/10.1136/gutjnl-2021-325564>
- Augoff, K., Hryniewicz-Jankowska, A., Tabola, R., & Stach, K. (2022). MMP9: A Tough Target for Targeted Therapy for Cancer. *Cancers*, *14*(7). <https://doi.org/10.3390/cancers14071847>
- Barkan, D., & Green, J. E. (2011). An in vitro system to study tumor dormancy and the switch to metastatic growth. *Journal of Visualized Experiments : JoVE*, *54*. <https://doi.org/10.3791/2914>
- Barker, H. E., Chang, J., Cox, T. R., Lang, G., Bird, D., Nicolau, M., Evans, H. R., Gartland, A., & Erler, J. T. (2011). LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. *Cancer Research*, *71*(5), 1561–1572. <https://doi.org/10.1158/0008-5472.CAN-10-2868>
- Biswas, S. K., & Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature Immunology*, *11*(10), 889–896. <https://doi.org/10.1038/ni.1937>
- Borriello, L., Coste, A., Sharma, V. P., Karagiannis, G. S., Lin, Y., Ye, X., Duran, C. L., Chen, X., Dalla, E., Singh, D. K., Aguirre-Ghiso, J. A., Condeelis, J., & Entenberg, D. (2021). Primary tumor associated macrophages activate programs of invasion and dormancy in disseminating tumor cells. *BioRxiv*, 2021.02.04.429798. <https://doi.org/10.1038/s41467-022-28076-3>
- Bragado, P., Sosa, M. S., Keely, P., Condeelis, J., & Aguirre-Ghiso, J. A. (2012). Microenvironments dictating tumor cell dormancy. *Recent Results in Cancer Research. Fortschritte Der Krebsforschung. Progres Dans Les Recherches Sur Le Cancer*, *195*, 25–39. https://doi.org/10.1007/978-3-642-28160-0_3
- Chen, Q., Zhang, X. H.-F., & Massagué, J. (2011). Macrophage Binding to Receptor VCAM-1 Transmits Survival Signals in Breast Cancer Cells that Invade the Lungs. *Cancer Cell*, *20*(4), 538–549. <https://doi.org/10.1016/j.ccr.2011.08.025>
- Condeelis, J., & Pollard, J. W. (2006). Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis. *Cell*, *124*(2), 263–266. <https://doi.org/10.1016/j.cell.2006.01.007>
- Cox, T. R., Bird, D., Baker, A.-M., Barker, H. E., Ho, M. W.-Y., Lang, G., & Erler, J. T. (2013). LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. *Cancer Research*, *73*(6), 1721–1732. <https://doi.org/10.1158/0008-5472.CAN-12-2233>
- Dai, J., Cimino, P. J., Gouin, K. H., Grzelak, C. A., Barrett, A., Lim, A. R., Long, A., Weaver, S., Saldin, L. T., Uzamere, A., Schulte, V., Clegg, N., Pisarsky, L., Lyden, D., Bissell, M. J., Knott, S., Welm, A. L., Bielas, J. H., Hansen, K. C., ... Ghajar, C. M. (2022). Astrocytic laminin-211

- drives disseminated breast tumor cell dormancy in brain. *Nature Cancer*, 3(1), 25–42.
<https://doi.org/10.1038/s43018-021-00297-3>
- Doak, G. R., Schwertfeger, K. L., & Wood, D. K. (2018). Distant Relations: Macrophage Functions in the Metastatic Niche. *Trends in Cancer*, 4(6), 445–459.
<https://doi.org/10.1016/j.trecan.2018.03.011>
- Ferreira, S., Saraiva, N., Rijo, P., & Fernandes, A. S. (2021). LOXL2 Inhibitors and Breast Cancer Progression. *Antioxidants (Basel, Switzerland)*, 10(2). <https://doi.org/10.3390/antiox10020312>
- Gonzalez-Avila, G., Sommer, B., Mendoza-Posada, D. A., Ramos, C., Garcia-Hernandez, A. A., & Falfan-Valencia, R. (2019). Matrix metalloproteinases participation in the metastatic process and their diagnostic and therapeutic applications in cancer. *Critical Reviews in Oncology/Hematology*, 137, 57–83. <https://doi.org/10.1016/j.critrevonc.2019.02.010>
- Grasset, E. M., Bertero, T., Bozec, A., Friard, J., Bourget, I., Pisano, S., Lecacheur, M., Maiel, M., Bailleux, C., Emelyanov, A., Ilie, M., Hofman, P., Meneguzzi, G., Durantou, C., Bulavin, D. v., & Gaggioli, C. (2018). Matrix Stiffening and EGFR Cooperate to Promote the Collective Invasion of Cancer Cells. *Cancer Research*, 78(18), 5229–5242. <https://doi.org/10.1158/0008-5472.CAN-18-0601>
- Harper, K. L., Sosa, M. S., Entenberg, D., Hosseini, H., Cheung, J. F., Nobre, R., Avivar-Valderas, A., Nagi, C., Girnius, N., Davis, R. J., Farias, E. F., Condeelis, J., Klein, C. A., & Aguirre-Ghiso, J. A. (2016). Mechanism of early dissemination and metastasis in Her2+ mammary cancer. *Nature*, 540(7634), 588–592. <https://doi.org/10.1038/nature20609>
- Kaplan, R. N., Rafii, S., & Lyden, D. (2006). Preparing the ‘soil’: the premetastatic niche. *Cancer Research*, 66(23), 11089–11093. <https://doi.org/10.1158/0008-5472.CAN-06-2407>
- Kessenbrock, K., Plaks, V., & Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*, 141(1), 52–67. <https://doi.org/10.1016/j.cell.2010.03.015>
- Kirschmann, D. A., Seftor, E. A., Fong, S. F. T., Nieva, D. R. C., Sullivan, C. M., Edwards, E. M., Sommer, P., Csiszar, K., & Hendrix, M. J. C. (2002). A molecular role for lysyl oxidase in breast cancer invasion. *Cancer Research*, 62(15), 4478–4483.
<http://www.ncbi.nlm.nih.gov/pubmed/12154058>
- Krall, J. A., Reinhardt, F., Mercury, O. A., Pattabiraman, D. R., Brooks, M. W., Dougan, M., Lambert, A. W., Bierie, B., Ploegh, H. L., Dougan, S. K., & Weinberg, R. A. (2018). The systemic response to surgery triggers the outgrowth of distant immune-controlled tumors in mouse models of dormancy. *Science Translational Medicine*, 10(436).
<https://doi.org/10.1126/scitranslmed.aan3464>
- Lamagna, C., Aurrand-Lions, M., & Imhof, B. A. (2006). Dual role of macrophages in tumor growth and angiogenesis. *Journal of Leukocyte Biology*, 80(4), 705–713.
<https://doi.org/10.1189/jlb.1105656>
- Larsen, M., Artym, V. v., Green, J. A., & Yamada, K. M. (2006). The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Current Opinion in Cell Biology*, 18(5), 463–471. <https://doi.org/10.1016/j.ceb.2006.08.009>
- Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F. T., Csiszar, K., Giaccia, A., Weninger, W., Yamauchi, M., Gasser, D. L., & Weaver, V. M. (2009). Matrix crosslinking forces tumor progression by enhancing integrin signaling. *Cell*, 139(5), 891–906.
<https://doi.org/10.1016/j.cell.2009.10.027>
- Lin, C.-H., Pelissier, F. A., Zhang, H., Lakins, J., Weaver, V. M., Park, C., & LaBarge, M. A. (2015). Microenvironment rigidity modulates responses to the HER2 receptor tyrosine kinase inhibitor

- lapatinib via YAP and TAZ transcription factors. *Molecular Biology of the Cell*, 26(22), 3946–3953. <https://doi.org/10.1091/mbc.E15-07-0456>
- Lin, Y., Xu, J., & Lan, H. (2019). Tumor-associated macrophages in tumor metastasis: biological roles and clinical therapeutic applications. *Journal of Hematology & Oncology*, 12(1), 76. <https://doi.org/10.1186/s13045-019-0760-3>
- Liotta, L. A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C. M., & Shafie, S. (1980). Metastatic potential correlates with enzymatic degradation of basement membrane collagen. *Nature*, 284(5751), 67–68. <https://doi.org/10.1038/284067a0>
- Liu, D., Aguirre Ghiso, J., Estrada, Y., & Ossowski, L. (2002). EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell*, 1(5), 445–457. [https://doi.org/10.1016/s1535-6108\(02\)00072-7](https://doi.org/10.1016/s1535-6108(02)00072-7)
- Liu, Y., Zhang, P., Wu, Q., Fang, H., Wang, Y., Xiao, Y., Cong, M., Wang, T., He, Y., Ma, C., Tian, P., Liang, Y., Qin, L.-X., Yang, Q., Yang, Q., Liao, L., & Hu, G. (2021). Long non-coding RNA NR2F1-AS1 induces breast cancer lung metastatic dormancy by regulating NR2F1 and ΔNp63. *Nature Communications*, 12(1), 5232. <https://doi.org/10.1038/s41467-021-25552-0>
- Liu, Z., Li, L., Yang, Z., Luo, W., Li, X., Yang, H., Yao, K., Wu, B., & Fang, W. (2010). Increased expression of MMP9 is correlated with poor prognosis of nasopharyngeal carcinoma. *BMC Cancer*, 10, 270. <https://doi.org/10.1186/1471-2407-10-270>
- Martinez, F. O., & Gordon, S. (2015). The evolution of our understanding of macrophages and translation of findings toward the clinic. *Expert Review of Clinical Immunology*, 11(1), 5–13. <https://doi.org/10.1586/1744666X.2015.985658>
- Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J.-L., Mosser, D. M., Natoli, G., Saeij, J. P., Schultze, J. L., Shirey, K. A., ... Wynn, T. A. (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*, 41(1), 14–20. <https://doi.org/10.1016/j.immuni.2014.06.008>
- Newby, A. C. (2016). Metalloproteinase production from macrophages - a perfect storm leading to atherosclerotic plaque rupture and myocardial infarction. *Experimental Physiology*, 101(11), 1327–1337. <https://doi.org/10.1113/EP085567>
- Nielsen, S. R., Quaranta, V., Linford, A., Emeagi, P., Rainer, C., Santos, A., Ireland, L., Sakai, T., Sakai, K., Kim, Y.-S., Engle, D., Campbell, F., Palmer, D., Ko, J. H., Tuveson, D. A., Hirsch, E., Mielgo, A., & Schmid, M. C. (2016). Macrophage-secreted granulins supports pancreatic cancer metastasis by inducing liver fibrosis. *Nature Cell Biology*, 18(5), 549–560. <https://doi.org/10.1038/ncb3340>
- Niland, S., Riscanevo, A. X., & Eble, J. A. (2021). Matrix Metalloproteinases Shape the Tumor Microenvironment in Cancer Progression. *International Journal of Molecular Sciences*, 23(1). <https://doi.org/10.3390/ijms23010146>
- Nobre, A. R., Dalla, E., Yang, J., Huang, X., Wullkopf, L., Risson, E., Razghandi, P., Anton, M. L., Zheng, W., Seoane, J. A., Curtis, C., Kenigsberg, E., Wang, J., & Aguirre-Ghiso, J. A. (2022). ZFP281 drives a mesenchymal-like dormancy program in early disseminated breast cancer cells that prevents metastatic outgrowth in the lung. *Nature Cancer*. <https://doi.org/10.1038/s43018-022-00424-8>
- Pickup, M. W., Mouw, J. K., & Weaver, V. M. (2014). The extracellular matrix modulates the hallmarks of cancer. *EMBO Reports*, 15(12), 1243–1253. <https://doi.org/10.15252/embr.201439246>

- Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews Cancer*, 4(1), 71–78. <https://doi.org/10.1038/nrc1256>
- Qian, B.-Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., Kaiser, E. A., Snyder, L. A., & Pollard, J. W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*, 475(7355), 222–225. <https://doi.org/10.1038/nature10138>
- Qian, B.-Z., & Pollard, J. W. (2010). Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell*, 141(1), 39–51. <https://doi.org/10.1016/j.cell.2010.03.014>
- Ren, F., Tang, R., Zhang, X., Madushi, W. M., Luo, D., Dang, Y., Li, Z., Wei, K., & Chen, G. (2015). Overexpression of MMP Family Members Functions as Prognostic Biomarker for Breast Cancer Patients: A Systematic Review and Meta-Analysis. *PloS One*, 10(8), e0135544. <https://doi.org/10.1371/journal.pone.0135544>
- Retsky, M., & Demicheli, R. (2014). Multimodal hazard rate for relapse in breast cancer: quality of data and calibration of computer simulation. *Cancers*, 6(4), 2343–2355. <https://doi.org/10.3390/cancers6042343>
- Ruffell, B., Affara, N. I., & Coussens, L. M. (2012). Differential macrophage programming in the tumor microenvironment. *Trends in Immunology*, 33(3), 119–126. <https://doi.org/10.1016/j.it.2011.12.001>
- Samuel, M. S., Lopez, J. I., McGhee, E. J., Croft, D. R., Strachan, D., Timpson, P., Munro, J., Schröder, E., Zhou, J., Brunton, V. G., Barker, N., Clevers, H., Sansom, O. J., Anderson, K. I., Weaver, V. M., & Olson, M. F. (2011). Actomyosin-mediated cellular tension drives increased tissue stiffness and β -catenin activation to induce epidermal hyperplasia and tumor growth. *Cancer Cell*, 19(6), 776–791. <https://doi.org/10.1016/j.ccr.2011.05.008>
- Saxena, M., Liu, S., Yang, B., Hajal, C., Changede, R., Hu, J., Wolfenson, H., Hone, J., & Sheetz, M. P. (2017). EGFR and HER2 activate rigidity sensing only on rigid matrices. *Nature Materials*, 16(7), 775–781. <https://doi.org/10.1038/nmat4893>
- Sica, A., Schioppa, T., Mantovani, A., & Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. *European Journal of Cancer*, 42(6), 717–727. <https://doi.org/10.1016/j.ejca.2006.01.003>
- Trieu, K. G., Tsai, S.-Y., Eberl, M., Ju, V., Ford, N. C., Doane, O. J., Peterson, J. K., Veniaminova, N. A., Grachtchouk, M., Harms, P. W., Swartling, F. J., Dlugosz, A. A., & Wong, S. Y. (2022). Basal cell carcinomas acquire secondary mutations to overcome dormancy and progress from microscopic to macroscopic disease. *Cell Reports*, 39(5), 110779. <https://doi.org/10.1016/j.celrep.2022.110779>
- Weckermann, D., Müller, P., Wawroschek, F., Harzmann, R., Riethmüller, G., & Schlimok, G. (2001). Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. *The Journal of Urology*, 166(2), 699–703. <http://www.ncbi.nlm.nih.gov/pubmed/11458120>
- Weidenfeld, K., Schif-Zuck, S., Abu-Tayeh, H., Kang, K., Kessler, O., Weissmann, M., Neufeld, G., & Barkan, D. (2016). Dormant tumor cells expressing LOXL2 acquire a stem-like phenotype mediating their transition to proliferative growth. *Oncotarget*, 7(44), 71362–71377. <https://doi.org/10.18632/oncotarget.12109>
- Wu, S., Zheng, Q., Xing, X., Dong, Y., Wang, Y., You, Y., Chen, R., Hu, C., Chen, J., Gao, D., Zhao, Y., Wang, Z., Xue, T., Ren, Z., & Cui, J. (2018). Matrix stiffness-upregulated LOXL2 promotes fibronectin production, MMP9 and CXCL12 expression and BMDCs recruitment to assist pre-

metastatic niche formation. *Journal of Experimental & Clinical Cancer Research : CR*, 37(1), 99. <https://doi.org/10.1186/s13046-018-0761-z>

Xiao, Y., Cong, M., Li, J., He, D., Wu, Q., Tian, P., Wang, Y., Yang, S., Liang, C., Liang, Y., Wen, J., Liu, Y., Luo, W., Lv, X., He, Y., Cheng, D.-D., Zhou, T., Zhao, W., Zhang, P., ... Hu, G. (2021). Cathepsin C promotes breast cancer lung metastasis by modulating neutrophil infiltration and neutrophil extracellular trap formation. *Cancer Cell*, 39(3), 423-437.e7. <https://doi.org/10.1016/j.ccell.2020.12.012>

Discussion

Breast cancer is the most frequently diagnosed malignant tumor in women worldwide. Neoadjuvant therapeutic management protocol includes chemotherapy cycles followed by surgical removal of the cancerous mass. Although this first line of breast cancer treatment is usually considered to be successful, some cancer cells can manage to escape from these attempts and undergo dormancy (Peyvandi et al., 2019). Dormant cancer cells are viable, non-proliferative cells which can preserve this status for years or even decades. Moreover, due to their low proliferative activity, dormant cells can evade therapy regimens and survive, while the patient can be falsely considered as clinically free of cancer (Damen et al., 2021). However, despite the various therapy efforts, cancer can come back after several years of disease-free condition, and can lead to recurrence, which frequently has fatal outcomes.

Recently, increased scientific efforts have been focused on the identification of the mechanisms which induce the re-activation of dormant cancer cells, as it has been recognized that dormancy escape can drive cancer recurrence and metastasis (Park & Nam, 2020). For example, it has been observed that microenvironmental changes can trigger dormant cells to resume proliferation which then leads to tumor relapse (Santos-de-Frutos & Djouder, 2021). Also, it has been described that chronic inflammation can drive the re-activation of dormant cells suggesting that the immune system can regulate recurrence (Pierce et al., 2009). Furthermore, Albregues and his collaborators demonstrated that neutrophil extracellular traps can induce dormancy escape by remodelling the extracellular matrix and thus altering integrin signalling, further confirming the importance of immune microenvironment in this process (Albregues et al., 2018a).

Macrophages have been long recognized as active components of the tumor microenvironment and their involvement in the progression of the primary tumor has been well characterized (Condeelis & Pollard, 2006; Lamagna et al., 2006; Pollard, 2004; B.-Z. Qian & Pollard, 2010; Ruffell et al., 2012; Sica et al., 2006). However, our knowledge on their contribution to dormancy and dormancy escape is very limited. In 2022, Borriello and his collaborators demonstrated that macrophages within the primary tumor microenvironment can prime disseminating tumor cells for a dormant phenotype. These primed DTCs were shown to perform increased speed of extravasation and better survival rates compared to experimentally metastasized cancer cells. However, depletion of macrophages at the primary tumor site resulted in a significantly decreased level of dormant cancer cells, thus confirming the contribution of macrophages to the success of the metastatic process (Borriello et al., 2021).

Macrophages were also implicated in dormancy escape, in an in-direct manner. It was shown in a pancreatic ductal adenocarcinoma model that macrophages favour the formation of a fibrotic microenvironment, through the secretion of granulin, which initiates the conversion of resident stellate cells into myofibroblasts. These myofibroblasts are responsible for the generation of the fibrotic environment, which triggers the awakening of dormant cancer cells *via* activation of integrin signalling (Nielsen et al., 2016).

It has been shown that those breast cancer patients who undergo surgical removal of the primary tumor, face an elevated risk for metastatic recurrence, which peaks at 12 to 18 months following the surgery. Surgical injury is known to trigger a systemic inflammation, which generally accompanied by increased production of inflammatory mediators, such as the main monocyte chemoattractant CCL2. Krall et al. demonstrated in 2018 that accumulation of myeloid cells – as a part of the inflammatory response following surgery – correlates with increased metastatic recurrence (Krall et al., 2018). Additionally, in a breast cancer mouse model, the downregulation of Her2 leads to an increased CCL5 secretion by the tumor cells, which resulted in the accumulation of CCR5+ macrophages within the tumor and was associated to increased recurrence. The authors suggest that macrophages might promote recurrence through collagen deposition, however, the molecular mechanisms need to be elucidated (Walens, DiMarco, et al., 2019).

It has never been sufficiently demonstrated whether direct interactions between dormant cancer cells and macrophages could lead to cancer relapse. Thus, the main objective of my thesis work was to investigate **whether there is a functional interaction between dormant cancer cells and macrophages** and to test **whether macrophages can trigger the re-activation of dormant cancer cells**. Understanding the molecular mechanisms of such interactions are particularly important since they can serve as potential targets for the development of targeted therapies to prevent recurrence.

To test the above-mentioned hypothesis, we used the D2.OR cell line – an accepted model for cancer dormancy both *in vitro* and *in vivo* – which is derived from spontaneous malignant neoplasms of Balb/C mouse mammary gland. D2.OR cells are dormant when cultured on basement membrane matrix (Matrigel) in DMEM media containing 0,5% serum or when injected in mice (Barkan & Green, 2011). Therefore, this cell line can be used to identify factors involved in the awakening of dormant cells.

Taking advantage of this model, we developed an *in vitro* co-culture system of bone marrow-derived, M-CSF-activated macrophages and dormant cancer cells to study whether macrophages themselves could contribute to the awakening of the dormant cells. For this, morphometric quantification of cancer cell outgrowth as well as proliferation measurements based on D2.OR bioluminescence was applied. Strikingly, we found that in such co-cultures, M-CSF-activated primary macrophages are able to induce the awakening of dormant D2.OR cells. However, neither cancer outgrowth nor proliferation were detected in co-cultures with non-activated bone marrow-derived cells, indicating that the previously observed awakening is macrophage-dependent (Fig. 1B-D).

To further confirm that in the presence of M-CSF-activated macrophages, dormant cancer cell awakening is accompanied with increased cell proliferation, we performed immunofluorescence staining for Ki67. Ki67 protein is known to be present in the nucleus of cells during all active phases of the cell cycle (G₁, S, G₂ and M), while is absent in quiescent cells (G₀), therefore can be used as a marker for cell proliferation (Bruno & Darzynkiewicz, 1992). Although morphologically we observed the formation of cellular outgrowths, it does not imply the re-activation of the cell proliferation. Therefore, performing Ki67 staining was essential to confirm that the macrophage-induced phenotype is coupled to an elevated proliferative status. Notably, we did not observe Ki67⁺ D2.OR cells in control conditions or when D2.OR cells were co-cultured with non-differentiated bone marrow-derived cells, which indicate that these D2.OR cells are in a quiescent, dormant state. However, around 33% of all D2.OR cells were Ki67⁺ in the presence of M-CSF activated macrophages, confirming that they switch from a quiescent to a proliferative status (Fig. 1E and 1F). **Thus, we demonstrated for the first time, that functional interactions between macrophages and dormant cancer cells can result in dormancy escape.**

Next, we were wondering whether differentiating macrophages into different subtypes would affect their ability to awaken dormant cancer cells. Therefore, we differentiated

macrophages from bone marrow-derived cells, using M-CSF (we considered these macrophages as M0-like macrophage), then transdifferentiated these M0-like macrophages into M1-like macrophages using LPS and IFN γ or into M2-like macrophages using IL-4 and IL-13 (Supp. Fig.1A) . Interestingly, when D2.OR cells were co-cultured with M1-like macrophages, we didn't observe the previously seen cancer cell outgrowths and in line with this, the bioluminescence of the D2.OR cells didn't increase, suggesting that M1-like macrophages do not have the ability to awaken dormant cancer cells. On the other hand, when D2.OR cells were co-cultured with M2-like macrophages, we could observe cancer cell awakening. However, we found that M-CSF-activated macrophages are the most potent inducers of dormant cancer cell awakening (Supp. Fig. 1B-E). It is important to note that those M-CSF-activated macrophages that we consider as M0, have been proposed to show rather M2-like phenotypic and functional properties. These M0 macrophages are known to be more involved in scavenging, phagocytosis and secrete anti-inflammatory cytokines, such as IL-10 (Orekhov et al., 2019). Also, M-CSF is frequently overexpressed by tumors, to activate the pro-tumoral functions of macrophages, thus generating tumor-associated macrophages (Laoui et al., 2014). Therefore, M-CSF generated macrophages should rather be considered as tumor promoting macrophages. Whereas, GM-CSF, another cytokine that is able to trigger macrophage differentiation, generates macrophages which belong to M1-like phenotype. These GM-CSF-activated macrophages participate in antigen presentation and secrete inflammatory cytokines, such as IL-12, IL-23 and TNF- α (Fleetwood et al., 2007). In all my experiments, I used M-CSF to generate macrophages *in vitro*, however, in order to better characterize how the different phenotypic variants influence dormancy escape, it would be important to test GM-CSF-activated macrophages as well within these experimental setups. As GM-CSF-activated macrophages exert M1-like characteristics and in our setups M1-like macrophages don't have potency to awaken dormant cancer cells, one could expect that GM-CSF-activated macrophages wouldn't trigger cancer cell awakening either.

Further experiments are required to evaluate whether macrophage polarization from M0 macrophages to M1- and M2-like phenotype was successful in our experimental setup. It could be done by comparing the expression patterns of different M1 (IL-1 β , CD86, CD80, TNF, IL-6) and M2 (CD163, CD206, IL-10, CXCR1, Arginase) marker genes by qPCR (Jayasingam et al., 2020). Additionally, it was suggested that M2-like macrophages should be further categorized into subgroups. The IL-4/IL-13 method, which have been applied here, is known to polarize

macrophages into M2a phenotype, whereas IL-1 β and LPS co-stimulation induces the polarization of M0 cells toward M2b. Finally, IL-10, TGF- β or glucocorticoid hormones promote M0 to M2c polarization (Ferrante & Leibovich, 2012). In my hands, M-CSF-activated (M2-like) macrophages had the greatest potency to awaken dormant cancer cells, while M2a polarization of M0 macrophages limited this ability. Therefore, we decided to uncover how these M-CSF-activated macrophages can induce the awakening of the dormant cancer cells.

To identify those macrophage-derived mechanisms which trigger D2.OR awakening, we first tested whether macrophages stimulate dormant cells *via* direct cell-cell interactions or through the secretion of soluble factors. Therefore, we treated quiescent D2.OR cells with cell-free conditioned medium obtained from undifferentiated bone marrow-derived cells or from activated macrophages. Since treatments with the macrophage conditioned medium (M0_CM) triggered the activation of D2.OR proliferation, we concluded that secreted factors mediate the macrophage-induced awakening. In addition, boiling the conditioned medium prevented its awakening potency, suggesting that macrophages act on dormant cells *via* soluble proteins. To identify those macrophage-secreted factors which are responsible for the awakening of the dormant D2.OR cells we performed a proteome profiling screen on bone marrow-derived cell and macrophage conditioned mediums. Using a membrane array kit, we identified a total of 8 molecules (CD14, LDLR, CCL6, CXCL10, CXCL16, Endostatin, HGF, MMP9) showing upregulation upon MCSF-activation of bone marrow-derived cells (Fig. 2C and 2D). Using recombinant proteins, blocking antibodies and inhibitor treatments, we aimed to validate whether among these molecules we can identify those ones which are responsible for the macrophage-mediated cancer cell awakening (Supp.Fig. 2A-C).

Zymography tests showed MMP9 activity within the M0 CM, confirming that these cells are expressing this matrix metalloprotease (Supp.Fig. 2D). Additionally, we found that blocking MMP activity with a pan-MMP inhibitor (Figure 17) or MMP9 activity with a specific MMP9 inhibitor (MMP9 inhibitor 1) significantly decreased the awakening potency of the macrophage conditioned media, indicating that **macrophage-derived MMP9 is involved in the macrophage-mediated cancer cell awakening**. However, treatment with recombinant either pro- or active MMP9 is not sufficient to resume the proliferation of dormant cancer cells, indicating that MMP9 co-operates with other factors to mediate dormancy escape (Fig. 2E and 2F). The importance of

MMP9-mediated matrix modifications in cancer cell awakening has been already demonstrated by previous works. Albregues et al. showed that cigarette smoke exposure or LPS-induced inflammation cause the formation of neutrophil extracellular traps (NETs) in the lung, which are DNA scaffolds that can release different enzymes and proteases. Among these, NETs release two proteases, MMP9 and neutrophil elastase, which were shown to cleave and remodel laminin which is then able to stimulate the proliferation of dormant D2.OR cells, through the activation of integrin $\alpha3\beta1$ and FAK/ERK/MLCK/YAP signalling. In this work, the authors demonstrate also, that neither MMP9 nor neutrophil elastase is sufficient alone to induce the awakening of dormant cancer cells (Albregues et al., 2018a).

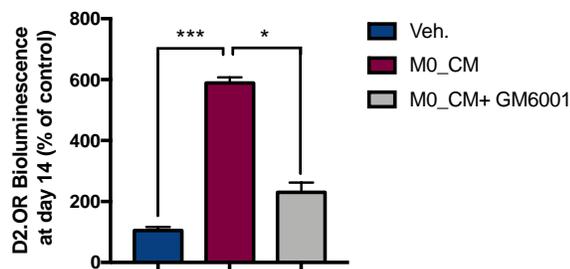


Figure 17. Pan-MMP inhibitor significantly decreased the awakening potency of M0 CM
Quantification of D2.OR bioluminescence on Matrigel at day 14 in 0,5% DMEM (Veh.) or upon treatment with M0_CM in the presence or absence of GM6001, a pan-MMP inhibitor shows, that GM6001 significantly decrease the awakening by M0_CM.

The role of the extracellular matrix has been already implied in dormancy escape. Several studies demonstrated that the transition from cellular dormancy to metastatic expansion both *in vitro* and *in vivo* is strongly regulated by integrin signalling (Albregues et al., 2018a; Barkan et al., 2008; Shibue & Weinberg, 2009). Additionally, collagen binding was shown to activate the non-canonical DDR1 signalling, which is known to promote cancer cell growth at metastatic regions (Gao et al., 2016). Furthermore, Tenascin-C has been shown to promote the metastatic expansion of breast cancer cells by enhancing Wnt signalling (Malladi et al., 2016). These findings suggest that the ECM can trigger activation of signalling pathways that enable the spread of metastatic disease and dormancy escape. Considering that we found MMP9 essential in the macrophage-mediated cancer cell awakening, we were wondering, whether macrophages trigger dormancy escape exclusively through matrix modifications. To test this hypothesis, we set up a

3D culture system. We primed Matrigel matrix with M0 or BMD conditioned media (CM), then seeded the D2.OR cells on these remodelled matrices (Fig. 3A). Interestingly, we found that these M0 CM-primed matrices are sufficient to trigger cancer cells awakening, whereas D2.OR cells showed dormant phenotype on BMD CM-primed Matrigel, suggesting that **macrophages mediate dormancy escape via matrix modifications** (Fig. 3B-E).

In order to identify which macrophage-mediated matrix modification could lead to cancer cell awakening, we performed a mini screen. Macrophages known to secrete different cysteine and serine proteases. It was previously published that MMP9 can cooperate with neutrophil elastase, a serine protease, to induce the dormancy escape of D2.OR cells (Albregues et al., 2018a). Therefore, we were wondering whether macrophages mediate cancer cell awakening through similar mechanisms. Thus, we tested protease nexin, a pan-serine protease inhibitor in our co-culture system, however, this inhibitor did not affect the macrophage-mediated awakening (Supp. Fig. 3C and 3D). In their work, Albregues and his colleagues found that *via* protease secretion, NETs reveal an epitope on the ECM within the lung, which is then recognised by the dormant cells. This interaction with the dormant cancer cells and the modified matrix then leads to the neutrophil-triggered dormancy escape. Importantly, they found that a custom-made antibody, antibody 28, which recognises the revealed epitopes of the MMP9/neutrophil elastase-primed matrix blocks D2.OR awakening (Figure 18). We had the opportunity to test whether this antibody 28 could affect the macrophage-mediated cancer cell awakening as well. We found that antibody 28 did not inhibit the macrophage-mediated dormancy escape, suggesting that macrophages promote cancer cell re-activation through different matrix modifications (Supp. Fig. 3G and 3H). Additionally, we tested a pan-cysteine protease inhibitor, called E64, which also proved to be ineffective to block macrophage-mediated cancer cell awakening (Supp. Fig. 3A and 3B).

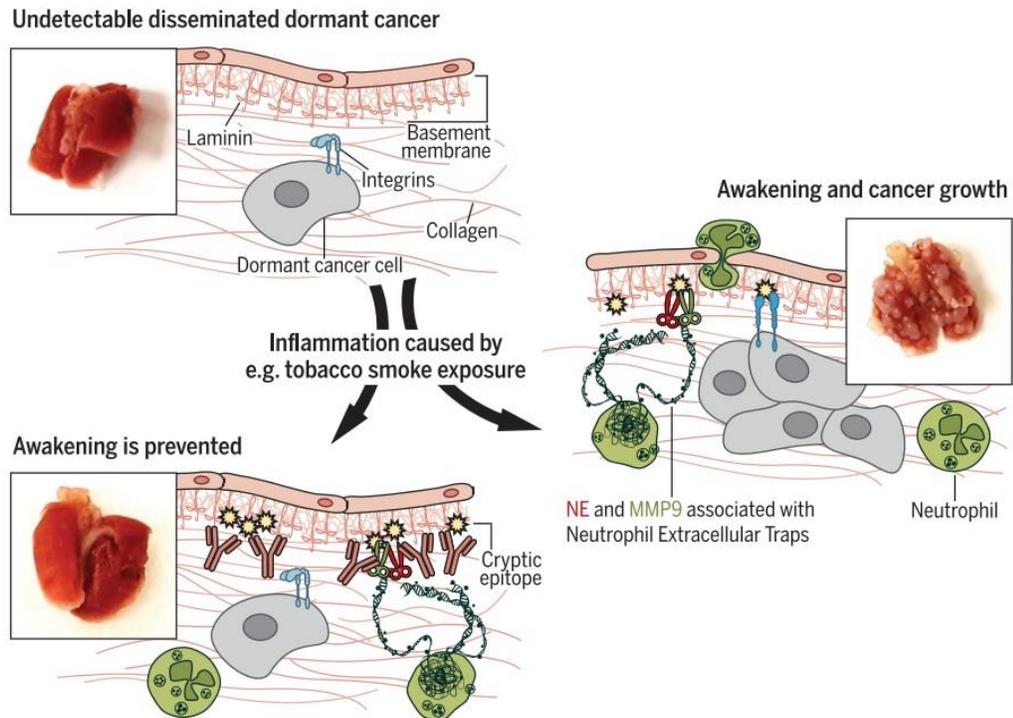


Figure 18. Schematic representation of the NETs-dependent dormancy escape

Neutrophil extracellular traps formed upon lung inflammation awaken dormant cancer cells through the secretion of neutrophil elastase (NE) and MMP9. These two proteases cleave specific epitopes on the extracellular matrix protein laminin, which trigger the awakening of dormant cells. Antibodies blocking this epitope (Antibody 28) prevent the inflammation-triggered awakening.

Adapted from: (Albregues et al., 2018b)

Clinical data shows that in breast cancer patients the presence of LOXL2 is associated with a decreased time of relapse-free survival (Weidenfeld et al., 2016). Additionally, Hollosi and his collaborators demonstrated that MCF-7 breast cancer cells show dormant phenotype *in vivo* within the lung, however when MCF-7 cells were stably transduced to express LOXL2, they observed metastatic outgrowth (Hollosi et al., 2009). Furthermore, according to Levental and colleagues, lysyl-oxidase-mediated collagen crosslinking contributes to the stiffening of the extracellular matrix, which subsequently enables tumor cells to behave more invasively. In more details, increased tension caused by a stiffer matrix triggers integrin clustering and leads to the phosphorylation of the focal adhesion kinase (pFAK), thus promoting the activation of several

downstream signalling pathways, such as the AKT, Rho and ERK pathways. The activation of these pathways leads to cytoskeletal changes within the breast cancer cells accompanied with increased proliferation and migration (Levental et al., 2009). These observations implicate that lysyl oxidases and their matrix modifications could influence dormancy. Evidence suggests that macrophages can trigger matrix modifications through lysyl oxidases as well (Alonso-Nocelo et al., 2022; Huang et al., 2021). Therefore, we compared the LOX activity within M0 and BMD CM. We found that LOX activity was significantly higher in the M0 CM, compared to the BMD CM. To test whether the LOX activity of M-CSF-activated macrophages contributes to their awakening potency, we used β -Aminopropionitrile (BAPN), an inhibitor of LOX activity. BAPN significantly decreased the effect of the M0 CM, suggesting that **LOX activity is involved in the macrophage-mediated dormancy escape** (Fig. 3D-H).

Interestingly, the relative expression of LOX and MMP9 in tissue specimens from gastric cancer patients showed a positive correlation. Furthermore, when BGC-823 gastric cancer cells were treated *in vitro* with BAPN to inhibit LOX, both protein concentration and enzyme activity of MMP9 decreased in the culture supernatants. However, the treatment of BGC-823 cells with exogenous LOX resulted in an increased MMP9 protein concentration and enzyme activity (Zhao et al., 2019). Correlations were also observed between LOX and MMP9 in non-small cell lung cancer (NSCLC) by comparing the mRNA and protein expression of LOX and MMP9 in samples from 30 NSCLC patients. In comparison to their non-cancerous counterparts, the relative mRNA and protein expression of LOX was significantly higher in NSCLC tumor tissues. In addition, higher LOX expression was strongly correlated with MMP9 expression, as well as with increased tumor size and lymph node metastasis. The authors suggest that LOX might promote NSCLC metastasis *via* enhancing MMP9 expression (J. Liu et al., 2014). These observations suggest that LOX may have a role in controlling MMP9 activity, therefore using BAPN to inhibit LOX, might affect MMP9 as well.

Considering that LOXL2 has already been implied in dormancy escape and macrophages were already shown to secrete this specific amine oxidase, we were wondering whether recombinant, APMA-activated MMP9 and recombinant LOXL2 treatment would be sufficient to induce the awakening of dormant D2.OR cells (Hollosi et al., 2009; Levental et al., 2009; Ramos et al., 2022; Weidenfeld et al., 2016). We found that this combinational treatment could not trigger dormancy escape (Figure 19). It is possible, that M-CSF-activated macrophages secrete a different

LOX isoform. In order to identify which isoform is expressed by MCSF-activated macrophages, in the future we will compare the expression levels of LOX, LOXL1, LOXL2, LOXL3 and LOXL4 using qPCR. After specifically determining which LOX enzyme is expressed by the M-CSF-activated macrophages, the same experiment should be performed, but using this recombinant form of LOX protein. However, it cannot be excluded that not only MMP9 and LOX activities are responsible for the macrophage-mediated cancer cell awakening.

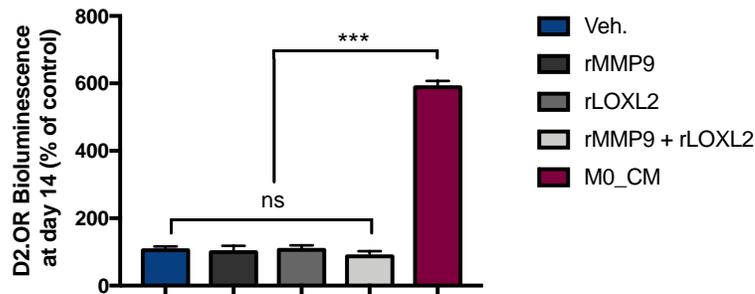


Figure 19. Combinational treatment with rLOXL2 and rMMP9 is not sufficient to induce the awakening of dormant D2.OR cells

Quantification of D2.OR bioluminescence on Matrigel at day 14 in 0,5% DMEM (Veh.) or upon treatment with M0_CM or recombinant MMP9, recombinant LOXL2 or both.

Next, we aimed to identify those matrix modifications which might be responsible for the macrophage mediated dormant cell awakening. It has been already shown that MMP9 has collagenase activity (Kumar et al., 2000; Zeng et al., 1999). Therefore, using a fluorescent reporter assay, we decided to test whether the observed awakening is coupled to increased hydrolysis of Collagene IV – the dominant collagen form of the Matrigel matrix we used. We didn’t observe any fluorescent signal in control conditions or when D2.OR cells were treated with BMD CM, indicating that D2.OR cells by themselves do not secrete any enzymes with collagenase activity. However, we saw a significant increase in the fluorescent signal when D2.OR cells were treated with M0 CM. As expected, MMP9 inhibitor treatments decreased the collagenase activity of the applied conditioned media. However, treatments with the LOX inhibitor BAPN alone and also in

combination with MMP9 inhibitor led to a similar degree of collagenase inhibition, suggesting that LOX is an upstream regulator of MMP9 (Fig. 3I and 3J).

To test, whether the increased collagen degradation was a result of enzyme activity derived from the macrophage conditioned media or was dependent on the presence of D2.OR cells, we measured the fluorescent intensity generated by the different conditioned media in the absence of the D2.OR cells. Importantly, we found that M0 macrophage conditioned medium has high collagenase IV activity, however, this activity was further elevated when D2.OR cells were present (Figure 20). These results suggest that upon stimulation with macrophage conditioned media, D2.OR cells might secrete further proteases, which could contribute to those matrix modifications which subsequently lead to the awakening of dormant cancer cells. Further experiments are required to study whether the secretome of D2.OR cells are changing upon stimulation with M0 CM or when D2.OR cells are seeded on the different primed matrices. Such experiments could bring us closer to the better understanding of those matrix modifications which can trigger dormancy escape.

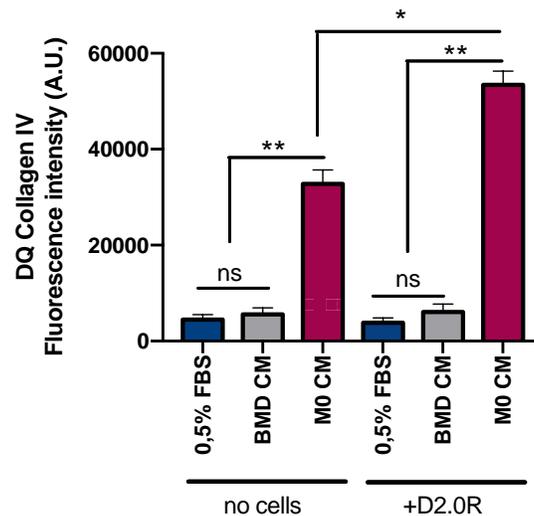


Figure 20. DQ Collagen IV degradation in the presence and absence of D2.OR cells

Quantification of the fluorescence intensity derived from the degradation of DQ Collagen IV upon stimulation with the indicated CM in the presence or absence of D2.OR cells.

To better understand the macrophage-mediated dormancy escape, we also investigated the molecular mechanisms which are triggered in the dormant cancer cells. The mechanical cues arising from the extracellular matrix are known to be transduced by integrins (Barkan & Green, 2011). Several studies provided evidence about the importance of integrins, particularly β -1 integrin in the transition from cellular dormancy to metastasis formation (Albregues et al., 2018a; Barkan et al., 2008, 2010; Barkan & Chambers, 2011; Barkan & Green, 2011; Shibue & Weinberg, 2009). Notably, the downregulation of the uPA-UPAR interaction was shown to decrease β -1 integrin signalling, thus leading to the transition from proliferative to dormant phenotype (Aguirre-Ghiso et al., 2003). Additionally, β -1 integrin signalling was found to be particularly important in the regulation of metastasis formation in D2.OR cells, as upregulating β -1 signalling allowed dormant cancer cells to re-enter the cell cycle (Barkan et al., 2008). Integrins promote the recruitment and activation of signalling proteins such as non-receptor tyrosine kinases including focal adhesion kinase (FAK) and c-Src that form a dual kinase complex and initiate biochemical signalling to regulate cell proliferation, survival and motility (Mitra & Schlaepfer, 2006). It was shown that collagen binding to integrin receptors can initiate the activation of FAK and Src complex and trigger ERK phosphorylation, thus promoting the metastatic outgrowth (Boyerinas et al., 2013). Therefore, we aimed to test whether the macrophage-mediated matrix modifications can also trigger similar integrin signalling activation and subsequent FAK-Src activation. For this, we used specific blocking antibodies against integrin β -1, and small inhibitory molecules to target FAK (PF573228) and Src (SU6656). Remarkably, these treatments blocked the macrophage-mediated cancer cell awakening; therefore, we suggest that **macrophages induces matrix modifications, which trigger β -1 integrin- and subsequent FAK-Src activation.**

We found that the dormancy state of D2.OR cells on Matrigel is strongly depend on the available nutrients and growth factors, such as EGF. D2.OR cells displayed dormant phenotype only in low serum (0,5%) containing media, whereas formed outgrowth when 10% serum containing media was applied (results not shown). Proteases, including MMP9 are known to cleave proteins from the ECM, thus releasing matrix-bound growth factors, cytokines and matrikines (Winkler et al., 2020). The term matrikines refers to those proteins that were released *via* partial proteolysis from the macromolecules of the ECM and possess the ability to control cellular activity

(Maquart et al., 2004). One could imagine that upon M0 CM treatment, MMP9 activity could trigger the release of growth factors or matrikines from the Matrigel, which could contribute to the dormancy escape of D2.OR cells. However, it would not explain the awakening of D2.OR cells on M0 conditioned media-primed matrices, as following the pre-conditioning of the Matrigel with the different conditioned media, we performed washing steps which could remove factors that may become soluble. On the other hand, it cannot be excluded that macrophage-mediated matrix modifications reveal a new epitope, which could trigger integrin activation, thus leading to dormancy escape.

We identified EGF also to be a potent inducer of the dormancy escape of the D2.OR cells (Fig. 4A and 4B). Since macrophages were shown to secrete EGF upon M-CSF stimulation in order to promote cancer cell progression (Condeelis & Pollard, 2006; Wyckoff et al., 2004), we sought to investigate, whether EGF is involved in the macrophage-mediated cancer cell awakening. Since the proteome profiler that we used to compare the secretome of M0 and BMD cells did not detect any EGF secretion upon M-CSF stimulation of macrophages, we used ELISA to quantify the level of EGF within the different M0, M1, M2 and BMD CMs. However, we found that none of these tested media contained EGF, indicating that the observed D2.OR reactivation is not mediated by macrophage-secreted EGF (Supp.Fig. 4C). Despite that the macrophage-conditioned media did not contain EGF, we found that EGFR signalling is essential for the macrophage-mediated cancer cell awakening. Using the antibody Cetuximab, a specific inhibitor of the ligand-dependent activation of EGFR and the two tyrosine kinase inhibitors Ag1478 and Gefitinib, we were able to demonstrate that **macrophage-mediated matrix modifications trigger EGFR activation in dormant D2.OR cells in a ligand independent manner** (Fig. 4C-E). It has been long recognised that integrins can stimulate the tyrosine phosphorylation of the EGFR and its family member receptor Her2 in the absence of any receptor ligands, thus leading to ERK activation (Miyamoto et al., 1996; Moro, 1998; S. E. Wang et al., 2009; Yoon et al., 2006). These studies demonstrate that integrin clustering could result in the clustering and phosphorylation of EGFR/ErbB2 and shows that FAK represents the link between integrin and receptor tyrosine kinases, including the ErbB family (Sieg et al., 2000; S. E. Wang et al., 2009). Additionally, similar mechanisms have been already implied in dormancy escape. Notably, it was observed, that fibronectin-derived signals trigger integrin activation in dormant, uPAR-rich squamous carcinoma cells, which leads to a FAK-dependent EGFR phosphorylation in the absence of EGF receptor

ligands. Then EGFR signalling will result in ERK phosphorylation and subsequent dormancy escape and cancer cell proliferation (Ki67+ cells). The authors also show that this dormancy escape is accompanied by p38 inactivation, which is mediated by CDC42 (Aguirre-Ghiso, 2007b; Aguirre-Ghiso et al., 2001; D. Liu et al., 2002). In line with this, we showed that Her2/ErbB2, FAK, ERK1 and ERK2 phosphorylation is increased when D2.OR cells were seeded on M0 CM-primed matrix (Fig. 4F-I). Therefore, we suggest that **macrophage-mediated matrix modifications could trigger β 1-integrin- and FAK dependent EGFR activation which then culminates to ERK signalling and increased proliferation**. Currently, we are evaluating how these phosphorylation patterns are changing in the presence of the different inhibitors (MMP9 inhibitor, BAPN, CD29 inhibitor, SU6656, Ag1478, Gefitinib, Cetuximab, PF573228, UO126) in order to better characterize the regulatory network of macrophage-mediated signalling pathways leading to dormancy escape.

An important follow-up of this work would be the *in vivo* validation of my results. For this, I suggest a liver metastasis model, as it was shown that 45% of Her2+ breast cancer cells metastasize to this organ (Harbeck et al., 2019). Additionally, it has been shown that macrophages recruited to the liver promote metastasis formation of pancreatic ductal adenocarcinoma indirectly by the activation of hepatic stellate cells into myofibroblasts, which triggers the generation of a fibrotic microenvironment that promotes the metastatic outgrowth of PDAC cells (Nielsen et al., 2016). Importantly, it has been shown that D2.OR cells preserve their dormant phenotype within the liver (Goddard et al., 2016). Additionally, macrophages are known to accumulate within the liver upon CCL4-induced fibrosis (Binatti et al., 2022; Manuelpillai et al., 2012). Therefore, based on my results, it would be important to investigate whether macrophages recruited to the liver upon CCL4-induced fibrosis could trigger dormancy escape of breast cancer cells. Moreover, we would also study whether the administration of MMP9 inhibitor, BAPN or EGFR tyrosine kinase inhibitors (Ag1478 and Gefitinib) would influence the dormancy state of D2.OR cells in this experimental setup. These *in vivo* studies could validate my *in vitro* findings regarding the importance of macrophage-derived mechanisms in dormancy escape and could contribute to the improvement of therapeutic approaches to prevent the spread of breast cancer.

Another possible follow-up for this work could be an obesity study. Clinical studies show that obesity represents an increased risk for breast cancer recurrence, suggesting that the

microenvironment that is formed upon obesity favors the re-activation of dormant tumor cells (Blair et al., 2019). During weight gain, because of the intensive lipid accumulation, the average size of the adipocytes is increasing. This can eventually lead to adipocyte necrosis which culminates to the rupture of the plasma membrane and the release of the adipocyte content. The cellular contents released into the microenvironment can trigger the accumulation of phagocytic macrophages to scavenge lipids and the cellular debris. It has been observed that the recruited macrophages encircle the necrotic adipocytes, thus form the so-called crown-like structures (CLS), which became the histological hallmarks of adipose tissue inflammation upon obesity (Weisberg et al., 2003). Since mammary tissue is enriched in adipocytes, such changes in breast adipose homeostasis upon obesity might have a determinant effect on breast cancer progression, therapy outcome and relapse as well (Vaysse et al., 2017). Accordingly, histology analysis of breast cancer samples indicated that the presence of CLS is associated to an increased risk for recurrence and mortality, suggesting that changes in the immune microenvironment of the obese breast adipose tissue might affect dormant cancer cells (Maliniak et al., 2021). Based on my results, an important issue would be to study the contribution of obesity-associated macrophages to dormancy escape.

Clinical implications

Elevated MMP9 levels has been associated to poor prognosis in patients with breast, colorectal, ovarian and non-small lung cancer, which makes MMP9 an attractive target for anticancer therapies (Jiang & Li, 2021; Martins et al., 2019; Peltonen et al., 2021; Shao et al., 2011). Several MMP9 inhibitors have been already developed and tested in clinical trials, however so far all of them failed mainly due to poor inhibitor selectivity, dose-limiting toxicity or insufficient efficacy (Augoff et al., 2022). My results further highlight the potential of specific MMP9 inhibitors as important targets for targeted therapies. Therefore, in order to exploit MMP9 inhibitors as crucial tools in anticancer therapy, it is necessary to continue the search for selective, safe, and more potent MMP9 inhibitors.

LOX enzymes have been also showed to be critical in the progression and metastasis formation of breast cancer. Although, several LOX inhibitors are under preclinical trials, which are encouraging, clinical data in breast cancer is non-existent so far (Ferreira et al., 2021).

Patient relapse can be prevented by keeping the disseminated cancer cells in a dormant state. Our data suggests that tyrosine kinase inhibitors, such as Gefitinib could contribute to the dormancy state of cancer cells. Gefitinib was shown to be a well-tolerated EGFR inhibitor, with promising results in lung cancer (Wakeling et al., 2002; Ye et al., 2020). Gefitinib was discovered to have a synergistic impact with tamoxifen treatment, inhibiting the proliferation of endocrine-resistant MCF-7 breast cancer cells *via* lowering AKT and MAPK phosphorylation (Gee et al., 2003; Normanno et al., 2006). Although multiple studies have noted the effectiveness of Gefitinib, there is still insufficient data to support routine clinical use of Gefitinib supplementation in patients with breast cancer.

Taken together, our results contribute to the better understanding of the molecular regulation of dormancy escape, thereby highlighting MMP9, LOX and EGFR inhibitors as potential therapeutic targets to prevent cancer cell awakening.

Concluding remarks

As a conclusion, my work identified macrophages as major actors in dormancy escape and provided evidence that macrophages mediate cancer cell awakening *via* LOX and MMP9-dependent matrix modifications. These matrix modifications are transduced via integrin- β 1/Src/FAK axis and trigger a ligand-independent EGFR phosphorylation, which leads to ERK activation, thus to the proliferation of cancer cells. One possible way to avoid patient relapse is to block the awakening of dormant cancer cells. For this, there is an urgent need to better characterize those mechanisms which could re-activate dormant cancer cells, as they could facilitate the development of clinical cancer management. By uncovering this, we could open new avenues toward future clinical strategies by targeting dormant cancer cells.

Bibliography

- Acerbi, I., Cassereau, L., Dean, I., Shi, Q., Au, A., Park, C., Chen, Y. Y., Liphardt, J., Hwang, E. S., & Weaver, V. M. (2015). Human breast cancer invasion and aggression correlates with ECM stiffening and immune cell infiltration. *Integrative Biology*, 7(10), 1120–1134. <https://doi.org/10.1039/c5ib00040h>
- Adam, A. P., George, A., Schewe, D., Bragado, P., Iglesias, B. v., Ranganathan, A. C., Kourtidis, A., Conklin, D. S., & Aguirre-Ghiso, J. A. (2009). Computational identification of a p38 SAPK-regulated transcription factor network required for tumor cell quiescence. *Cancer Research*, 69(14), 5664–5672. <https://doi.org/10.1158/0008-5472.CAN-08-3820>
- Ades, F., Zardavas, D., Bozovic-Spasojevic, I., Pugliano, L., Fumagalli, D., de Azambuja, E., Viale, G., Sotiriou, C., & Piccart, M. (2014). Luminal B breast cancer: Molecular characterization, clinical management, and future perspectives. *Journal of Clinical Oncology*, 32(25), 2794–2803. <https://doi.org/10.1200/JCO.2013.54.1870>
- Afik, R., Zigmond, E., Vugman, M., Klepfish, M., Shimshoni, E., Pasmanik-Chor, M., Shenoy, A., Bassat, E., Halpern, Z., Geiger, T., Sagi, I., & Varol, C. (2016). Tumor macrophages are pivotal constructors of tumor collagenous matrix. *Journal of Experimental Medicine*, 213(11), 2315–2331. <https://doi.org/10.1084/jem.20151193>
- Aguirre-Ghiso, J. A. (2007a). Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews Cancer*, 7(11), 834–846. <https://doi.org/10.1038/nrc2256>
- Aguirre-Ghiso, J. A. (2007b). Models, mechanisms and clinical evidence for cancer dormancy. *Nature Reviews Cancer*, 7(11), 834–846. <https://doi.org/10.1038/nrc2256>
- Aguirre-Ghiso, J. A., Estrada, Y., Liu, D., & Ossowski, L. (2003). ERKMAPK activity as a determinant of tumor growth and dormancy; regulation by p38SAPK. *Cancer Research*, 63(7), 1684–1695. <https://doi.org/10.1016/j.urolonc.2003.12.012>
- Aguirre-Ghiso, J. A., Estrada, Y., Liu, D., & Ossowski, L. (2003). ERKMAPK activity as a determinant of tumor growth and dormancy; regulation by p38SAPK. *Cancer Research*, 63(7), 1684–1695. <https://doi.org/10.1016/j.urolonc.2003.12.012>
- Aguirre-Ghiso, J. A., Liu, D., Mignatti, A., Kovalski, K., & Ossowski, L. (2001). Urokinase receptor and fibronectin regulate the ERKMAPK to p38MAPK activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Molecular Biology of the Cell*, 12(4), 863–879. <https://doi.org/10.1091/mbc.12.4.863>
- Akkari, L., Gocheva, V., Kester, J. C., Hunter, K. E., Quick, M. L., Sevenich, L., Wang, H.-W., Peters, C., Tang, L. H., Klimstra, D. S., Reinheckel, T., & Joyce, J. A. (2014). Distinct functions of macrophage-derived and cancer cell-derived cathepsin Z combine to promote tumor malignancy via interactions with the extracellular matrix. *Genes & Development*, 28(19), 2134–2150. <https://doi.org/10.1101/gad.249599.114>
- Albregues, J., Shields, M. A., Ng, D., Park, C. G., Ambrico, A., Poindexter, M. E., Upadhyay, P., Uyeminami, D. L., Pommier, A., Küttner, V., Bružas, E., Maiorino, L., Bautista, C., Carmona, E. M., Gimotty, P. A., Fearon, D. T., Chang, K., Lyons, S. K., Pinkerton, K. E., ... Egeblad, M. (2018a). Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*, 361(6409). <https://doi.org/10.1126/science.aao4227>
- Albregues, J., Shields, M. A., Ng, D., Park, C. G., Ambrico, A., Poindexter, M. E., Upadhyay, P., Uyeminami, D. L., Pommier, A., Küttner, V., Bružas, E., Maiorino, L., Bautista, C., Carmona, E. M., Gimotty, P. A., Fearon, D. T., Chang, K., Lyons, S. K., Pinkerton, K. E., ... Egeblad, M. (2018b). Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science (New York, N.Y.)*, 361(6409). <https://doi.org/10.1126/science.aao4227>

- Almog, N., Ma, L., Raychowdhury, R., Schwager, C., Erber, R., Short, S., Hlatky, L., Vajkoczy, P., Huber, P. E., Folkman, J., & Abdollahi, A. (2009). *Transcriptional Switch of Dormant Tumors to Fast-Growing Angiogenic Phenotype*. 3, 836–845. <https://doi.org/10.1158/0008-5472.CAN-08-2590>
- Almog, N., Ma, L., Schwager, C., Brinkmann, B. G., Beheshti, A., Vajkoczy, P., Folkman, J., Hlatky, L., & Abdollahi, A. (2012). Consensus Micro RNAs Governing the Switch of Dormant Tumors to the Fast-Growing Angiogenic Phenotype. *PLoS ONE*, 7(8), e44001. <https://doi.org/10.1371/journal.pone.0044001>
- Alonso-Nocelo, M., Ruiz-Cañas, L., Sancho, P., Görgülü, K., Alcalá, S., Pedrero, C., Vallespinos, M., López-Gil, J. C., Ochando, M., García-García, E., David Trabulo, S. M., Martinelli, P., Sánchez-Tomero, P., Sánchez-Palomo, C., Gonzalez-Santamaría, P., Yuste, L., Wörmann, S. M., Kabacaoğlu, D., Earl, J., ... Sainz, B. (2022). Macrophages direct cancer cells through a LOXL2-mediated metastatic cascade in pancreatic ductal adenocarcinoma. *Gut*. <https://doi.org/10.1136/gutjnl-2021-325564>
- Amin, M. B., Greene, F. L., Edge, S. B., Compton, C. C., Gershenwald, J. E., Brookland, R. K., Meyer, L., Gress, D. M., Byrd, D. R., & Winchester, D. P. (2017). The Eighth Edition AJCC Cancer Staging Manual: Continuing to build a bridge from a population-based to a more “personalized” approach to cancer staging. In *CA: A Cancer Journal for Clinicians* (Vol. 67, Issue 2, pp. 93–99). <https://doi.org/10.3322/caac.21388>
- Augoff, K., Hryniewicz-Jankowska, A., Tabola, R., & Stach, K. (2022). MMP9: A Tough Target for Targeted Therapy for Cancer. *Cancers*, 14(7). <https://doi.org/10.3390/cancers14071847>
- Barkan, D., & Chambers, A. F. (2011). β 1-integrin: A potential therapeutic target in the battle against cancer recurrence. *Clinical Cancer Research*, 17(23), 7219–7223. <https://doi.org/10.1158/1078-0432.CCR-11-0642>
- Barkan, D., el Touny, L. H., Michalowski, A. M., Smith, J. A., Chu, I., Davis, A. S., Webster, J. D., Hoover, S., Simpson, R. M., Gaudie, J., & Green, J. E. (2010). Metastatic Growth from Dormant Cells Induced by a Col-I–Enriched Fibrotic Environment. *Cancer Research*, 70(14), 5706–5716. <https://doi.org/10.1158/0008-5472.CAN-09-2356>
- Barkan, D., & Green, J. E. (2011). An in vitro system to study tumor dormancy and the switch to metastatic growth. *Journal of Visualized Experiments: JoVE*, 54. <https://doi.org/10.3791/2914>
- Barkan, D., Kleinman, H., Simmons, J. L., Asmussen, H., Kamaraju, A. K., Hoenorhoff, M. J., Liu, Z., Costes, S. v., Cho, E. H., Lockett, S., Khanna, C., Chambers, A. F., & Green, J. E. (2008). Inhibition of Metastatic Outgrowth from Single Dormant Tumor Cells by Targeting the Cytoskeleton. *Cancer Research*, 68(15), 6241–6250. <https://doi.org/10.1158/0008-5472.CAN-07-6849>
- Benz, C. C. (2008). Impact of aging on the biology of breast cancer. *Critical Reviews in Oncology/Hematology*, 66(1), 65–74. <https://doi.org/10.1016/j.critrevonc.2007.09.001>
- Bertazza, L., & Mocellin, S. (2010). The Dual Role of Tumor Necrosis Factor (TNF) in Cancer Biology. *Current Medicinal Chemistry*, 17(29), 3337–3352. <https://doi.org/10.2174/092986710793176339>
- Bezbradica, J. S., Gordy, L. E., Stanic, A. K., Dragovic, S., Hill, T., Hawiger, J., Unutmaz, D., van Kaer, L., & Joyce, S. (2006). Granulocyte-Macrophage Colony-Stimulating Factor Regulates Effector Differentiation of Invariant Natural Killer T Cells during Thymic Ontogeny. *Immunity*, 25(3), 487–497. <https://doi.org/10.1016/j.immuni.2006.06.017>

- Bhowmick, N. A., Chytil, A., Plieth, D., Gorska, A. E., Dumont, N., Shappell, S., Washington, M. K., Neilson, E. G., & Moses, H. L. (2004). TGF- β Signaling in Fibroblasts Modulates the Oncogenic Potential of Adjacent Epithelia. *Science*, *303*(5659), 848–851. <https://doi.org/10.1126/science.1090922>
- Binatti, E., Gerussi, A., Barisani, D., & Invernizzi, P. (2022). The Role of Macrophages in Liver Fibrosis: New Therapeutic Opportunities. *International Journal of Molecular Sciences*, *23*(12), 6649. <https://doi.org/10.3390/ijms23126649>
- Bissell, M. J., & Hines, W. C. (2011). Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature Medicine*, *17*(3), 320–329. <https://doi.org/10.1038/nm.2328>
- Biswas, S. K., & Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature Immunology*, *11*(10), 889–896. <https://doi.org/10.1038/ni.1937>
- Blair, C. K., Wiggins, C. L., Nibbe, A. M., Storlie, C. B., Prossnitz, E. R., Royce, M., Lomo, L. C., & Hill, D. A. (2019). Obesity and survival among a cohort of breast cancer patients is partially mediated by tumor characteristics. *Npj Breast Cancer*, *5*(1), 33. <https://doi.org/10.1038/s41523-019-0128-4>
- Bloom, H. J., & Richardson, W. W. (1957). Histological grading and prognosis in breast cancer a study of 1409 cases of which 359 have been followed for 15 years. *British Journal of Cancer*, *11*(3), 359–377. <https://doi.org/10.1038/bjc.1957.43>
- Bonde, A.-K., Tischler, V., Kumar, S., Soltermann, A., & Schwendener, R. A. (2012). Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. *BMC Cancer*, *12*(1), 35. <https://doi.org/10.1186/1471-2407-12-35>
- Borriello, L., Coste, A., Sharma, V. P., Karagiannis, G. S., Lin, Y., Ye, X., Duran, C. L., Chen, X., Dalla, E., Singh, D. K., Aguirre-Ghiso, J. A., Condeelis, J., & Entenberg, D. (2021). Primary tumor associated macrophages activate programs of invasion and dormancy in disseminating tumor cells. *BioRxiv*, 2021.02.04.429798. <https://doi.org/10.1038/s41467-022-28076-3>
- Bowers, L. W., Maximo, I. X. F., Brenner, A. J., Beeram, M., Hursting, S. D., Price, R. S., Tekmal, R. R., Jolly, C. A., & DeGraffenried, L. A. (2014). NSAID use reduces breast cancer recurrence in overweight and obese women: Role of prostaglandin-aromatase interactions. *Cancer Research*, *74*(16), 4446–4457. <https://doi.org/10.1158/0008-5472.CAN-13-3603>
- Boyerinas, B., Zafrir, M., Yesilkanal, A. E., Price, T. T., Hyjek, E. M., & Sipkins, D. A. (2013). Adhesion to osteopontin in the bone marrow niche regulates lymphoblastic leukemia cell dormancy. *Blood*, *121*(24), 4821–4831. <https://doi.org/10.1182/blood-2012-12-475483>
- Bragado, P., Estrada, Y., Parikh, F., Krause, S., Capobianco, C., Farina, H. G., Schewe, D. M., & Aguirre-Ghiso, J. A. (2013). TGF- β 2 dictates disseminated tumour cell fate in target organs through TGF- β -RIII and p38 α / β signalling. *Nature Cell Biology*, *15*(11), 1351–1361. <https://doi.org/10.1038/ncb2861>
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, *68*(6), 394–424. <https://doi.org/10.3322/caac.21492>
- Breast cancer and hormone-replacement therapy in the Million Women Study. (2003). *The Lancet*, *362*(9382), 419–427. [https://doi.org/10.1016/S0140-6736\(03\)14065-2](https://doi.org/10.1016/S0140-6736(03)14065-2)

- Brewer, H. R., Jones, M. E., Schoemaker, M. J., Ashworth, A., & Swerdlow, A. J. (2017). Family history and risk of breast cancer: an analysis accounting for family structure. *Breast Cancer Research and Treatment*, *165*(1), 193–200. <https://doi.org/10.1007/s10549-017-4325-2>
- Bruno, S., & Darzynkiewicz, Z. (1992). Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells. *Cell Proliferation*, *25*(1), 31–40. <https://doi.org/10.1111/j.1365-2184.1992.tb01435.x>
- Burke, R. M., Madden, K. S., Perry, S. W., Zettel, M. L., & Brown, E. B. (2013). Tumor-associated macrophages and stromal TNF- α regulate collagen structure in a breast tumor model as visualized by second harmonic generation. *Journal of Biomedical Optics*, *18*(8), 086003. <https://doi.org/10.1117/1.JBO.18.8.086003>
- Cabrera, R. M., Mao, S. P. H., Surve, C. R., Condeelis, J. S., & Segall, J. E. (2018). A novel neuregulin – jagged1 paracrine loop in breast cancer transendothelial migration. *Breast Cancer Research*, *20*(1), 24. <https://doi.org/10.1186/s13058-018-0960-8>
- Cardones, A. R., Murakami, T., & Hwang, S. T. (2003). CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via beta(1) integrin. *Cancer Research*, *63*(20), 6751–6757. <http://www.ncbi.nlm.nih.gov/pubmed/14583470>
- Cardoso, F., Kyriakides, S., Ohno, S., Penault-Llorca, F., Poortmans, P., Rubio, I. T., Zackrisson, S., & Senkus, E. (2019). Early breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of Oncology*, *30*(8), 1194–1220. <https://doi.org/10.1093/annonc/mdz173>
- Catsburg, C., Miller, A. B., & Rohan, T. E. (2015). Active cigarette smoking and risk of breast cancer. *International Journal of Cancer*, *136*(9), 2204–2209. <https://doi.org/10.1002/ijc.29266>
- Chaffer, C. L., & Weinberg, R. A. (2011). A perspective on cancer cell metastasis. *Science*, *331*(6024), 1559–1564. <https://doi.org/10.1126/science.1203543>
- Chambard, J. C., Lefloch, R., Pouyssegur, J., & Lenormand, P. (2007). ERK implication in cell cycle regulation. *Biochimica et Biophysica Acta - Molecular Cell Research*, *1773*(8), 1299–1310. <https://doi.org/10.1016/j.bbamcr.2006.11.010>
- Chen, Q., Zhang, X. H.-F., & Massagué, J. (2011). Macrophage Binding to Receptor VCAM-1 Transmits Survival Signals in Breast Cancer Cells that Invade the Lungs. *Cancer Cell*, *20*(4), 538–549. <https://doi.org/10.1016/j.ccr.2011.08.025>
- Chen, S., & Parmigiani, G. (2007). Meta-analysis of BRCA1 and BRCA2 penetrance. *Journal of Clinical Oncology*, *25*(11), 1329–1333. <https://doi.org/10.1200/JCO.2006.09.1066>
- Chevrier, S., Levine, J. H., Zanutelli, V. R. T., Silina, K., Schulz, D., Bacac, M., Ries, C. H., Ailles, L., Jewett, M. A. S., Moch, H., van den Broek, M., Beisel, C., Stadler, M. B., Gedye, C., Reis, B., Pe'er, D., & Bodenmiller, B. (2017). An Immune Atlas of Clear Cell Renal Cell Carcinoma. *Cell*, *169*(4), 736–749.e18. <https://doi.org/10.1016/j.cell.2017.04.016>
- Chiarugi, P. (2013). Cancer-associated fibroblasts and macrophages. *OncoImmunology*, *2*(9), e25563. <https://doi.org/10.4161/onci.25563>
- Cole, S. W. (2009). Chronic inflammation and breast cancer recurrence. *Journal of Clinical Oncology*, *27*(21), 3418–3419. <https://doi.org/10.1200/JCO.2009.21.9782>
- Coller, H. A., Sang, L., & Roberts, J. M. (2006). *A New Description of Cellular Quiescence*. *4*(3). <https://doi.org/10.1371/journal.pbio.0040083>
- Condeelis, J., & Pollard, J. W. (2006). Macrophages: Obligate Partners for Tumor Cell Migration, Invasion, and Metastasis. *Cell*, *124*(2), 263–266. <https://doi.org/10.1016/j.cell.2006.01.007>

- Considine, M. J., & Considine, J. A. (2016). *On the language and physiology of dormancy and quiescence in plants*. 1–15. <https://doi.org/10.1093/jxb/erw138>
- Costa-Silva, B., Aiello, N. M., Ocean, A. J., Singh, S., Zhang, H., Thakur, B. K., Becker, A., Hoshino, A., Mark, M. T., Molina, H., Xiang, J., Zhang, T., Theilen, T.-M., García-Santos, G., Williams, C., Ararso, Y., Huang, Y., Rodrigues, G., Shen, T.-L., ... Lyden, D. (2015). Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nature Cell Biology*, *17*(6), 816–826. <https://doi.org/10.1038/ncb3169>
- Cox, T. R., Rumney, R. M. H., Schoof, E. M., Perryman, L., Høye, A. M., Agrawal, A., Bird, D., Latif, N. A., Forrest, H., Evans, H. R., Huggins, I. D., Lang, G., Linding, R., Gartland, A., & Erler, J. T. (2015). The hypoxic cancer secretome induces pre-metastatic bone lesions through lysyl oxidase. *Nature*, *522*(7554), 106–110. <https://doi.org/10.1038/nature14492>
- Damen, M. P. F., van Rheenen, J., & Scheele, C. L. G. J. (2021). Targeting dormant tumor cells to prevent cancer recurrence. *FEBS Journal*, *288*(21), 6286–6303. <https://doi.org/10.1111/febs.15626>
- Danaei, G., vander Hoorn, S., Lopez, A. D., Murray, C. J. L., & Ezzati, M. (2005). Causes of cancer in the world: Comparative risk assessment of nine behavioural and environmental risk factors. *Lancet*, *366*(9499), 1784–1793. [https://doi.org/10.1016/S0140-6736\(05\)67725-2](https://doi.org/10.1016/S0140-6736(05)67725-2)
- Davies, L. C., Jenkins, S. J., Allen, J. E., & Taylor, P. R. (2013). Tissue-resident macrophages. *Nature Immunology*, *14*(10), 986–995. <https://doi.org/10.1038/ni.2705>
- Deligne, C., & Midwood, K. S. (2021). Macrophages and Extracellular Matrix in Breast Cancer: Partners in Crime or Protective Allies? *Frontiers in Oncology*, *11*(February), 1–12. <https://doi.org/10.3389/fonc.2021.620773>
- Deligne, C., Murdamoothoo, D., Gammage, A. N., Gschwandtner, M., Erne, W., Loustau, T., Marzeda, A. M., Carapito, R., Paul, N., Velazquez-Quesada, I., Mazzier, I., Sun, Z., Orend, G., & Midwood, K. S. (2020). Matrix-Targeting Immunotherapy Controls Tumor Growth and Spread by Switching Macrophage Phenotype. *Cancer Immunology Research*, *8*(3), 368–382. <https://doi.org/10.1158/2326-6066.CIR-19-0276>
- Deng, C. X. (2006). BRCA1: Cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. *Nucleic Acids Research*, *34*(5), 1416–1426. <https://doi.org/10.1093/nar/gkl010>
- Dhillon, A. S., Hagan, S., Rath, O., & Kolch, W. (2007). MAP kinase signalling pathways in cancer. *Oncogene*, *26*(22), 3279–3290. <https://doi.org/10.1038/sj.onc.1210421>
- Doak, G. R., Schwertfeger, K. L., & Wood, D. K. (2018). Distant Relations: Macrophage Functions in the Metastatic Niche. *Trends in Cancer*, *4*(6), 445–459. <https://doi.org/10.1016/j.trecan.2018.03.011>
- Drăgănescu, M., & Carmocan, C. (2017). Hormone therapy in breast cancer. *Chirurgia (Romania)*, *112*(4), 413–417. <https://doi.org/10.21614/chirurgia.112.4.413>
- Duffy, S. A., Taylor, J. M. G., Terrell, J. E., Islam, M., Li, Y., Fowler, K. E., Wolf, G. T., & Teknos, T. N. (2008). Interleukin-6 predicts recurrence and survival among head and neck cancer patients. In *Cancer* (Vol. 113, Issue 4, pp. 750–757). <https://doi.org/10.1002/cncr.23615>
- Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., & Schreiber, R. D. (2002). *Cancer immunoediting : from immuno- surveillance to tumor escape*. *3*(11), 991–998.
- Dunn, G. P., Old, L. J., Schreiber, R. D., Louis, S., Burnet, F. M., & Thomas, L. (2004). *of Cancer Immun-surveillance and Immunoediting*. *21*, 137–148.

- Egeblad, M., Rasch, M. G., & Weaver, V. M. (2010). Dynamic interplay between the collagen scaffold and tumor evolution. *Current Opinion in Cell Biology*, 22(5), 697–706. <https://doi.org/10.1016/j.ceb.2010.08.015>
- ELSTON, C. W., & ELLIS, I. O. (1991). pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology*, 19(5), 403–410. <https://doi.org/10.1111/j.1365-2559.1991.tb00229.x>
- Er, E. E., Valiente, M., Ganesh, K., Zou, Y., Agrawal, S., Hu, J., Griscom, B., Rosenblum, M., Boire, A., Brogi, E., Giancotti, F. G., Schachner, M., Malladi, S., & Massagué, J. (2018). Pericyte-like spreading by disseminated cancer cells activates YAP and MRTF for metastatic colonization. *Nature Cell Biology*, 20(8), 966–978. <https://doi.org/10.1038/s41556-018-0138-8>
- Erler, J. T., Bennewith, K. L., Cox, T. R., Lang, G., Bird, D., Koong, A., Le, Q.-T., & Giaccia, A. J. (2009). Hypoxia-Induced Lysyl Oxidase Is a Critical Mediator of Bone Marrow Cell Recruitment to Form the Premetastatic Niche. *Cancer Cell*, 15(1), 35–44. <https://doi.org/10.1016/j.ccr.2008.11.012>
- Eroles, P., Bosch, A., Alejandro Pérez-Fidalgo, J., & Lluch, A. (2012). Molecular biology in breast cancer: Intrinsic subtypes and signaling pathways. *Cancer Treatment Reviews*, 38(6), 698–707. <https://doi.org/10.1016/j.ctrv.2011.11.005>
- Esbona, K., Inman, D., Saha, S., Jeffery, J., Schedin, P., Wilke, L., & Keely, P. (2016). COX-2 modulates mammary tumor progression in response to collagen density. *Breast Cancer Research*, 18(1), 35. <https://doi.org/10.1186/s13058-016-0695-3>
- Eyles, J., Puaux, A., Wang, X., Toh, B., Prakash, C., Hong, M., Tan, T. G., Zheng, L., Ong, L. C., Jin, Y., Kato, M., Prévost-blondel, A., Chow, P., Yang, H., & Abastado, J. (2010). *Tumor cells disseminate early , but immunosurveillance limits metastatic outgrowth , in a mouse model of melanoma*. 120(6). <https://doi.org/10.1172/JCI42002DS1>
- Fane, M. E., Chhabra, Y., Alicea, G. M., Maranto, D. A., Douglass, S. M., Webster, M. R., Rebecca, V. W., Marino, G. E., Almeida, F., Ecker, B. L., Zabransky, D. J., Hüser, L., Beer, T., Tang, H.-Y., Kossenkov, A., Herlyn, M., Speicher, D. W., Xu, W., Xu, X., ... Weeraratna, A. T. (2022). Stromal changes in the aged lung induce an emergence from melanoma dormancy. *Nature*, 606(7913), 396–405. <https://doi.org/10.1038/s41586-022-04774-2>
- Ferrante, C. J., & Leibovich, S. J. (2012). Regulation of Macrophage Polarization and Wound Healing. *Advances in Wound Care*, 1(1), 10–16. <https://doi.org/10.1089/wound.2011.0307>
- Ferreira, S., Saraiva, N., Rijo, P., & Fernandes, A. S. (2021). LOXL2 Inhibitors and Breast Cancer Progression. *Antioxidants (Basel, Switzerland)*, 10(2). <https://doi.org/10.3390/antiox10020312>
- Fleetwood, A. J., Lawrence, T., Hamilton, J. A., & Cook, A. D. (2007). Granulocyte-Macrophage Colony-Stimulating Factor (CSF) and Macrophage CSF-Dependent Macrophage Phenotypes Display Differences in Cytokine Profiles and Transcription Factor Activities: Implications for CSF Blockade in Inflammation. *The Journal of Immunology*, 178(8), 5245–5252. <https://doi.org/10.4049/jimmunol.178.8.5245>
- Folkman, J. (1989). *What Is the Evidence That Tumors Are Angiogenesis Dependent ?* 7(23), 8–11.
- Friedl, P., & Alexander, S. (2011). Cancer Invasion and the Microenvironment: Plasticity and Reciprocity. *Cell*, 147(5), 992–1009. <https://doi.org/10.1016/j.cell.2011.11.016>

- Gallerand, A., Stunault, M. I., Merlin, J., Luehmann, H. P., Sultan, D. H., Firulyova, M. M., Magnone, V., Khedher, N., Jalil, A., Dolfi, B., Castiglione, A., Dumont, A., Ayrault, M., Vaillant, N., Gilleron, J., Barbry, P., Dombrowicz, D., Mack, M., Masson, D., ... Ivanov, S. (2021). Brown adipose tissue monocytes support tissue expansion. *Nature Communications*, *12*(1), 5255. <https://doi.org/10.1038/s41467-021-25616-1>
- Galon, J., Pagès, F., Marincola, F. M., Thurin, M., Trinchieri, G., Fox, B. A., Gajewski, T. F., & Ascierto, P. A. (2012). The immune score as a new possible approach for the classification of cancer. *Journal of Translational Medicine*, *10*(1), 1. <https://doi.org/10.1186/1479-5876-10-1>
- Gao, H., Chakraborty, G., Zhang, Z., Akalay, I., Gadiya, M., Gao, Y., Sinha, S., Hu, J., Jiang, C., Akram, M., Brogi, E., Leitinger, B., & Giancotti, F. G. (2016). Multi-organ Site Metastatic Reactivation Mediated by Non-canonical Discoidin Domain Receptor 1 Signaling. *Cell*, *166*(1), 47–62. <https://doi.org/10.1016/j.cell.2016.06.009>
- Gee, J. M. W., Harper, M. E., Hutcheson, I. R., Madden, T. A., Barrow, D., Knowlden, J. M., McClelland, R. A., Jordan, N., Wakeling, A. E., & Nicholson, R. I. (2003). The Antiepidermal Growth Factor Receptor Agent Gefitinib (ZD1839/Iressa) Improves Antihormone Response and Prevents Development of Resistance in Breast Cancer in Vitro. *Endocrinology*, *144*(11), 5105–5117. <https://doi.org/10.1210/en.2003-0705>
- Gelao, L., Criscitiello, C., Fumagalli, L., Locatelli, M., Manunta, S., Esposito, A., Minchella, I., Goldhirsch, A., & Curigliano, G. (2013). Tumour dormancy and clinical implications in breast cancer. *Ecancermedicalscience*, *7*(1). <https://doi.org/10.3332/ecancer.2013.320>
- Giblin, S. P., Schwenzler, A., & Midwood, K. S. (2020). Alternative splicing controls cell lineage-specific responses to endogenous innate immune triggers within the extracellular matrix. *Matrix Biology*, *93*, 95–114. <https://doi.org/10.1016/j.matbio.2020.06.003>
- Gnant, M., Filipits, M., Greil, R., Stoeger, H., Rudas, M., Bago-Horvath, Z., Mlineritsch, B., Kwasny, W., Knauer, M., Singer, C., Jakesz, R., Dubsy, P., Fitzal, F., Bartsch, R., Steger, G., Balic, M., Ressler, S., Cowens, J. W., Storhoff, J., ... Nielsen, T. O. (2014). Predicting distant recurrence in receptor-positive breast cancer patients with limited clinicopathological risk: Using the PAM50 Risk of Recurrence score in 1478 postmenopausal patients of the ABCSG-8 trial treated with adjuvant endocrine therapy alone. *Annals of Oncology*, *25*(2), 339–345. <https://doi.org/10.1093/annonc/mdt494>
- Goddard, E. T., Fischer, J., & Schedin, P. (2016). A Portal Vein Injection Model to Study Liver Metastasis of Breast Cancer. *Journal of Visualized Experiments*, *118*. <https://doi.org/10.3791/54903>
- Gupta, G. P., & Massagué, J. (2006). Cancer Metastasis: Building a Framework. *Cell*, *127*(4), 679–695. <https://doi.org/10.1016/j.cell.2006.11.001>
- Hamajima, N., Hirose, K., Tajima, K., Rohan, T., Calle, E. E., Heath, C. W., Coates, R. J., Liff, J. M., Talamini, R., Chantarakul, N., Koetsawang, S., Rachawat, D., Morabia, A., Schuman, L., Stewart, W., Szklo, M., Bain, C., Schofield, F., Siskind, V., ... van Leeuwen, M. (2002). Alcohol, tobacco and breast cancer - Collaborative reanalysis of individual data from 53 epidemiological studies, including 58 515 women with breast cancer and 95 067 women without the disease. *British Journal of Cancer*, *87*(11), 1234–1245. <https://doi.org/10.1038/sj.bjc.6600596>
- Hamilton, J. A. (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nature Reviews Immunology*, *8*(7), 533–544. <https://doi.org/10.1038/nri2356>

- Hanahan, D., & Folkman, J. (1996). *Patterns and Emerging Mechanisms of the Angiogenic Switch during Tumorigenesis*. 86, 353–364.
- Hanby, A. M., & Walker, C. (2004). Tavassoli FA, Devilee P: Pathology and Genetics: Tumours of the Breast and Female Genital Organs. WHO Classification of Tumours series - volume IV. Lyon, France: IARC Press: 2003. 250pp. ISBN 92 832 2412 4. *Breast Cancer Research*, 6(3), 133–134. <https://doi.org/10.1186/bcr788>
- Harbeck, N., Penault-Llorca, F., Cortes, J., Gnant, M., Houssami, N., Poortmans, P., Ruddy, K., Tsang, J., & Cardoso, F. (2019). Breast cancer. In *Nature Reviews Disease Primers* (Vol. 5, Issue 1). <https://doi.org/10.1038/s41572-019-0111-2>
- Harney, A. S., Arwert, E. N., Entenberg, D., Wang, Y., Guo, P., Qian, B.-Z., Oktay, M. H., Pollard, J. W., Jones, J. G., & Condeelis, J. S. (2015). Real-Time Imaging Reveals Local, Transient Vascular Permeability, and Tumor Cell Intravasation Stimulated by TIE2hi Macrophage-Derived VEGFA. *Cancer Discovery*, 5(9), 932–943. <https://doi.org/10.1158/2159-8290.CD-15-0012>
- Hastings, J. F., Skhinas, J. N., Fey, D., Croucher, D. R., & Cox, T. R. (2019). The extracellular matrix as a key regulator of intracellular signalling networks. *British Journal of Pharmacology*, 176(1), 82–92. <https://doi.org/10.1111/bph.14195>
- Headley, M. B., Bins, A., Nip, A., Roberts, E. W., Looney, M. R., Gerard, A., & Krummel, M. F. (2016a). Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature*, 531(7595), 513–517. <https://doi.org/10.1038/nature16985>
- Headley, M. B., Bins, A., Nip, A., Roberts, E. W., Looney, M. R., Gerard, A., & Krummel, M. F. (2016b). Visualization of immediate immune responses to pioneer metastatic cells in the lung. *Nature*, 531(7595), 513–517. <https://doi.org/10.1038/nature16985>
- Herschkowitz, J. I., Simin, K., Weigman, V. J., Mikaelian, I., Usary, J., Hu, Z., Rasmussen, K. E., Jones, L. P., Assefnia, S., Chandrasekharan, S., Backlund, M. G., Yin, Y., Khramtsov, A. I., Bastein, R., Quackenbush, J., Glazer, R. I., Brown, P. H., Green, J. E., Kopelovich, L., ... Perou, C. M. (2007). Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biology*, 8(5), 1–17. <https://doi.org/10.1186/gb-2007-8-5-r76>
- Hiratsuka, S., Nakamura, K., Iwai, S., Murakami, M., Itoh, T., Kijima, H., Shipley, J. M., Senior, R. M., & Shibuya, M. (2002). MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell*, 2(4), 289–300. [https://doi.org/10.1016/S1535-6108\(02\)00153-8](https://doi.org/10.1016/S1535-6108(02)00153-8)
- Hiratsuka, S., Watanabe, A., Aburatani, H., & Maru, Y. (2006). Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nature Cell Biology*, 8(12), 1369–1375. <https://doi.org/10.1038/ncb1507>
- Hollosi, P., Yakushiji, J. K., Fong, K. S. K., Csiszar, K., & Fong, S. F. T. (2009). Lysyl oxidase-like 2 promotes migration in noninvasive breast cancer cells but not in normal breast epithelial cells. *International Journal of Cancer*, 125(2), 318–327. <https://doi.org/10.1002/ijc.24308>
- Holmgren, L., O'reilly, M. S., & Folkman, J. (1995). Dormancy of micrometastases: Balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature Medicine*, 1(2), 149–153. <https://doi.org/10.1038/nm0295-149>
- Holzmann, B., Gossler, U., & Bittner, M. (1998). *$\alpha 4$ Integrins and Tumor Metastasis* (pp. 125–141). https://doi.org/10.1007/978-3-642-71987-5_8

- Horn, J., & Vatten, L. J. (2017). Reproductive and hormonal risk factors of breast cancer: A historical perspective. *International Journal of Women's Health*, 9, 265–272. <https://doi.org/10.2147/IJWH.S129017>
- Howlander, N., Altekruse, S. F., Li, C. I., Chen, V. W., Clarke, C. A., Ries, L. A. G., & Cronin, K. A. (2014). US incidence of breast cancer subtypes defined by joint hormone receptor and HER2 status. *Journal of the National Cancer Institute*, 106(5). <https://doi.org/10.1093/jnci/dju055>
- Huang, A., Lin, Y., Kao, L., Chiou, Y., Lee, G., Lin, H., Wu, C., Chang, C., Lee, K., Hsueh, Y., Tsai, P., Tang, M., & Tsai, Y. (2021). Inflammation-induced macrophage lysyl oxidase in adipose stiffening and dysfunction in obesity. *Clinical and Translational Medicine*, 11(9). <https://doi.org/10.1002/ctm2.543>
- Hughes, R., Qian, B. Z., Rowan, C., Muthana, M., Keklikoglou, I., Olson, O. C., Tazzyman, S., Danson, S., Addison, C., Clemons, M., Gonzalez-Angulo, A. M., Joyce, J. A., de Palma, M., Pollard, J. W., & Lewis, C. E. (2015). Perivascular M2 macrophages stimulate tumor relapse after chemotherapy. *Cancer Research*, 75(17), 3479–3491. <https://doi.org/10.1158/0008-5472.CAN-14-3587>
- Humphrey, J. D., Dufresne, E. R., & Schwartz, M. A. (2014). Mechanotransduction and extracellular matrix homeostasis. *Nature Reviews Molecular Cell Biology*, 15(12), 802–812. <https://doi.org/10.1038/nrm3896>
- Hüsemann, Y., Geigl, J. B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., Forni, G., Eils, R., Fehm, T., Riethmüller, G., & Klein, C. A. (2008). Systemic Spread Is an Early Step in Breast Cancer. *Cancer Cell*, 13(1), 58–68. <https://doi.org/10.1016/j.ccr.2007.12.003>
- Ingman, W. v., Wyckoff, J., Gouon-Evans, V., Condeelis, J., & Pollard, J. W. (2006). Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Developmental Dynamics*, 235(12), 3222–3229. <https://doi.org/10.1002/dvdy.20972>
- Jayadev, R., & Sherwood, D. R. (2017). Basement membranes. *Current Biology*, 27(6), R207–R211. <https://doi.org/10.1016/j.cub.2017.02.006>
- Jayasingam, S. D., Citartan, M., Thang, T. H., Mat Zin, A. A., Ang, K. C., & Ch'ng, E. S. (2020). Evaluating the Polarization of Tumor-Associated Macrophages Into M1 and M2 Phenotypes in Human Cancer Tissue: Technicalities and Challenges in Routine Clinical Practice. *Frontiers in Oncology*, 9. <https://doi.org/10.3389/fonc.2019.01512>
- Jena, M. K., & Janjanam, J. (2018). Role of extracellular matrix in breast cancer development: a brief update. *F1000Research*, 7, 274. <https://doi.org/10.12688/f1000research.14133.2>
- Jiang, H., & Li, H. (2021). Prognostic values of tumoral MMP2 and MMP9 overexpression in breast cancer: a systematic review and meta-analysis. *BMC Cancer*, 21(1), 149. <https://doi.org/10.1186/s12885-021-07860-2>
- Jonathan Yang, T., & Ho, A. Y. (2013). Radiation Therapy in the Management of Breast Cancer. *Surgical Clinics of North America*, 93(2), 455–471. <https://doi.org/10.1016/j.suc.2013.01.002>
- Joshi, S. C., Khan, F. A., Pant, I., & Shukla, A. (2007). Role of radiotherapy in early breast cancer: an overview. *International Journal of Health Sciences*, 1(2), 259–264. <http://www.ncbi.nlm.nih.gov/pubmed/21475437> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3068638>
- Joyce, J. A., & Pollard, J. W. (2009). Microenvironmental regulation of metastasis. *Nature Reviews Cancer*, 9(4), 239–252. <https://doi.org/10.1038/nrc2618>

- Kaplan, R. N., Riba, R. D., Zacharoulis, S., Bramley, A. H., Vincent, L., Costa, C., MacDonald, D. D., Jin, D. K., Shido, K., Kerns, S. A., Zhu, Z., Hicklin, D., Wu, Y., Port, J. L., Altorki, N., Port, E. R., Ruggero, D., Shmelkov, S. v., Jensen, K. K., ... Lyden, D. (2005). VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature*, *438*(7069), 820–827. <https://doi.org/10.1038/nature04186>
- Keeratichamroen, S., Lirdprapamongkol, K., & Svasti, J. (2018). Mechanism of ECM-induced dormancy and chemoresistance in A549 human lung carcinoma cells. *Oncology Reports*. <https://doi.org/10.3892/or.2018.6258>
- Keklikoglou, I., Cianciaruso, C., Güç, E., Squadrito, M. L., Spring, L. M., Tazzyman, S., Lambein, L., Poissonnier, A., Ferraro, G. B., Baer, C., Cassarà, A., Guichard, A., Iruela-Arispe, M. L., Lewis, C. E., Coussens, L. M., Bardia, A., Jain, R. K., Pollard, J. W., & de Palma, M. (2019). Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models. *Nature Cell Biology*, *21*(2), 190–202. <https://doi.org/10.1038/s41556-018-0256-3>
- Key, T. (2013). Sex hormones and risk of breast cancer in premenopausal women: A collaborative reanalysis of individual participant data from seven prospective studies. *The Lancet Oncology*, *14*(10), 1009–1019. [https://doi.org/10.1016/S1470-2045\(13\)70301-2](https://doi.org/10.1016/S1470-2045(13)70301-2)
- Kirk, D., Szalay, M. F., & Kaighn, M. E. (1981). Modulation of Growth of a Human Prostatic Cancer Cell Line (PC-3) in Agar Culture by Normal Human Lung Fibroblasts. *Cancer Research*, *41*(3), 1100–1103.
- Kitamura, T., Qian, B.-Z., Soong, D., Cassetta, L., Noy, R., Sugano, G., Kato, Y., Li, J., & Pollard, J. W. (2015). CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *Journal of Experimental Medicine*, *212*(7), 1043–1059. <https://doi.org/10.1084/jem.20141836>
- Klein, C. A. (2009). Parallel progression of primary tumours and metastases. *Nature Reviews Cancer*, *9*(4), 302–312. <https://doi.org/10.1038/nrc2627>
- Klein, C. A. (2011). Framework models of tumor dormancy from patient-derived observations. *Current Opinion in Genetics and Development*, *21*(1), 42–49. <https://doi.org/10.1016/j.gde.2010.10.011>
- Klepfish, M., Gross, T., Vugman, M., Afratis, N. A., Havusha-Laufer, S., Brazowski, E., Solomonov, I., Varol, C., & Sagi, I. (2020). LOXL2 Inhibition Paves the Way for Macrophage-Mediated Collagen Degradation in Liver Fibrosis. *Frontiers in Immunology*, *11*. <https://doi.org/10.3389/fimmu.2020.00480>
- Kmieciak, M., Payne, K. K., Wang, X. Y., & Manjili, M. H. (2013). IFN- γ R α is a key determinant of CD8+ T cell-mediated tumor elimination or tumor escape and relapse in FVB mouse. *PLoS ONE*, *8*(12), 1–8. <https://doi.org/10.1371/journal.pone.0082544>
- Koboldt, D. C., Fulton, R. S., McLellan, M. D., Schmidt, H., Kalicki-Veizer, J., McMichael, J. F., Fulton, L. L., Dooling, D. J., Ding, L., Mardis, E. R., Wilson, R. K., Ally, A., Balasundaram, M., Butterfield, Y. S. N., Carlsen, R., Carter, C., Chu, A., Chuah, E., Chun, H. J. E., ... Palchik, J. D. (2012). Comprehensive molecular portraits of human breast tumours. *Nature*, *490*(7418), 61–70. <https://doi.org/10.1038/nature11412>
- Krall, J. A., Reinhardt, F., Mercury, O. A., Pattabiraman, D. R., Brooks, M. W., Dougan, M., Lambert, A. W., Bierie, B., Ploegh, H. L., Dougan, S. K., & Weinberg, R. A. (2018). The systemic response to surgery triggers the outgrowth of distant immune-controlled tumors in mouse models of dormancy. *Science Translational Medicine*, *10*(436). <https://doi.org/10.1126/scitranslmed.aan3464>

- Kumar, A., Collins, H. M., Scholefield, J. H., & Watson, S. A. (2000). Increased type-IV collagenase (MMP-2 and MMP-9) activity following preoperative radiotherapy in rectal cancer. *British Journal of Cancer*, 82(4), 960–965. <https://doi.org/10.1054/bjoc.1999.1025>
- Kuziel, G., Thompson, V., D'Amato, J. v., & Arendt, L. M. (2020). Stromal CCL2 Signaling Promotes Mammary Tumor Fibrosis through Recruitment of Myeloid-Lineage Cells. *Cancers*, 12(8), 2083. <https://doi.org/10.3390/cancers12082083>
- Lamagna, C., Aurrand-Lions, M., & Imhof, B. A. (2006). Dual role of macrophages in tumor growth and angiogenesis. *Journal of Leukocyte Biology*, 80(4), 705–713. <https://doi.org/10.1189/jlb.1105656>
- Lambert, A. W., Pattabiraman, D. R., & Weinberg, R. A. (2017). Emerging Biological Principles of Metastasis. *Cell*, 168(4), 670–691. <https://doi.org/10.1016/j.cell.2016.11.037>
- Lan, Q., Peyvandi, S., Duffey, N., Huang, Y. T., Barras, D., Held, W., Richard, F., Delorenzi, M., Sotiriou, C., Desmedt, C., Lorusso, G., & Rüegg, C. (2019). Type I interferon/IRF7 axis instigates chemotherapy-induced immunological dormancy in breast cancer. *Oncogene*, 38(15), 2814–2829. <https://doi.org/10.1038/s41388-018-0624-2>
- Langley, R. R., & Fidler, I. J. (2011). The seed and soil hypothesis revisited-The role of tumor-stroma interactions in metastasis to different organs. *International Journal of Cancer*, 128(11), 2527–2535. <https://doi.org/10.1002/ijc.26031>
- Laoui, D., van Overmeire, E., de Baetselier, P., van Ginderachter, J. A., & Raes, G. (2014). Functional Relationship between Tumor-Associated Macrophages and Macrophage Colony-Stimulating Factor as Contributors to Cancer Progression. *Frontiers in Immunology*, 5. <https://doi.org/10.3389/fimmu.2014.00489>
- Lecarpentier, Y., Schussler, O., Hébert, J. L., & Vallée, A. (2019). Multiple Targets of the Canonical WNT/ β -Catenin Signaling in Cancers. *Frontiers in Oncology*, 9(November), 1–17. <https://doi.org/10.3389/fonc.2019.01248>
- Levental, K. R., Yu, H., Kass, L., Lakins, J. N., Egeblad, M., Erler, J. T., Fong, S. F. T., Csiszar, K., Giaccia, A., Weninger, W., Yamauchi, M., Gasser, D. L., & Weaver, V. M. (2009). Matrix Crosslinking Forces Tumor Progression by Enhancing Integrin Signaling. *Cell*, 139(5), 891–906. <https://doi.org/10.1016/j.cell.2009.10.027>
- Lewis, C. E., & Pollard, J. W. (2006). Distinct Role of Macrophages in Different Tumor Microenvironments. *Cancer Research*, 66(2), 605–612. <https://doi.org/10.1158/0008-5472.CAN-05-4005>
- Linde, N., Fluegen, G., & Aguirre-Ghiso, J. A. (2016). The Relationship Between Dormant Cancer Cells and Their Microenvironment. In *Advances in Cancer Research* (1st ed., Vol. 132). Elsevier Inc. <https://doi.org/10.1016/bs.acr.2016.07.002>
- Liou, G. Y., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research*, 44(5), 479–496. <https://doi.org/10.3109/10715761003667554>
- Liu, D., Aguirre Ghiso, J., Estrada, Y., & Ossowski, L. (2002). EGFR is a transducer of the urokinase receptor initiated signal that is required for in vivo growth of a human carcinoma. *Cancer Cell*, 1(5), 445–457. [https://doi.org/10.1016/s1535-6108\(02\)00072-7](https://doi.org/10.1016/s1535-6108(02)00072-7)
- Liu, J., Ping, W., Zu, Y., & Sun, W. (2014). Correlations of lysyl oxidase with MMP2/MMP9 expression and its prognostic value in non-small cell lung cancer. *International Journal of Clinical and Experimental Pathology*, 7(9), 6040–6047. <http://www.ncbi.nlm.nih.gov/pubmed/25337249>

- Lorusso, G., & Rüegg, C. (2012). New insights into the mechanisms of organ-specific breast cancer metastasis. *Seminars in Cancer Biology*, 22(3), 226–233. <https://doi.org/10.1016/j.semcancer.2012.03.007>
- Luis-Ravelo, D., Antón, I., Vicent, S., Hernández, I., Valencia, K., Zanduetta, C., Martínez-Canarias, S., Gúrpide, A., & Lecanda, F. (2011). Tumor–stromal interactions of the bone microenvironment: in vitro findings and potential in vivo relevance in metastatic lung cancer models. *Clinical & Experimental Metastasis*, 28(8), 779–791. <https://doi.org/10.1007/s10585-011-9409-5>
- Łukasiewicz, S., Czezelewski, M., Forma, A., Baj, J., Sitarz, R., & Stanisławek, A. (2021). Breast cancer—epidemiology, risk factors, classification, prognostic markers, and current treatment strategies—An updated review. *Cancers*, 13(17), 1–30. <https://doi.org/10.3390/cancers13174287>
- Machida, H., de Zoysa, M. Y., Takiuchi, T., Hom, M. S., Tierney, K. E., & Matsuo, K. (2017). Significance of Monocyte Counts at Recurrence on Survival Outcome of Women with Endometrial Cancer. *International Journal of Gynecological Cancer*, 27(2), 302–310. <https://doi.org/10.1097/IGC.0000000000000865>
- Madsen, D. H., Jürgensen, H. J., Siersbæk, M. S., Kuczek, D. E., Grey Cloud, L., Liu, S., Behrendt, N., Grøntved, L., Weigert, R., & Bugge, T. H. (2017). Tumor-Associated Macrophages Derived from Circulating Inflammatory Monocytes Degrade Collagen through Cellular Uptake. *Cell Reports*, 21(13), 3662–3671. <https://doi.org/10.1016/j.celrep.2017.12.011>
- Mahmood, N., Mihalcioiu, C., & Rabbani, S. A. (2018). Multifaceted Role of the Urokinase-Type Plasminogen Activator (uPA) and Its Receptor (uPAR): Diagnostic, Prognostic, and Therapeutic Applications. *Frontiers in Oncology*, 8. <https://doi.org/10.3389/fonc.2018.00024>
- Malanchi, I., Santamaria-Martínez, A., Susanto, E., Peng, H., Lehr, H.-A., Delaloye, J.-F., & Huelsken, J. (2012). Interactions between cancer stem cells and their niche govern metastatic colonization. *Nature*, 481(7379), 85–89. <https://doi.org/10.1038/nature10694>
- Maliniak, M. L., Miller-Kleinhenz, J., Cronin-Fenton, D. P., Lash, T. L., Gogineni, K., Janssen, E. A. M., & McCullough, L. E. (2021). Crown-like structures in breast adipose tissue: Early evidence and current issues in breast cancer. *Cancers*, 13(9). <https://doi.org/10.3390/cancers13092222>
- Malladi, S., MacAlinao, D. G., Jin, X., He, L., Basnet, H., Zou, Y., de Stanchina, E., & Massagué, J. (2016). Metastatic Latency and Immune Evasion through Autocrine Inhibition of WNT. *Cell*, 165(1), 45–60. <https://doi.org/10.1016/j.cell.2016.02.025>
- Manuelpillai, U., Lourensz, D., Vaghjiani, V., Tchongue, J., Lacey, D., Tee, J. Y., Murthi, P., Chan, J., Hodge, A., & Sievert, W. (2012). Human Amniotic epithelial cell transplantation induces markers of alternative macrophage activation and reduces established hepatic fibrosis. *PLoS ONE*, 7(6), 1–11. <https://doi.org/10.1371/journal.pone.0038631>
- Maquart, F.-X., Pasco, S., Ramont, L., Hornebeck, W., & Monboisse, J.-C. (2004). An introduction to matrikines: extracellular matrix-derived peptides which regulate cell activity. *Critical Reviews in Oncology/Hematology*, 49(3), 199–202. <https://doi.org/10.1016/j.critrevonc.2003.06.007>
- Marinovich, M. L., Hunter, K. E., Macaskill, P., & Houssami, N. (2018). Breast cancer screening using tomosynthesis or mammography: A meta-analysis of cancer detection and recall. *Journal of the National Cancer Institute*, 110(9), 942–949. <https://doi.org/10.1093/jnci/djy121>

- Marmot, M., Altman, D. G., Cameron, D. A., Dewar, J. A., Thompson, S. G., & Wilcox, M. (2012). The benefits and harms of breast cancer screening: An independent review. *The Lancet*, 380(9855), 1778–1786. [https://doi.org/10.1016/S0140-6736\(12\)61611-0](https://doi.org/10.1016/S0140-6736(12)61611-0)
- Martinez, F. O., & Gordon, S. (2015). The evolution of our understanding of macrophages and translation of findings toward the clinic. *Expert Review of Clinical Immunology*, 11(1), 5–13. <https://doi.org/10.1586/1744666X.2015.985658>
- Martinez, J. S., von Nicolai, C., Kim, T., Ehlén, Å., Mazin, A. v., Kowalczykowski, S. C., & Carreira, A. (2016). BRCA2 regulates DMC1-mediated recombination through the BRC repeats. *Proceedings of the National Academy of Sciences of the United States of America*, 113(13), 3515–3520. <https://doi.org/10.1073/pnas.1601691113>
- Martins, L. M., de Melo Escorcio Dourado, C. S., Campos-Verdes, L. M., Sampaio, F. A., Revoredo, C. M. S., Costa-Silva, D. R., da Conceição Barros-Oliveira, M., de Jesus Nery Junior, E., do Rego-Medeiros, L. M., Gebrim, L. H., Alves-Ribeiro, F. A., Rodrigues, G. P., Chagas, D. C., do Nascimento Marreiro, D., & da Silva, B. B. (2019). Expression of matrix metalloproteinase 2 and 9 in breast cancer and breast fibroadenoma: a randomized, double-blind study. *Oncotarget*, 10(64), 6879–6884. <https://doi.org/10.18632/oncotarget.27347>
- Mason, S. D., & Joyce, J. A. (2011). Proteolytic networks in cancer. *Trends in Cell Biology*, 21(4), 228–237. <https://doi.org/10.1016/j.tcb.2010.12.002>
- Mebratu, Y., & Tesfaigzi, Y. (2009). Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). *Cell Cycle*, 8(8), 1168–1175. <http://www.landesbioscience.com/journals/cc/article/8147>
- Meijers-Heijboer, H., van den Ouweland, A., Klijn, J., Wasielewski, M., de Shoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., van Veghel-Plandsoen, M., Elstrodt, F., van Duijn, C., Barreels, C., Meijerste, C., Schutte, M., McGuffog, L., Thompson, D., Easton, D. F., Sodha, N., ... Stratton, M. R. (2002). Low-penetrance susceptibility to breast cancer due to CHEK2*1100delC in noncarriers of BRCA1 or BRCA2 mutations: The CHEK2-breast cancer consortium. *Nature Genetics*, 31(1), 55–59. <https://doi.org/10.1038/ng879>
- Metge, B. J., Frost, Æ. A. R., King, Æ. J. A., Lynn, D., Danny, D. Æ., Rajeev, R. W. Æ., & Shevde, L. A. (2008). Epigenetic silencing contributes to the loss of BRMS1 expression in breast cancer. 753–763. <https://doi.org/10.1007/s10585-008-9187-x>
- Milanese, T. R., Hartmann, L. C., Sellers, T. A., Frost, M. H., Vierkant, R. A., Maloney, S. D., Pankratz, V. S., Degnim, A. C., Vachon, C. M., Reynolds, C. A., Thompson, R. A., Melton, L. J., Goode, E. L., & Visscher, D. W. (2006). Age-related lobular involution and risk of breast cancer. *Journal of the National Cancer Institute*, 98(22), 1600–1607. <https://doi.org/10.1093/jnci/djj439>
- Mitra, S. K., & Schlaepfer, D. D. (2006). Integrin-regulated FAK–Src signaling in normal and cancer cells. *Current Opinion in Cell Biology*, 18(5), 516–523. <https://doi.org/10.1016/j.ceb.2006.08.011>
- Miyamoto, S., Teramoto, H., Gutkind, J. S., & Yamada, K. M. (1996). Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors. *Journal of Cell Biology*, 135(6), 1633–1642. <https://doi.org/10.1083/jcb.135.6.1633>
- Moro, L. (1998). Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *The EMBO Journal*, 17(22), 6622–6632. <https://doi.org/10.1093/emboj/17.22.6622>

- Morrow, M., White, J., Moughan, J., Owen, J., Pajack, T., Sylvester, J., Wilson, J. F., & Winchester, D. (2001). Factors predicting the use of breast-conserving therapy in stage I and II breast carcinoma. *Journal of Clinical Oncology*, *19*(8), 2254–2262. <https://doi.org/10.1200/JCO.2001.19.8.2254>
- Murray, P. J., Allen, J. E., Biswas, S. K., Fisher, E. A., Gilroy, D. W., Goerdt, S., Gordon, S., Hamilton, J. A., Ivashkiv, L. B., Lawrence, T., Locati, M., Mantovani, A., Martinez, F. O., Mege, J.-L., Mosser, D. M., Natoli, G., Saeij, J. P., Schultze, J. L., Shirey, K. A., ... Wynn, T. A. (2014). Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*, *41*(1), 14–20. <https://doi.org/10.1016/j.immuni.2014.06.008>
- Naba, A., Clauser, K. R., Ding, H., Whittaker, C. A., Carr, S. A., & Hynes, R. O. (2016). The extracellular matrix: Tools and insights for the “omics” era. *Matrix Biology*, *49*, 10–24. <https://doi.org/10.1016/j.matbio.2015.06.003>
- Naldini, L., Vigna, E., Bardelli, A., Follenzi, A., Galimi, F., & Comoglio, P. M. (1995). Biological Activation of pro-HGF (Hepatocyte Growth Factor) by Urokinase Is Controlled by a Stoichiometric Reaction. *Journal of Biological Chemistry*, *270*(2), 603–611. <https://doi.org/10.1074/jbc.270.2.603>
- Naumov, G. N., Akslen, L. A., Folkman, J., Naumov, G. N., Akslen, L. A., Folkman, J., Naumov, G. N., Akslen, L. A., & Folkman, J. (2016). *Role of Angiogenesis in Human Tumor Dormancy ND ES SC RIB. 4101*(January). <https://doi.org/10.4161/cc.5.16.3018>
- Naumov, G. N., Bender, E., Zurakowski, D., Kang, S., Flynn, E., Watnick, R. S., Straume, O., Lars, A., Folkman, J., & Almog, N. (2006). *A Model of Human Tumor Dormancy: An Angiogenic Switch From the Nonangiogenic Phenotype.* *98*(5). <https://doi.org/10.1093/jnci/djj068>
- Newman, L. A., Reis-Filho, J. S., Morrow, M., Carey, L. A., & King, T. A. (2015). The 2014 Society of Surgical Oncology Susan G. Komen for the Cure Symposium: Triple-Negative Breast Cancer. *Annals of Surgical Oncology*, *22*(3), 874–882. <https://doi.org/10.1245/s10434-014-4279-0>
- Nielsen, S. R., Quaranta, V., Linford, A., Emeagi, P., Rainer, C., Santos, A., Ireland, L., Sakai, T., Sakai, K., Kim, Y.-S., Engle, D., Campbell, F., Palmer, D., Ko, J. H., Tuveson, D. A., Hirsch, E., Mielgo, A., & Schmid, M. C. (2016). Macrophage-secreted granulin supports pancreatic cancer metastasis by inducing liver fibrosis. *Nature Cell Biology*, *18*(5), 549–560. <https://doi.org/10.1038/ncb3340>
- Normanno, N., Luca, A. de, Maiello, M. R., Campiglio, M., Napolitano, M., Mancino, M., Carotenuto, A., Viglietto, G., & Menard, S. (2006). The MEK/MAPK pathway is involved in the resistance of breast cancer cells to the EGFR tyrosine kinase inhibitor gefitinib. *Journal of Cellular Physiology*, *207*(2), 420–427. <https://doi.org/10.1002/jcp.20588>
- Okubo, M., Kioi, M., Nakashima, H., Sugiura, K., Mitsudo, K., Aoki, I., Taniguchi, H., & Tohnai, I. (2016). M2-polarized macrophages contribute to neovasculogenesis, leading to relapse of oral cancer following radiation. *Scientific Reports*, *6*(May), 1–12. <https://doi.org/10.1038/srep27548>
- Orekhov, A. N., Orekhova, V. A., Nikiforov, N. G., Myasoedova, V. A., Grechko, A. v., Romanenko, E. B., Zhang, D., & Chistiakov, D. A. (2019). Monocyte differentiation and macrophage polarization. *Vessel Plus*, *3*. <https://doi.org/10.20517/2574-1209.2019.04>
- Osborn, L., Hession, C., Tizard, R., Vassallo, C., Luhowskyj, S., Chi-Rosso, G., & Lobb, R. (1989). Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced

- endothelial protein that binds to lymphocytes. *Cell*, 59(6), 1203–1211. [https://doi.org/10.1016/0092-8674\(89\)90775-7](https://doi.org/10.1016/0092-8674(89)90775-7)
- Oskarsson, T., Acharyya, S., Zhang, X. H.-F., Vanharanta, S., Tavazoie, S. F., Morris, P. G., Downey, R. J., Manova-Todorova, K., Brogi, E., & Massagué, J. (2011). Breast cancer cells produce tenascin C as a metastatic niche component to colonize the lungs. *Nature Medicine*, 17(7), 867–874. <https://doi.org/10.1038/nm.2379>
- Paget, S. (1989). The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Reviews*, 8(2), 98–101. <http://www.ncbi.nlm.nih.gov/pubmed/2673568>
- Pantel, K., & Brakenhoff, R. H. (2004). Dissecting the metastatic cascade. *Nature Reviews Cancer*, 4(6), 448–456. <https://doi.org/10.1038/nrc1370>
- Paolillo, & Schinelli. (2019). Extracellular Matrix Alterations in Metastatic Processes. *International Journal of Molecular Sciences*, 20(19), 4947. <https://doi.org/10.3390/ijms20194947>
- Park, S. Y., & Nam, J. S. (2020). The force awakens: metastatic dormant cancer cells. *Experimental and Molecular Medicine*, 52(4), 569–581. <https://doi.org/10.1038/s12276-020-0423-z>
- Pastushenko, I., Brisebarre, A., Sifrim, A., Fioramonti, M., Revenco, T., Boumahdi, S., van Keymeulen, A., Brown, D., Moers, V., Lemaire, S., de Clercq, S., Minguijón, E., Balsat, C., Sokolow, Y., Dubois, C., de Cock, F., Scozzaro, S., Sopena, F., Lanas, A., ... Blanpain, C. (2018). Identification of the tumour transition states occurring during EMT. *Nature*, 556(7702), 463–468. <https://doi.org/10.1038/s41586-018-0040-3>
- Peinado, H., Zhang, H., Matei, I. R., Costa-Silva, B., Hoshino, A., Rodrigues, G., Psaila, B., Kaplan, R. N., Bromberg, J. F., Kang, Y., Bissell, M. J., Cox, T. R., Giaccia, A. J., Ertler, J. T., Hiratsuka, S., Ghajar, C. M., & Lyden, D. (2017). Pre-metastatic niches: Organ-specific homes for metastases. *Nature Reviews Cancer*, 17(5), 302–317. <https://doi.org/10.1038/nrc.2017.6>
- Peltonen, R., Hagström, J., Tervahartiala, T., Sorsa, T., Haglund, C., & Isoniemi, H. (2021). High Expression of MMP-9 in Primary Tumors and High Preoperative MPO in Serum Predict Improved Prognosis in Colorectal Cancer with Operable Liver Metastases. *Oncology*, 99(3), 144–160. <https://doi.org/10.1159/000510609>
- Perou, C. M., Sørile, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Ress, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., Fluge, Ø., Pergammenschlkov, A., Williams, C., Zhu, S. X., Lønning, P. E., Børresen-Dale, A. L., Brown, P. O., & Botstein, D. (2000). Molecular portraits of human breast tumours. *Nature*, 406(6797), 747–752. <https://doi.org/10.1038/35021093>
- Peyvandi, S., Lan, Q., Lorusso, G., & Røedegg, C. (2019). Chemotherapy-induced immunological breast cancer dormancy: a new function for old drugs? *Journal of Cancer Metastasis and Treatment*, 2019. <https://doi.org/10.20517/2394-4722.2019.16>
- Piccinini, A. M., Zuliani-Alvarez, L., Lim, J. M. P., & Midwood, K. S. (2016). Distinct microenvironmental cues stimulate divergent TLR4-mediated signaling pathways in macrophages. *Science Signaling*, 9(443). <https://doi.org/10.1126/scisignal.aaf3596>
- Pierce, B. L., Ballard-Barbash, R., Bernstein, L., Baumgartner, R. N., Neuhaus, M. L., Wener, M. H., Baumgartner, K. B., Gilliland, F. D., Sorensen, B. E., McTiernan, A., & Ulrich, C. M. (2009). Elevated Biomarkers of Inflammation Are Associated With Reduced Survival Among Breast Cancer Patients. *Journal of Clinical Oncology*, 27(21), 3437–3444. <https://doi.org/10.1200/JCO.2008.18.9068>

- Plasilova, M. L., Hayse, B., Killelea, B. K., Horowitz, N. R., Chagpar, A. B., & Lannin, D. R. (2016). Features of triple-negative breast cancer Analysis of 38,813 cases from the national cancer database. *Medicine (United States)*, 95(35). <https://doi.org/10.1097/MD.0000000000004614>
- Pollard, J. W. (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nature Reviews Cancer*, 4(1), 71–78. <https://doi.org/10.1038/nrc1256>
- Pollard, J. W. (2008). Macrophages define the invasive microenvironment in breast cancer. *Journal of Leukocyte Biology*, 84(3), 623–630. <https://doi.org/10.1189/jlb.1107762>
- Pozzi, A., Yurchenco, P. D., & Iozzo, R. v. (2017). The nature and biology of basement membranes. *Matrix Biology*, 57–58, 1–11. <https://doi.org/10.1016/j.matbio.2016.12.009>
- Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J. I., He, X., & Perou, C. M. (2010). Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Research*, 12(5). <https://doi.org/10.1186/bcr2635>
- Prat, A., & Perou, C. M. (2011). Deconstructing the molecular portraits of breast cancer. *Molecular Oncology*, 5(1), 5–23. <https://doi.org/10.1016/j.molonc.2010.11.003>
- Qian, B., Deng, Y., Im, J. H., Muschel, R. J., Zou, Y., Li, J., Lang, R. A., & Pollard, J. W. (2009). A Distinct Macrophage Population Mediates Metastatic Breast Cancer Cell Extravasation, Establishment and Growth. *PLoS ONE*, 4(8), e6562. <https://doi.org/10.1371/journal.pone.0006562>
- Qian, B.-Z., Li, J., Zhang, H., Kitamura, T., Zhang, J., Campion, L. R., Kaiser, E. A., Snyder, L. A., & Pollard, J. W. (2011). CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature*, 475(7355), 222–225. <https://doi.org/10.1038/nature10138>
- Qian, B.-Z., & Pollard, J. W. (2010). Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell*, 141(1), 39–51. <https://doi.org/10.1016/j.cell.2010.03.014>
- Qin, X., Yan, M., Zhang, J., Wang, X., Shen, Z., Lv, Z., Li, Z., Wei, W., & Chen, W. (2016). TGFβ3-mediated induction of Periostin facilitates head and neck cancer growth and is associated with metastasis. *Scientific Reports*, 6(1), 20587. <https://doi.org/10.1038/srep20587>
- Rahman, G. A. (2011). Editorial: Breast conserving therapy: A surgical technique where little can mean more. *Journal of Surgical Technique and Case Report*, 3(1), 1–4. <https://doi.org/10.4103/2006-8808.78459>
- Rahman, N., Seal, S., Thompson, D., Kelly, P., Renwick, A., Elliott, A., Reid, S., Spanova, K., Barfoot, R., Chagtai, T., Jayatilake, H., McGuffog, L., Hanks, S., Evans, D. G., Eccles, D., Easton, D. F., & Stratton, M. R. (2007). PALB2, which encodes a BRCA2-interacting protein, is a breast cancer susceptibility gene. *Nature Genetics*, 39(2), 165–167. <https://doi.org/10.1038/ng1959>
- Ramos, S., Ferreira, S., Fernandes, A. S., & Saraiva, N. (2022). Lysyl Oxidases Expression and Breast Cancer Progression: A Bioinformatic Analysis. *Frontiers in Pharmacology*, 13. <https://doi.org/10.3389/fphar.2022.883998>
- Renwick, A., Thompson, D., Seal, S., Kelly, P., Chagtai, T., Ahmed, M., North, B., Jayatilake, H., Barfoot, R., Spanova, K., McGuffog, L., Evans, D. G., Eccles, D., Easton, D. F., Stratton, M. R., & Rahman, N. (2006). ATM mutations that cause ataxia-telangiectasia are breast cancer susceptibility alleles. *Nature Genetics*, 38(8), 873–875. <https://doi.org/10.1038/ng1837>
- Reymond, N., D'Água, B. B., & Ridley, A. J. (2013). Crossing the endothelial barrier during metastasis. *Nature Reviews Cancer*, 13(12), 858–870. <https://doi.org/10.1038/nrc3628>
- Rhim, A. D., Mirek, E. T., Aiello, N. M., Maitra, A., Bailey, J. M., McAllister, F., Reichert, M., Beatty, G. L., Rustgi, A. K., Vonderheide, R. H., Leach, S. D., & Stanger, B. Z. (2012). EMT

- and dissemination precede pancreatic tumor formation. *Cell*, 148(1–2), 349–361. <https://doi.org/10.1016/j.cell.2011.11.025>
- Riggio, A. I., Varley, K. E., & Welm, A. L. (2021). The lingering mysteries of metastatic recurrence in breast cancer. *British Journal of Cancer*, 124(1), 13–26. <https://doi.org/10.1038/s41416-020-01161-4>
- Rouzier, R., Perou, C. M., Symmans, W. F., Ibrahim, N., Cristofanilli, M., Anderson, K., Hess, K. R., Stec, J., Ayers, M., Wagner, P., Morandi, P., Fan, C., Rabiul, I., Ross, J. S., Hortobagyi, G. N., & Pusztai, L. (2005). Breast cancer molecular subtypes respond differently to preoperative chemotherapy. *Clinical Cancer Research*, 11(16), 5678–5685. <https://doi.org/10.1158/1078-0432.CCR-04-2421>
- Rucker, R. B., Kosonen, T., Clegg, M. S., Mitchell, A. E., Rucker, B. R., Uriu-Hare, J. Y., & Keen, C. L. (1998). Copper, lysyl oxidase, and extracellular matrix protein cross-linking. *The American Journal of Clinical Nutrition*, 67(5), 996S–1002S. <https://doi.org/10.1093/ajcn/67.5.996S>
- Ruddy, K. J., & Winer, E. P. (2013). Male breast cancer: risk factors, biology, diagnosis, treatment, and survivorship. *Annals of Oncology*, 24(6), 1434–1443. <https://doi.org/10.1093/annonc/mdt025>
- Ruffell, B., Affara, N. I., & Coussens, L. M. (2012). Differential macrophage programming in the tumor microenvironment. *Trends in Immunology*, 33(3), 119–126. <https://doi.org/10.1016/j.it.2011.12.001>
- Said, N., Smith, S., Sanchez-Carbayo, M., & Theodorescu, D. (2011). Tumor endothelin-1 enhances metastatic colonization of the lung in mouse xenograft models of bladder cancer. *Journal of Clinical Investigation*, 121(1), 132–147. <https://doi.org/10.1172/JCI42912>
- Sánchez, H., Paul, M. W., Grosbart, M., van Rossum-Fikkert, S. E., Lebbink, J. H. G., Kanaar, R., Houtsmuller, A. B., & Wyman, C. (2017). Architectural plasticity of human BRCA2-RAD51 complexes in DNA break repair. *Nucleic Acids Research*, 45(8), 4507–4518. <https://doi.org/10.1093/nar/gkx084>
- Santos-de-Frutos, K., & Djouder, N. (2021). When dormancy fuels tumour relapse. *Communications Biology*, 4(1). <https://doi.org/10.1038/s42003-021-02257-0>
- Saslow, D., Boetes, C., Burke, W., Harms, S., Leach, M. O., Lehman, C. D., Morris, E., Pisano, E., Schnall, M., Sener, S., Smith, R. A., Warner, E., Yaffe, M., Andrews, K. S., & Russell, C. A. (2007). American cancer society guidelines for breast screening with MRI as an adjunct to mammography. In *Obstetrical and Gynecological Survey* (Vol. 62, Issue 7, pp. 458–460). <https://doi.org/10.1097/01.ogx.0000269073.50925.38>
- Seal, S., Thompson, D., Renwick, A., Elliott, A., Kelly, P., Barfoot, R., Chagtai, T., Jayatilake, H., Ahmed, M., Spanova, K., North, B., McGuffog, L., Evans, D. G., Eccles, D., Easton, D. F., Stratton, M. R., & Rahman, N. (2006). Truncating mutations in the Fanconi anemia J gene BRIP1 are low-penetrance breast cancer susceptibility alleles. *Nature Genetics*, 38(11), 1239–1241. <https://doi.org/10.1038/ng1902>
- Seltzer, S., Corrigan, M., & O'Reilly, S. (2020). The clinicomolecular landscape of de novo versus relapsed stage IV metastatic breast cancer. In *Experimental and Molecular Pathology* (Vol. 114). <https://doi.org/10.1016/j.yexmp.2020.104404>
- Sethi, G. (2008). TNF: A master switch for inflammation to cancer. *Frontiers in Bioscience, Volume(13)*, 5094. <https://doi.org/10.2741/3066>
- Shao, W., Wang, W., Xiong, X., Cao, C., Yan, T. D., Chen, G., Chen, H., Yin, W., Liu, J., Gu, Y., Mo, M., & He, J. (2011). Prognostic impact of MMP-2 and MMP-9 expression in pathologic

- stage IA non-small cell lung cancer. *Journal of Surgical Oncology*, 104(7), 841–846. <https://doi.org/10.1002/jso.22001>
- Sheinboim, D., Parikh, S., Manich, P., Markus, I., Dahan, S., Parikh, R., Stubbs, E., Cohen, G., Zemser-Werner, V., Bell, R. E., Arciniegas Ruiz, S., Percik, R., Brenner, R., Leibou, S., Vaknine, H., Arad, G., Gerber, Y., Keinan Boker, L., Shimony, T., ... Levy, C. (2022). An exercise-induced metabolic shield in distant organs blocks cancer progression and metastatic dissemination. *Cancer Research*. <https://doi.org/10.1158/0008-5472.CAN-22-0237>
- Shiao, S. L., Preethi Ganesan, A., Rugo, H. S., & Coussens, L. M. (2011). Immune microenvironments in solid tumors: New targets for therapy. In *Genes and Development* (Vol. 25, Issue 24, pp. 2559–2572). <https://doi.org/10.1101/gad.169029.111>
- Shibata, Y., Berclaz, P.-Y., Chroneos, Z. C., Yoshida, M., Whitsett, J. A., & Trapnell, B. C. (2001). GM-CSF Regulates Alveolar Macrophage Differentiation and Innate Immunity in the Lung through PU.1. *Immunity*, 15(4), 557–567. [https://doi.org/10.1016/S1074-7613\(01\)00218-7](https://doi.org/10.1016/S1074-7613(01)00218-7)
- Shibue, T., & Weinberg, R. A. (2009). Integrin β 1-focal adhesion kinase signaling directs the proliferation of metastatic cancer cells disseminated in the lungs. *Proceedings of the National Academy of Sciences of the United States of America*, 106(25), 10290–10295. <https://doi.org/10.1073/pnas.0904227106>
- Shrotriya, S., Walsh, D., Nowacki, A. S., Lorton, C., Aktas, A., Hullihen, B., Benanni-Baiti, N., Hauser, K., Ayvaz, S., & Estfan, B. (2018). Serum c-reactive protein is an important and powerful prognostic biomarker in most adult solid tumors. *PLoS ONE*, 13(8), 1–12. <https://doi.org/10.1371/journal.pone.0202555>
- Sica, A., Schioppa, T., Mantovani, A., & Allavena, P. (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: Potential targets of anti-cancer therapy. *European Journal of Cancer*, 42(6), 717–727. <https://doi.org/10.1016/j.ejca.2006.01.003>
- Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H., & Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nature Cell Biology*, 2(5), 249–256. <https://doi.org/10.1038/35010517>
- Sosa, M. S., Avivar-Valderas, A., Bragado, P., Wen, H. C., & Aguirre-Ghiso, J. A. (2011). ERK1/2 and p38 α / β signaling in tumor cell quiescence: Opportunities to control dormant residual disease. *Clinical Cancer Research*, 17(18), 5850–5857. <https://doi.org/10.1158/1078-0432.CCR-10-2574>
- Sosa, M. S., Bragado, P., & Aguirre-Ghiso, J. A. (2014). Mechanisms of disseminated cancer cell dormancy: An awakening field. *Nature Reviews Cancer*, 14(9), 611–622. <https://doi.org/10.1038/nrc3793>
- STOKER, M. G. P., SHEARER, M., & O'NEILL, C. (1966). Growth Inhibition of Polyoma-Transformed Cells by Contact with Static Normal Fibroblasts. *Journal of Cell Science*, 1(3), 297–310. <https://doi.org/10.1242/jcs.1.3.297>
- Su, S., Liu, Q., Chen, J., Chen, J., Chen, F., He, C., Huang, D., Wu, W., Lin, L., Huang, W., Zhang, J., Cui, X., Zheng, F., Li, H., Yao, H., Su, F., & Song, E. (2014). A Positive Feedback Loop between Mesenchymal-like Cancer Cells and Macrophages Is Essential to Breast Cancer Metastasis. *Cancer Cell*, 25(5), 605–620. <https://doi.org/10.1016/j.ccr.2014.03.021>
- Suarez-Carmona, M., Lesage, J., Cataldo, D., & Gilles, C. (2017). EMT and inflammation: inseparable actors of cancer progression. *Molecular Oncology*, 11(7), 805–823. <https://doi.org/10.1002/1878-0261.12095>

- Sun, X., Glynn, D. J., Hodson, L. J., Huo, C., Britt, K., Thompson, E. W., Woolford, L., Evdokiou, A., Pollard, J. W., Robertson, S. A., & Ingman, W. v. (2017). CCL2-driven inflammation increases mammary gland stromal density and cancer susceptibility in a transgenic mouse model. *Breast Cancer Research*, *19*(1), 4. <https://doi.org/10.1186/s13058-016-0796-z>
- Takahashi, A., Ohtani, N., Yamakoshi, K., Iida, S., Tahara, H., Nakayama, K., Nakayama, K. I., Ide, T., Saya, H., & Hara, E. (2006). *Mitogenic signalling and the p16 INK4a – Rb pathway cooperate to enforce irreversible cellular senescence*. *8*(11). <https://doi.org/10.1038/ncb1491>
- TANIGUCHI, T., LEMOINE, N. R., & KAKKAR, A. K. (1998). Urokinase receptor is a key player in tumour progression. *Gut*, *43*(6), 739–740. <https://doi.org/10.1136/gut.43.6.739>
- Teng, M. W. L., Swann, J. B., Koebel, C. M., Schreiber, R. D., & Smyth, M. J. (2008). Immune-mediated dormancy: an equilibrium with cancer. *Journal of Leukocyte Biology*, *84*(4), 988–993. <https://doi.org/10.1189/jlb.1107774>
- Thods, M. E., DeSilets, L. D., Bennion, A., Forshaw, M. J., Design, H. P., Linssen, A. M., Joore, M. A., Minten, R. K. H., Leeuwen, Y. D. van, Anteunis, L. J. C., Beukes, G., Gamiieldien, F., Boud, D., South, N., Walker, D., Bennion, A., Forshaw, M. J., Eeee, C. D., Ccccc, A. B., ... Scholarship, F. O. R. E. (2006). *spe ci a l r ep or t Health Care in the 2008 Presidential Primaries*. *Health Care*, *37*(4), 148–149.
- Tiainen, S., Tumelius, R., Rilla, K., Hämäläinen, K., Tammi, M., Tammi, R., Kosma, V.-M., Oikari, S., & Auvinen, P. (2015). High numbers of macrophages, especially M2-like (CD163-positive), correlate with hyaluronan accumulation and poor outcome in breast cancer. *Histopathology*, *66*(6), 873–883. <https://doi.org/10.1111/his.12607>
- Titus, B., Frierson, H. F., Conaway, M., Ching, K., Guise, T., Chirgwin, J., Hampton, G., & Theodorescu, D. (2005). Endothelin Axis Is a Target of the Lung Metastasis Suppressor Gene RhoGDI2. *Cancer Research*, *65*(16), 7320–7327. <https://doi.org/10.1158/0008-5472.CAN-05-1403>
- Toiyama, Y., Inoue, Y., Saigusa, S., Kawamura, M., Kawamoto, A., Okugawa, Y., Hiro, J., Tanaka, K., Mohri, Y., & Kusunoki, M. (2013). C-Reactive protein as predictor of recurrence in patients with rectal cancer undergoing chemoradiotherapy followed by surgery. *Anticancer Research*, *33*(11), 5065–5074.
- Torre, L. A., Siegel, R. L., Ward, E. M., & Jemal, A. (2016). Global cancer incidence and mortality rates and trends - An update. *Cancer Epidemiology Biomarkers and Prevention*, *25*(1), 16–27. <https://doi.org/10.1158/1055-9965.EPI-15-0578>
- Tremont, A., Lu, J., & Cole, J. T. (2017). Endocrine Therapy for Early Breast Cancer: Updated Review. *The Ochsner Journal*, *17*(4), 405–411. <http://www.ncbi.nlm.nih.gov/pubmed/29230126>
- Uhr, J. W. (1995). *Doi-iiiancy*. *181*(April).
- Ushach, I., & Zlotnik, A. (2016). Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. *Journal of Leukocyte Biology*, *100*(3), 481–489. <https://doi.org/10.1189/jlb.3RU0316-144R>
- Valastyan, S., & Weinberg, R. A. (2011). Tumor Metastasis: Molecular Insights and Evolving Paradigms The Invasion-Metastasis Cascade. *Cell*, *147*(2), 275–292. <https://doi.org/10.1016/j.cell.2011.09.024>.Tumor
- Valiente, M., Obenauf, A. C., Jin, X., Chen, Q., Zhang, X. H.-F., Lee, D. J., Chافت, J. E., Kris, M. G., Huse, J. T., Brogi, E., & Massagué, J. (2014). Serpins Promote Cancer Cell Survival and

- Vascular Co-Option in Brain Metastasis. *Cell*, 156(5), 1002–1016. <https://doi.org/10.1016/j.cell.2014.01.040>
- van Landeghem, A. A., Poortman, J., Nabuurs, M., & Thijssen, J. H. (1985). Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. *Cancer Research*, 45(6), 2900–2906. <http://www.ncbi.nlm.nih.gov/pubmed/3986816>
- Vanharanta, S., & Massagué, J. (2013). Origins of Metastatic Traits. *Cancer Cell*, 24(4), 410–421. <https://doi.org/10.1016/j.ccr.2013.09.007>
- Vara, J. Á. F., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., & González-Barón, M. (2004). PI3K/Akt signalling pathway and cancer. *Cancer Treatment Reviews*, 30(2), 193–204. <https://doi.org/10.1016/j.ctrv.2003.07.007>
- Vaysse, C., Lomo, J., Garred, O., Fjeldheim, F., Lofteroed, T., Schlichting, E., McTiernan, A., Frydenberg, H., Husoy, A., Lundgren, S., Fagerland, M. W., Richardsen, E., Wist, E. A., Muller, C., & Thune, I. (2017). Inflammation of mammary adipose tissue occurs in overweight and obese patients exhibiting early-stage breast cancer. *Npj Breast Cancer*, 3(1), 1–9. <https://doi.org/10.1038/s41523-017-0015-9>
- Veisy, A., & Viga, M. (2018). The role of oral contraceptive pills on increased risk of breast cancer in North West of Iran. *Clinical Sciences Research and Reports*, 1(2), 294–301. <https://doi.org/10.15761/csrr.1000112>
- Vera-ramirez, L., & Hunter, K. W. (2017). *Tumor cell dormancy as an adaptive cell stress response mechanism [version 1 ; referees : 4 approved] Referee Status : 6(0), 1–9.* <https://doi.org/10.12688/f1000research.12174.1>
- Wakeling, A. E., Guy, S. P., Woodburn, J. R., Ashton, S. E., Curry, B. J., Barker, A. J., & Gibson, K. H. (2002). ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Research*, 62(20), 5749–5754. <http://www.ncbi.nlm.nih.gov/pubmed/12384534>
- Walens, A., DiMarco, A. v., Lupo, R., Kroger, B. R., Damrauer, J. S., & Alvarez, J. v. (2019). CCL5 promotes breast cancer recurrence through macrophage recruitment in residual tumors. *ELife*, 8, 1–26. <https://doi.org/10.7554/eLife.43653>
- Walens, A., DiMarco, A. v., Lupo, R., Kroger, B. R., Damrauer, J. S., & Alvarez, J. v. (2019). CCL5 promotes breast cancer recurrence through macrophage recruitment in residual tumors. *ELife*, 8. <https://doi.org/10.7554/eLife.43653>
- Wan, L., Pantel, K., & Kang, Y. (2013). Tumor metastasis: moving new biological insights into the clinic. *Nature Medicine*, 19(11), 1450–1464. <https://doi.org/10.1038/nm.3391>
- Wang, D. Y., Jiang, Z., Ben-David, Y., Woodgett, J. R., & Zacksenhaus, E. (2019). Molecular stratification within triple-negative breast cancer subtypes. *Scientific Reports*, 9(1), 1–10. <https://doi.org/10.1038/s41598-019-55710-w>
- Wang, H., Kaur, G., Sankin, A. I., Chen, F., Guan, F., & Zang, X. (2019). Immune checkpoint blockade and CAR-T cell therapy in hematologic malignancies. *Journal of Hematology and Oncology*, 12(1), 1–20. <https://doi.org/10.1186/s13045-019-0746-1>
- Wang, S. E., Xiang, B., Zent, R., Quaranta, V., Pozzi, A., & Arteaga, C. L. (2009). Transforming Growth Factor β Induces Clustering of HER2 and Integrins by Activating Src-Focal Adhesion Kinase and Receptor Association to the Cytoskeleton. *Cancer Research*, 69(2), 475–482. <https://doi.org/10.1158/0008-5472.CAN-08-2649>
- Weaver, V. M., Petersen, O. W., Wang, F., Larabell, C. A., Briand, P., Damsky, C., & Bissell, M. J. (1997). Reversion of the malignant phenotype of human breast cells in three-dimensional

- culture and in vivo by integrin blocking antibodies. *Journal of Cell Biology*, 137(1), 231–245. <https://doi.org/10.1083/jcb.137.1.231>
- Weidenfeld, K., Schif-Zuck, S., Abu-Tayeh, H., Kang, K., Kessler, O., Weissmann, M., Neufeld, G., & Barkan, D. (2016). Dormant tumor cells expressing LOXL2 acquire a stem-like phenotype mediating their transition to proliferative growth. *Oncotarget*, 7(44), 71362–71377. <https://doi.org/10.18632/oncotarget.12109>
- Weigelt, B., Baehner, F. L., & Reis-Filho, J. S. (2010). The contribution of gene expression profiling to breast cancer classification, prognostication and prediction: A retrospective of the last decade. In *Journal of Pathology* (Vol. 220, Issue 2, pp. 263–280). <https://doi.org/10.1002/path.2648>
- Weigelt, B., Geyer, F. C., & Reis-Filho, J. S. (2010). Histological types of breast cancer: How special are they? In *Molecular Oncology* (Vol. 4, Issue 3, pp. 192–208). <https://doi.org/10.1016/j.molonc.2010.04.004>
- Weigelt, B., Horlings, H., Kreike, B., Hayes, M., Hauptmann, M., Wessels, L., de Jong, D., van de Vijver, M., Veer, L. V., & Peterse, J. (2008). Refinement of breast cancer classification by molecular characterization of histological special types. *The Journal of Pathology*, 216(2), 141–150. <https://doi.org/10.1002/path.2407>
- Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., & Ferrante, A. W. (2003). Obesity is associated with macrophage accumulation in adipose tissue. *Journal of Clinical Investigation*, 112(12), 1796–1808. <https://doi.org/10.1172/JCI200319246>
- Weiss, A., Chavez-MacGregor, M., Lichtensztajn, D. Y., Yi, M., Tadros, A., Hortobagyi, G. N., Giordano, S. H., Hunt, K. K., & Mittendorf, E. A. (2018). Validation study of the American joint committee on cancer eighth edition prognostic stage compared with the anatomic stage in breast cancer. *JAMA Oncology*, 4(2), 203–209. <https://doi.org/10.1001/jamaoncol.2017.4298>
- White, D. E., Kurpios, N. A., Zuo, D., Hassell, J. A., Blaess, S., Mueller, U., & Muller, W. J. (2004). Targeted disruption of $\beta 1$ -integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer Cell*, 6(2), 159–170. <https://doi.org/10.1016/j.ccr.2004.06.025>
- Willis, R. A. (1935). The structure of teratomata. *The Journal of Pathology and Bacteriology*, 40(1), 1–36. <https://doi.org/10.1002/path.1700400102>
- Winkler, J., Abisoye-Ogunniyan, A., Metcalf, K. J., & Werb, Z. (2020). Concepts of extracellular matrix remodelling in tumour progression and metastasis. *Nature Communications*, 11(1), 1–19. <https://doi.org/10.1038/s41467-020-18794-x>
- Wortzel, I., Dror, S., Kenific, C. M., & Lyden, D. (2019). Exosome-Mediated Metastasis: Communication from a Distance. *Developmental Cell*, 49(3), 347–360. <https://doi.org/10.1016/j.devcel.2019.04.011>
- Wyckoff, J., Wang, W., Lin, E. Y., Wang, Y., Pixley, F., Stanley, E. R., Graf, T., Pollard, J. W., Segall, J., & Condeelis, J. (2004). A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors. *Cancer Research*, 64(19), 7022–7029. <https://doi.org/10.1158/0008-5472.CAN-04-1449>
- Yan, D., Wang, H.-W., Bowman, R. L., & Joyce, J. A. (2016). STAT3 and STAT6 Signaling Pathways Synergize to Promote Cathepsin Secretion from Macrophages via IRE1 α Activation. *Cell Reports*, 16(11), 2914–2927. <https://doi.org/10.1016/j.celrep.2016.08.035>

- Yang, H., Villani, R. M., Wang, H., Simpson, M. J., Roberts, M. S., Tang, M., & Liang, X. (2018). The role of cellular reactive oxygen species in cancer chemotherapy. *Journal of Experimental and Clinical Cancer Research*, *37*(1), 1–10. <https://doi.org/10.1186/s13046-018-0909-x>
- Ye, J., Tian, T., & Chen, X. (2020). The efficacy of gefitinib supplementation for breast cancer. *Medicine*, *99*(43), e22613. <https://doi.org/10.1097/MD.00000000000022613>
- Yoon, S.-O., Shin, S., & Lipscomb, E. A. (2006). A Novel Mechanism for Integrin-Mediated Ras Activation in Breast Carcinoma Cells: The $\alpha 6\beta 4$ Integrin Regulates ErbB2 Translation and Transactivates Epidermal Growth Factor Receptor/ErbB2 Signaling. *Cancer Research*, *66*(5), 2732–2739. <https://doi.org/10.1158/0008-5472.CAN-05-2941>
- Zeng, Z. S., Cohen, A. M., & Guillem, J. G. (1999). Loss of basement membrane type IV collagen is associated with increased expression of metalloproteinases 2 and 9 (MMP-2 and MMP-9) during human colorectal tumorigenesis. *Carcinogenesis*, *20*(5), 749–755. <https://doi.org/10.1093/carcin/20.5.749>
- Zhao, L., Niu, H., Liu, Y., Wang, L., Zhang, N., Zhang, G., Liu, R., & Han, M. (2019). LOX inhibition downregulates MMP-2 and MMP-9 in gastric cancer tissues and cells. *Journal of Cancer*, *10*(26), 6481–6490. <https://doi.org/10.7150/jca.33223>
- Zhou, L., Yang, K., Randall Wickett, R., & Zhang, Y. (2016). Dermal fibroblasts induce cell cycle arrest and block epithelial–mesenchymal transition to inhibit the early stage melanoma development. *Cancer Medicine*, *5*(7), 1566–1579. <https://doi.org/10.1002/cam4.707>

Collaborations

Neutrophil extracellular traps formed during chemotherapy confers treatment resistance via TGF- β activation. Alexandra Mousset, Enora Lecorgne, Isabelle Bourget, Pascal Lopez, Chloé Dominici, Kitti Jenovai, Julien Cherfils-Vicini, Mikkel Green Terp, Mikala Egeblad, Cédric Gaggioli*, Jean Albrengues*. *Cancer Cell*. Under revision.

Antifibrotic effect of mitomycin-C on human vocal cord fibroblasts. Diána Szabó, Dávid Kovács, Valéria Endrész, Nóra Igaz, Kitti Jenovai, Gabriella Spengler, László Tiszlavicz, József Molnár, Katalin Burián, Mónika Kiricsi, László Rovó. *The Laryngoscope*. 2019 July, doi: 10.1002/lary.27657

1st article:

Neutrophil extracellular traps formed during chemotherapy confers treatment resistance *via* TGF- β activation. Alexandra Mousset, Enora Lecorgne, Isabelle Bourget, Pascal Lopez, Chloé Dominici, Kitti Jenovai, Julien Cherfils-Vicini, Mikkel Green Terp, Mikala Egeblad, Cédric Gaggioli*, Jean Albrengues*.
Cancer Cell. Under revision.

Neutrophil extracellular traps formed during chemotherapy confers treatment resistance via TGF β activation

Authors: Alexandra Mousset¹, Enora Lecorgne¹, Isabelle Bourget¹, Pascal Lopez¹, Chloé Dominici¹, Kitti Jenovai¹, Julien Cherfils-Vicini¹, Mikkel Green Terp², Mikala Egeblad³, Cédric Gaggioli^{1*}, Jean Albregues^{1*}.

¹University Côte d'Azur, CNRS UMR7284, INSERM U1081, Institute for Research on Cancer and Aging, Nice (IRCAN), Nice, France.

²Department of Cancer and Inflammation Research, Institute of Molecular Medicine, University of Southern Denmark, Odense, Denmark.

³Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA.

*Contributed equally

Correspondence

jalbregues@unice.fr
gaggioli@unice.fr

Abstract

Metastasis is the major cause of cancer death and although most patients with metastases are treated with chemotherapy, the development of therapy resistance is common. The tumor microenvironment can confer chemoresistance, yet little is still known about how specific host cells influence therapy outcome. We show that chemotherapy induced neutrophil recruitment and Neutrophil Extracellular Trap (NET) formation which reduced therapy response in a mouse model of breast cancer lung metastasis. We found that chemotherapy-treated cancer cells released adenosine triphosphate causing other cancer cells to secrete IL-1 β , which in turn triggered neutrophils to form NETs. Two NET-associated proteins were required for NETs' ability to induce chemoresistance: first, integrin- $\alpha\beta$ 1 in NETs trapped latent TGF β . Then, matrix metalloproteinase 9 cleaved and activated the trapped latent TGF β . The NET-mediated TGF β activation caused cancer cell to undergo epithelial to mesenchymal transition and correlated with chemoresistance. Critically, pharmacologically targeting of IL-1 β , NETs, integrin- $\alpha\beta$ 1, MMP9, and TGF β all dramatically improved chemotherapy response in our mice model. Our work establishes a novel paradigm for how NETs regulate activities of neighbouring cells by trapping and activating cytokines. Additionally, our data suggest that chemotherapy resistance in the metastatic setting can be reduced or prevented by targeting the previously unrecognized IL-1 β -NET-TGF β axis.

2nd article:

Antifibrotic effect of mitomycin-C on human vocal cord fibroblasts. Diána Szabó, Dávid Kovács, Valéria Endrész, Nóra Igaz, Kitti Jenovai, Gabriella Spengler, László Tizslavicz, József Molnár, Katalin Burián, Mónika Kiricsi, László Rovó. *The Laryngoscope*. 2019 July, doi: 10.1002/lary.27657

Antifibrotic Effect of Mitomycin-C on Human Vocal Cord Fibroblasts

Diána Szabó, MD, PhD[†]; Dávid Kovács, PhD[†]; Valéria Endrész, PhD; Nóra Igaz, MSc;
 Kitti Jenovai, MSc; Gabriella Spengler, PhD; László Tiszlavicz, MD, PhD; József Molnár, MD, DSc;
 Katalin Burián, MD, PhD; Mónika Kiricsi, PhD[†]; László Rovó, MD, PhD[†]

Objective: Acquired laryngotracheal stenosis is a potentially life-threatening situation and a very difficult and challenging problem in laryngology. Therefore, new trends and innovative approaches based on antifibrotic drugs and minimally invasive regimens are being developed to attenuate laryngotracheal fibrosis and scarring. The purpose of this study was to examine the efficacy of mitomycin-C (MMC) to reverse the transforming growth factor (TGF)- β -induced differentiation of MRC-5 fibroblast and human primary vocal cord fibroblasts to reveal the possible applicability of MMC to laryngotracheal fibrotic conditions.

Methods: Human primary fibroblast cells were isolated from vocal cord specimens of patients undergoing total laryngectomy. The established primary vocal cord fibroblast cell cultures as well as the MRC-5 human fibroblast cells were treated with 5 ng/mL TGF- β alone and then with 0.5 μ g/mL MMC for 24 hours. Differentiation of fibroblasts was characterized by α -smooth muscle actin (α -SMA) immunohistochemistry, Western blot analysis, and real-time polymerase chain reaction. Cell motility was assessed by wound-healing assay.

Results: Elevated α -SMA mRNA and protein expression as well as increased cell motility were observed upon TGF- β exposures. However, after MMC treatments the TGF- β -induced fibroblasts exhibited a significant decrease in α -SMA expression and wound-healing activity. Therefore, TGF- β -stimulated fibroblast-myofibroblast transformation was reversed at least in part by MMC treatment. Histopathological examinations of tissue specimens of a laryngotracheal stenosis patient supported these findings.

Conclusion: Antifibrotic effects of MMC were demonstrated on the human MRC-5 cell line and on primary vocal cord fibroblast cultures. These results verify that MMC can be used with success to reverse upper airway stenosis by reverting the myofibroblast phenotype.

Key Words: Laryngotracheal stenosis, antifibrotic effects, mitomycin-C, primary vocal cord fibroblast, α -smooth muscle actin.

Level of Evidence: NA

Laryngoscope, 129:E255–E262, 2019

INTRODUCTION

In response to injury, inflammation, exposure to toxic substances, or physical trauma, tissues initiate healing processes; however, fibrotic diseases may develop if these reparative mechanisms are misregulated.^{1,2} Pathophysiological progression of fibrotic conditions eventually lead to organ failure; thus, fibrosis can be accounted for millions of deaths worldwide. Generally, fibrotic diseases are characterized by excessive differentiation of fibroblasts to myofibroblasts, resulting in a cell type capable of contraction due to elevated alpha-smooth muscle actin

(α -SMA) expression, as well as of accumulating extracellular matrix components following increased synthesis in the affected tissues. Although fibrosis can occur in various organs, and the underlying etiologies might be substantially different, mechanistically the pleiotropic transforming growth factor β (TGF- β) is the most frequent trigger for fibroblast differentiation in such conditions.

Based on own observations, although the highest incidence of fibrotic disease affects lungs, kidneys, and the liver, the frequency of fibrosis-related disorders in other organs such as the vocal cords and trachea has been progressively and alarmingly increased in recent years. Such an example is iatrogenic laryngotracheal stenosis (LTS), which is a serious end result of uncontrolled tracheal fibrotic processes.³ Previous investigations in our clinic revealed that the pathogenesis of LTS is multifactorial⁴; however, the most common cause is mechanical trauma derived from endotracheal intubation. Depending on the exact anatomical region, a sustained compression of the tracheal tube's cuff on the tracheal wall may cause blood circulation failure, inflammation, scarring in soft tissue, and even cartilage damage. Although minimally invasive treatment modalities have been implemented to manage LTS in most cases,^{5–7} surgical resection, tracheal

From the Department of Oto-Rhino-Laryngology and Head & Neck Surgery (D.S., L.R.); the Department of Medical Microbiology and Immunobiology (V.E., G.S., J.M., K.B.); the Department of Pathology (L.T.), Faculty of Medicine; and the Department of Biochemistry and Molecular Biology, Faculty of Science and Informatics (D.K., N.I., K.J., M.K.), University of Szeged, Szeged, Hungary.

Editor's Note: This Manuscript was accepted for publication on October 10, 2018.

[†]These authors contributed equally to this work.

This study received support from the National Research, Development and Innovation Office-NKFIH (GINOP-2.3.2-15-2016-00040).

The authors have no funding, financial relationships, or conflicts of interest to disclose.

Send correspondence to Diana Szabó, MD, PhD, Tisza Lajos bld 111, 6724 Szeged, Hungary. E-mail: diniklinik@freemail.hu

DOI: 10.1002/lary.27657

reconstructions, and end-to-end anastomosis must be applied as a definitive treatment even if these have moderate efficiency due to high risk of granulation tissue formation and restenosis.^{8,9} Considering several factors such as high risk of surgery, postoperative complications, extended hospitalization period after surgery, and substantial costs, new trends and innovative approaches of LTS treatment are mandatory.

For modulation of wound-healing processes and for attenuating scarring, one of the possible solutions described in the scientific literature is the application of antifibrotic drugs on primary lesions. For this purpose, various antifibrotic pharmaceutical agents such as corticosteroids,¹⁰ 5-fluorouracil,¹¹ carnitine, and mitomycin-C (MMC)^{12,13} have already been employed either alone in short-term local surface applications or as at our clinic in combination with preceding laser excision of cicatricial tissue in laryngotracheal stenosis treatment. Although MMC is a traditional chemotherapeutic drug, isolated from *Streptomyces sp.*, it has also been regarded as a compound with high potential to prevent tissue fibrosis. The antifibrotic activity of locally applied MMC is presumably due to its inhibiting effects on fibroblast proliferation¹⁴⁻¹⁶; however, clinical and experimental data considering its application in fibrosis and LTS treatment are still elusive.^{17,18} Although some clinical observations suggest the possible applicability of MMC for laryngotracheal fibrotic conditions, its efficacy on LTS requires more mechanistic and detailed investigations. Thus, using the human MRC-5 fibroblasts cell line and patient-derived primary laryngotracheal fibroblasts, we established TGF- β -induced fibrosis models to investigate the antifibrotic potential of MMC.¹⁹⁻²¹

MATERIALS AND METHODS

Culturing MRC-5 Fibroblasts

The MRC-5 human fibroblast cell line (Sigma-Aldrich, St. Louis, MO) was cultured in Eagle's Minimum Essential Medium (EMEM) complemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.001% streptomycin, and 0.005% penicillin under standard conditions.

Isolation and Culturing of Human Primary Fibroblasts

Human primary fibroblast cells were isolated from vocal cord specimens of human patients undergoing total laryngectomy due to laryngeal cancer. The larynx was removed, and the healthy part of the vocal cord was resected and placed in phosphate-buffered saline immediately. The tissue sample contained epithelium, lamina propria, and muscle tissue. The resected vocal cord was cut into 1 × 1 mm pieces, which were then placed into precoated T25 flasks in culture medium (EMEM supplemented with 10% FBS, 100 U/mL penicillin, 0.01 mg/mL streptomycin, 2 mM L-glutamine) and were kept at 37°C in 5% CO₂ atmosphere and 95% humidity. When fibroblast cultures started to grow, the medium was frequently changed. When 80% of the surface was covered with fibroblasts, the tissue pieces were removed, the cells were allowed to reach confluence, and then cells were trypsinized and passaged to new flasks. Cultured fibroblast was used between 4 to 8 passages.

Immunocytochemistry

To verify that the obtained patient-derived cells were fibroblasts, cells were stained for α -SMA and vimentin. For this, cells were grown on coverslips and fixed by 4% formaldehyde (PFA) solution. Following permeabilization with 0.3% Triton-X-100 (Sigma) solution, the samples were blocked in 5% bovine serum albumin (BSA) (Sigma) and stained with α -SMA- and vimentin-specific primary antibodies (Abcam) overnight. On the following day, fluorophore-conjugated secondary antibodies (Abcam) were applied and cell nuclei were counterstained with DAPI (Sigma). Samples were visualized by an Olympus BX51 fluorescent microscope equipped with Olympus DP70 camera (Tokyo, Japan).

Fibroblast Differentiation and Mitomycin-C Treatment

MRC-5 and primary vocal cord fibroblast cells were treated with 5 ng/mL TGF- β (Abcam) diluted in 2% serum-containing culture medium for 48 hours. After 24 hours, MMC was added to the TGF- β -containing medium in 0.5 μ g/mL concentration (determined by viability measurements), and cells were incubated for an additional 24 hours (Barcelona, Spain).

Measurement of Cytotoxicity, Proliferation, and Apoptosis

Impedance-based dynamic monitoring of living adherent cells was carried out with xCELLigence RTCA System (Roche Applied Science, Mannheim, Germany). 5,000 cells/well were loaded onto special 96-well E-plates with built-in gold electrodes and were covered with culture medium. After cells reached steady growth, they were treated with various concentrations (0.1, 0.3, 0.5, 1, 3, 5 μ g/mL) of MMC. To assess the toxicity of MMC (Inibsa) real-time, the impedance of the cells was registered every 10 minutes, and for each time point a cell index was calculated based on the measured values in the presence and in absence of cells. Based on these measurements, a nontoxic concentration for MMC could be determined, which was used throughout further cell treatments.

To detect cell proliferation, MRC-5 cells were seeded to 24-well plates and treated with 5 ng/mL TGF- β and/or MMC diluted in 2% serum containing medium. Cells were collected each day by trypsinization, and the number of cells/well was determined using a Vi-CELL XR cell counter (Beckman Coulter, Brea, CA). For cytotoxicity measurements, MRC-5 cells were seeded into 96-well plates in 5,000 cells/well density. On the following day, cells were treated with 5 ng/mL TGF- β diluted in 2% serum containing medium for 48 hours. After 48 hours, MMC was added to the TGF- β -containing medium, and cells were treated for further 48 hours. At the end of the treatment, cell viability was determined using CellTiter Aqueous One cell proliferation assay (Promega, Madison, WI), and absorbance was determined in a Synergy HTX multimode reader. Upon calculating cell viability, values corresponding to nonstimulated cells were considered 100%. For apoptosis detection, MRC-5 cells were seeded into black-wall 96-well plates (Thermo), and the same experimental protocol was used as described above. As a readout, Apo-ONE homogeneous Caspase-3/7 Assay (Promega) was applied using a FLUOstar Omega plate reader (BMG Labtech, Offenburg, Germany).

Western Blot

To analyze α -SMA protein levels, Western blot analysis was performed. MRC-5 and primary vocal cord fibroblast cells were grown in T25 culture flasks. Following treatments with either

TGF- β or MMC or both, cells were scraped, collected by centrifugation, and lysed in Radioimmunoprecipitation assay buffer (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Sodium dodecyl sulfate (SDS), 1% Triton-X-100). Protein concentrations were determined using the BCA method, and then 25 μ g total protein from each sample was resolved on 8% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham). The membranes were blocked with 5% nonfat dry milk-TBST solution, and then α -SMA-specific primary antibody (Abcam) was applied overnight in 1:2000 dilution and Tubulin-specific antibody (Sigma, 1:2000) was hybridized to the membranes to ensure equal loading. HRP-conjugated secondary antibodies (Dako) were used, and then membranes were developed using Immobilon Western HRP substrate (Millipore). Chemiluminescent signal was detected in a Li-Cor C-Digit blot scanner (Li-Cor Biotech., Cambridge, U.K.).

Scratch Assay

To measure the migration activity of MRC-5 and primary vocal cord fibroblasts, cells were left to grow in 6-well plates until they reached confluence. Next, cell layers were then treated with 5 ng/mL TGF- β for 24 hours, and then wounds were created using a P200 pipette tip. Cell-free zones were photographed with a phase-contrast microscope, and then 0.5 μ g/mL MMC was added to the cells. After 24-hour treatment, samples were photographed again, and the number of migrating cells were counted using ImageJ1.44 software.

qPCR

For real-time quantitative polymerase chain reaction (RT-qPCR) analysis, 6×10^5 cells were seeded into 6-cm diameter culture dishes. On the following day, cells were treated with TGF- β , and then 24 hours later MMC was added to the culture for an additional 24 hours of treatment. Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the guidelines of the manufacturer. Taqman Reverse Transcription Reagent (Applied Biosystems, Foster City, CA) was applied to generate cDNA using 1 μ g of total RNA, and relative levels of TGF- β , COL1A1, PAI-1, and CTGF transcripts were quantified in a Pico Real-Time qPCR System (Thermo Scientific) using gene-specific primers (Table I) and SYBR Green PCR Master Mix (Applied Biosystems). The level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-specific messengers was measured to calculate relative amount of each transcript using $\Delta\Delta$ Ct analysis. RT-qPCR measurements were carried out in triplicates.

Immunohistochemistry of Patient Samples

Samples were obtained from a patient undergoing laser excision of the posterior glottis stenosis and receiving topical

MMC treatment 1 week following scar excision. Tissue samples were fixed in 10% formaldehyde and 4- μ m thick paraffin sections were prepared. The specimens were incubated with α -SMA-specific primary antibody (Cellmarque Corp., Rocklin, CA) in 1:300 dilution at pH 9. Throughout the staining, the EnVision FLEX kit on Dako Autostainer Plus (Dako) protocol was used. α -SMA-positive cells were counted by ImageJ1.44 software with a CellCounter plugin on four independent ImageJ/sample.

Statistical Analysis

Statistical analysis and graphical visualization of the obtained data were carried out in a GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, CA) using *t* test and two-way analysis of variance ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$ and $\#P = 0.0006$ indicate statistical significance).

RESULTS

MMC Hampers TGF- β -Triggered MRC-5 Fibroblast Transformation

To test the antifibrotic potential of MMC, we first evaluated its cytotoxicity on MRC-5 human fibroblast cells. These cells represent a generally accepted and widely utilized in vitro model system for studying cellular and molecular mechanisms in the background of fibrotic diseases. MRC-5 cells received increasing concentrations of MMC, and the dose response of the treatments was obtained by real-time cell electronic sensing. Upon MMC exposure, fibroblast cells showed gradually decreasing cell indices suggesting a dose-dependent antiproliferative effect of MMC (Fig. 1A). Differences in the kinetic curves indicated that 5 μ g/mL MMC exhibited serious toxicity throughout the examined time period; however, MMC concentrations between the 0.1 and 1 μ g/mL range proved to be sublethal.

To test that MMC also exhibits similar effects on TGF- β -transformed fibrotic cells, MRC-5 cells were treated with either TGF- β or with MMC (in 0.5 or 5 μ g/mL concentrations) or with TGF- β +MMC combinations, and cell proliferation was monitored for 5 days (Fig. 1B). TGF- β -stimulated fibroblasts showed elevated proliferative activity compared to nonstimulated cells because the TGF- β -exposed cells reached the plateau phase already after 72 hours, whereas control fibroblasts accomplished this cell number only after 120 hours. Similar to what we observed earlier, 0.5 μ g/mL MMC exhibited cytostatic effect, whereas treatments with 5 μ g/mL MMC proved to be cytotoxic on nonstimulated

TABLE I.
Primer Sequences.

Target	Forward Primer	Reverse Primer
GAPDH	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGG-3'
TGF- β	5'-AGCAACAATTCCTGGCGATAC-3'	5'-CTAAGCGGAAAGCCCTCAAT-3'
COL1A1	5'-CATCTGGTGGTGGAGACTTGC-3'	5'-TCCTGGTTTCTCCTTTGG-3'
PAI-1	5'-AGTGGACTTTTCAGAGGTGGAG-3'	5'-GCCGTTGAAGTAGAGGGCATT-3'
CTGF	5'-GGAAATGCTGCGAGGAGTGG-3'	5'-GGCTCTAATCATAGTTGGGTCTGG-3'

CTGF = connective tissue growth factor; COL1A1 = collagen alpha-1(1); GAPDH = glyceraldehyde 3-phosphate dehydrogenase; PAI-1 = plasminogen activator inhibitor-1; TGF = transforming growth factor.

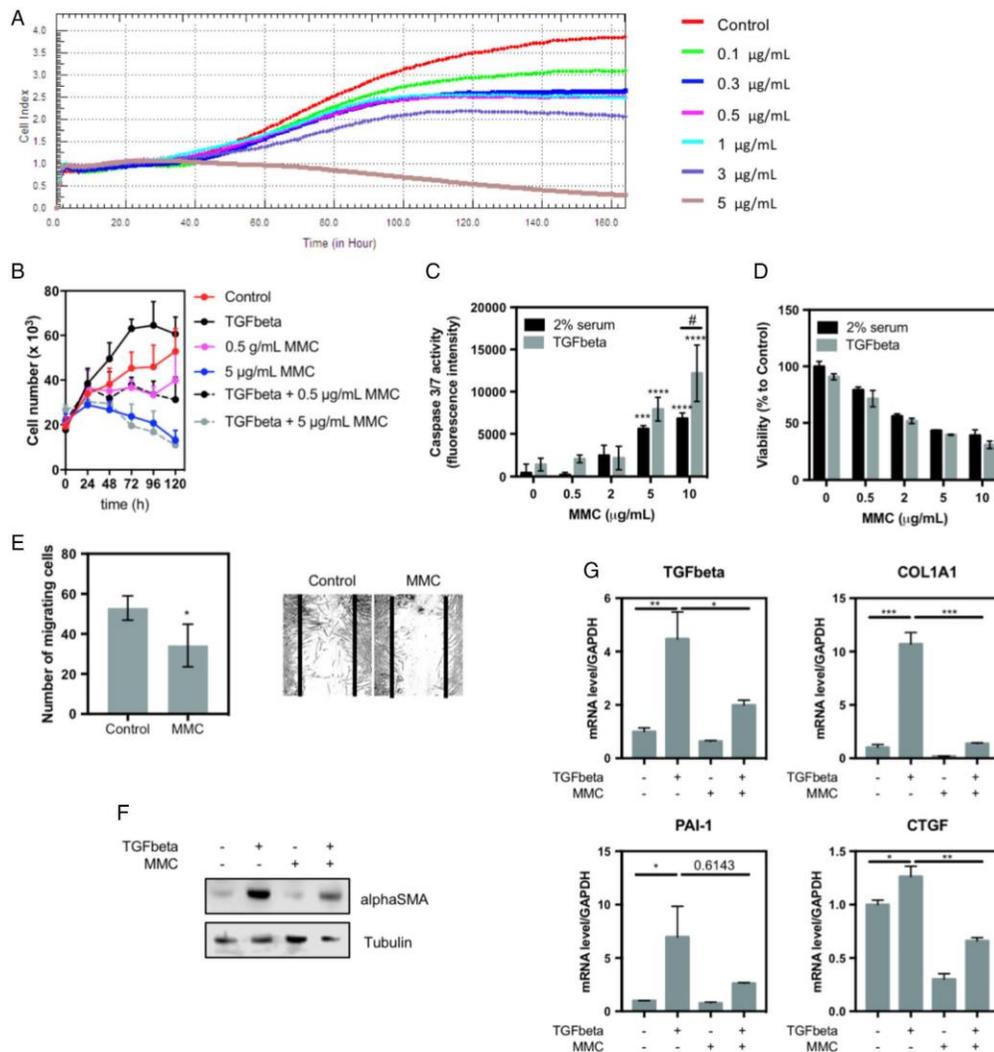


Fig. 1. (A–G) Dose response of MMC treatment (A) and effect of MMC on TGF- β -triggered MRC-5 fibroblast transformation, assessed by proliferation (B), Caspase 3/7 activity (C), viability (D), wound-healing assay (E), Western blot (F), and quantitative polymerase chain reaction (G). MMC = mitomycin-C; SMA = smooth muscle actin; TGF = transforming growth factor.

MRC-5 cells. Most importantly, MMC treatments exerted comparable effects on TGF- β -transformed cells as on nonstimulated cells, proving the antiproliferative activity of MMC on transformed fibroblasts as well. To compare the apoptotic and cytotoxic activity of MMC on fibrotic cells, nonstimulated and TGF- β -transformed cells were treated with MMC in various concentrations for 48 hours, and then Caspase 3/7 activity measurement (Fig. 1C) or cell viability assay (Fig. 1D) was performed. MMC treatments decreased cell viability in a dose-dependent manner both in naive and TGF- β -transformed fibroblasts. Five and 10 $\mu\text{g/mL}$ MMC

induced significant induction in Caspase 3/7 activity. Moreover, treatments with 10 $\mu\text{g/mL}$ MMC triggered Caspase 3/7 activity with a significantly higher degree in transformed fibroblasts than in the nonstimulated counterparts.

Because elevated cell migration during cicatrix formation is one of the characteristic hallmarks of fibrotic cells, we tested the migration capacity of scratch-activated MRC-5 fibroblasts upon MMC treatment. For this, MRC-5 cells were cultured until they reached confluence, and then wounds were scratched into the cell layers using sterile pipette tips. After scratching, cells were

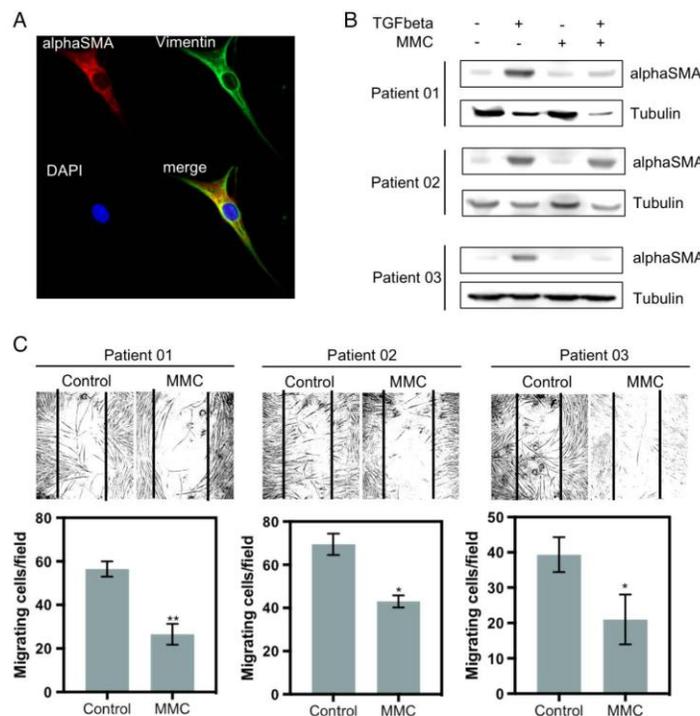


Fig. 2. (A–C) Verification of patient-derived fibroblast cells by immunocytochemistry (A). Effect of MMC on TGF- β -triggered patient-derived primary vocal cord fibroblasts transformation by Western blot (B) and wound-healing assay (C). MMC = mitomycin-C; SMA = smooth muscle actin; TGF = transforming growth factor.

incubated for 24 hours either in the presence or in the absence of 0.5 $\mu\text{g/mL}$ MMC. Cell-free zones were photographed, and then the number of cells that had migrated into the cell-free zones was determined using software-based ImageJ1 analysis. We found that MMC treatment significantly decreased the scratch-triggered migration of MRC-5 fibroblast cells (Fig. 1E).

Because elevated TGF- β secretion is responsible for maintaining fibroblasts in the activated phenotype during the development of fibrotic diseases,²² we tested whether MMC treatment can influence the TGF- β -provoked fibroblast-to-myofibroblast transformation. For this, serum-starved MRC-5 cells were treated with TGF- β in 0.5 ng/mL concentration for 24 hours, and then MMC was added to the culture and the cells were incubated for an additional 24 hours. To detect fibroblast transformation, α -SMA protein expression—a well-recognized marker of fibrotic fibroblasts—was monitored by Western blotting. As expected, TGF- β alone induced substantial fibroblast transformation because we observed highly elevated α -SMA expression in these cells compared to untreated fibroblasts. Although MMC alone did not influence α -SMA expression in nonstimulated fibroblasts, we found that MMC treatment effectively attenuated the fibroblast-transforming effect of TGF- β treatments (Fig. 1F). To verify this

antifibrotic activity of MMC, total RNA was isolated from TGF- β -stimulated and MMC-treated MRC-5 fibroblasts, and mRNA levels of fibrotic marker genes TGF- β , COL1A1, PAI-1, and CTGF were quantified by qPCR measurements. TGF- β alone induced significant upregulation in the expression of every examined fibrosis marker gene, although this effect was significantly abolished when TGF- β -exposed fibroblasts were treated with MMC as well (Fig. 1G). It is also noteworthy that MMC treatment alone resulted in lower expression levels of COL1A1 and CTGF in MMC-exposed cells compared to those of control MRC-5 fibroblasts.

MMC Exhibits Antifibrotic Effects in Patient-Derived Primary Vocal Cord Fibroblasts

Because we found that MMC displays a remarkable antifibrotic activity in MRC-5 fibroblast cells, we decided to test its efficiency on patient-derived primary vocal cord fibroblast cells as well. Three patients attending our clinic underwent total laryngectomy due to laryngeal cancer. Healthy parts of vocal cords were used to initiate primary human fibroblast cultures. Primary cells showed fibroblast characteristics such as spindle-like morphology; moreover, they stained positive for the fibroblast markers

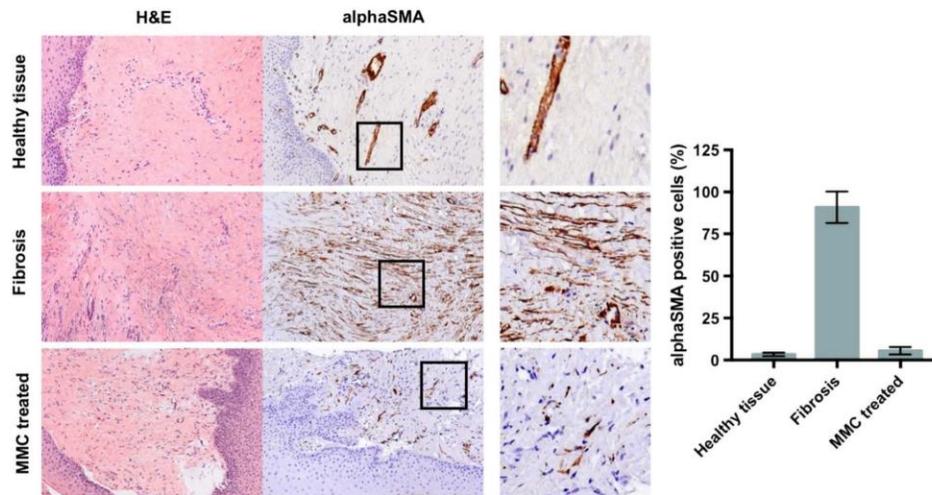


Fig. 3. Topical MMC treatment reduces laryngeal fibrosis in vivo verified by α -SMA immunohistochemistry. H&E = hematoxylin and eosin; MMC = mitomycin-C; SMA = smooth muscle actin.

α -SMA and vimentin and thus were proven to be primary fibroblast cultures (Fig. 2A).

To test the efficacy of MMC on the obtained primary fibroblasts, first, wound healing assays were performed. Primary fibroblasts were left to grow, and wounds were scratched when cells reached confluence. Similar to MRC-5 fibroblasts, patient-derived cells were also treated with MMC in 0.5 μ g/mL concentration for 24 hours, and then the number of cells that migrated to cell-free zones were counted. We found that MMC treatment significantly inhibited scratch-induced migration of all three of the examined patient-derived primary fibroblasts (Fig. 2B).

To examine whether MMC can influence the fibrosis-mediating potential of TGF- β in primary fibroblasts, likewise to the MRC-5 fibroblast cell line, serum-starved primary cells were treated with 5 ng/mL TGF- β . Twenty-four hours later, MMC was added to the medium in 0.5 μ g/mL concentration, and cells were incubated for further 24 hours. We detected the expression of the fibrotic fibroblast and myofibroblast marker α -SMA by Western blotting, which indicated that TGF- β triggered considerable fibroblast activation in all three of the treated primary fibroblast lines. Western blot data show that MMC treatment in primary fibroblasts hindered the fibrosis-inducing effect of TGF- β treatment in all three of the examined cases. Moreover, in cells obtained from patient 03, MMC treatments were able to completely restore the basal phenotype of TGF- β -stimulated fibroblasts because α -SMA level of the TGF- β +MMC-treated cells was comparable to that of the nontreated control cells (Fig. 2C).

Topical MMC Treatment Reduces Laryngeal Fibrosis In Vivo

Histopathological verification of highly effective MMC treatment on a patient diagnosed with posterior

commissure stenosis is shown in Figure 3 as a representative case. Prior to MMC treatment, tissue samples were obtained from the fibrotic area and also from the healthy adjacent tissue. Then, the patient received topical MMC treatment two times: after 1 week and 2 weeks after laser resection of posterior commissure stenosis. Two weeks after the second topical MMC treatment, specimens were resected from MMC-treated tissue and subjected to post-treatment histopathology. As expected, the healthy tissue region stained negative to the fibrosis marker α -SMA, whereas a strong positive staining was detected in the fibrotic area. Strikingly, after MMC treatment markedly lower expression of the fibrosis marker α -SMA was observed following immunohistochemical staining and subsequent pathological examination, and the staining intensity was comparable to that of the healthy tissue sample (Fig. 3.). Quantification of α -SMA-positive cells in tissue sections verified the high expression of α -SMA in fibrotic tissues, which was suppressed by MMC treatment.

DISCUSSION

Acquired laryngotracheal stenosis is a potentially life-threatening condition, induced mainly by traumatic and prolonged endotracheal intubation.²³ In most cases, LTS complications significantly influence the patient's life qualities because symptoms may vary from mild dyspnea and stridor to serious respiratory failure.²⁴ In the last decades, due to a significant increase in intensive care interventions worldwide, the frequency of iatrogenic LTS events has been substantially elevated and thus a demand for novel treatment modalities.²⁵

In our study, we proved that MMC is effective in attenuating TGF- β -induced fibroblast-to-myofibroblast transformation not only on the well-established human

MRC-5 fibroblast model system but also on patient-derived primary vocal cord fibroblasts. To verify this antifibrotic activity and the inhibitory action of MMC on fibroblast differentiation, the proliferation, wound-healing activity, apoptotic potential, α -SMA expression, and the mRNA levels of fibrotic marker genes (TGF- β , COL1A1, PAI-1, and CTGF) were quantified in TGF- β -stimulated and MMC-treated fibroblasts, respectively. Importantly, we demonstrated the beneficial effect of topical MMC treatment using histological staining results of a patient with posterior commissure stenosis, for which we observed a markedly lower expression level of the fibrosis marker α -SMA after MMC treatment and thereby confirmed the in vivo competence of MMC.

The antifibrotic potential of MMC has been tested in various human and animal in vivo, as well as in numerous in vitro model systems. MMC was shown to reduce pericardial fibrosis after cardiac surgery in rabbits²⁶ and to decrease the postoperative fibrosis after glaucoma surgery.²⁷ Its antifibrotic effect was confirmed in human Tenon's capsule fibroblast cultures in vitro,²⁸ in a rat Peyronie's disease model,²⁹ and in an experimentally induced urethral stricture model in rats.³⁰ Furthermore, MMC could prevent bronchial stenosis in a young cystic fibrosis patient following bilateral lung transplantation.³¹

We believe that the antifibrotic effect of MMC also can be exploited by LTS patients. According to our hypothesis, the maximal in vivo antifibrotic activity can be achieved if the treatment with MMC occurs in the granulation phase of the wound-healing process, which is when fibroblast proliferation is activated.^{32–37} In fact, rudimentary tissue generation relies mainly on fibroblasts that migrate into the wound area and ultimately transform into myofibroblasts, which is induced by the action of TGF- β .^{38–41} The presence of myofibroblasts in the regenerating tissue is a positive marker of progressive fibrosis, although their number usually decreases as the wound is closed. Experimental and clinical observations imply that topical MMC application should be started not earlier than 1 week after laser excision of stenotic tissue to inhibit myofibroblast overproduction and thereby prevent restenosis. Moreover, we expect that MMC treatment can reduce fibrosis with the highest efficiency by patients with LTS in a long-term application regime, in contrast to the moderate fibrosis inhibiting effect of early, short-term MMC application observed by Li et al.⁴² Considering the high-risk surgical procedures, the extended hospitalization periods, and various postoperative complications of tracheal stenosis, prevention of restenosis and minimalization of surgical intervention with the topical application of MMC might be successful treatment modality for laryngotracheal stenosis.

CONCLUSION

We demonstrated that MMC has the potential to reverse the TGF β -induced myofibroblast differentiation because we observed significant decrease in α -SMA expression and wound-healing activity in MMC-treated cells. Antifibrotic effects of MMC also were verified on human primary vocal cord fibroblast cell cultures. Our

results support that MMC can be applied to suppress upper airway stenosis by overturning the myofibroblast phenotype.

BIBLIOGRAPHY

- Nanthakumar CB, Hatley RJ, Lemma S, Gauldie J, Marshall RP, Macdonald SJ. Dissecting fibrosis: therapeutic insights from the small-molecule toolbox. *Nat Rev Drug Discov* 2015;14:693–720.
- Travers JG, Kamal FA, Robbins J, Yutzey KE, Blaxall BC. Cardiac fibrosis: the fibroblast awakens. *Circ Res* 2016;118:1021–1040.
- Singh T, Sandulache VC, Otterson TD, et al. Subglottic stenosis examined as a fibrotic response to airway injury characterized by altered mucosal fibroblast activity. *Arch Otolaryngol Head Neck Surg* 2010;136:163–170.
- Sztano B, Torkos A, Rovo L. The combined endoscopic management of congenital laryngeal web. *Int J Pediatr Otorhinolaryngol* 2010;74:212–215.
- Rovo L, Jori J, Brzozka M, Czigner J. Minimally invasive surgery for posterior glottis stenosis. *Otolaryngol Head Neck Surg* 1999;121:153–156.
- Rovo L, Venczel K, Torkos A, Majoros V, Sztano B, Jori J. Endoscopic arytenoid lateropexy for isolated posterior glottic stenosis. *Laryngoscope* 2008;118:1550–1555.
- Sztano B, Szakaacs L, Madani S, Toth F, Bere Zs, Castellanos PF, Rovo L. Comparison of endoscopic techniques designed for posterior glottic stenosis: a cadaver morphometric study. *Laryngoscope* 2014;124:705–710.
- Karsagiannis C, Velehorschi V, Obertrifler B, Macha HN, Linder A, Freitag L. High-level expression of matrix-associated transforming growth factor-beta1 in benign airway stenosis. *Chest* 2006;129:1298–1304.
- Simpson CB, James JC. The efficacy of mitomycin-C in the treatment of laryngotracheal stenosis. *Laryngoscope* 2006;116:1923–1925.
- Xi-Qiao W, Ying-Kai L, Chun Q, Shu-Liang L. A review of the effectiveness of antimitotic drug injections for hypertrophic scars and keloids. *Ann Plast Surg* 2009;63:688–692.
- Güven M, Turan F, Eyihibilen A, Akbas A, Erorkmaz U. A comparison of the efficacy of 5-fluorouracil/triamcinolone, carnitine and dexamethasone therapy on wound healing in tracheal injury: potential for preventing tracheal stenosis? *Eur Arch Otorhinolaryngol* 2012;269:201–206.
- Bast F, Risteska K, Jovanovic S, Sedlmaier B. The topical application of mitomycin C in the treatment of scar formation and stenosis in hollow organs of the head and neck: a field report. *Laryngorhinootologie* 2009;88:528–533.
- Camargo PA, Campos AC, Matias JE, Rispoli DZ, Przyssieny PE, Fonseca VR. Topical mitomycin C effect on swine vocal folds healing. *Braz J Otorhinolaryngol* 2006;72:601–604.
- Chen T, Kunnavatanas SS, Koch RJ. Effects of mitomycin-C on normal dermal fibroblasts. *Laryngoscope* 2006;116:514–517.
- Porter GT, Gadre SA, Calhoun KH. The effects of intradermal and topical mitomycin C on wound healing. *Otolaryngol Head Neck Surg* 2006;135:56–60.
- Kumar V, Ali MJ, Ramachandran C. Effect of mitomycin-C on contraction and migration of human nasal mucosa fibroblasts: implications in dacryocystorhinostomy. *Br J Ophthalmol* 2015;99:1295–1300.
- Roh JL, Koo BS, Yoon YH, Rha KS, Park CI. Effect of topical mitomycin C on the healing of surgical and laser wounds: a hint on clinical application. *Otolaryngol Head Neck Surg* 2005;133:851–856.
- Roediger FC, Orloff LA, Courey MS. Adult subglottic stenosis: management with laser incisions and mitomycin-C. *Laryngoscope* 2008;118:1542–1546.
- Morishima Y, Nomura A, Uchida Y, et al. Triggering the induction of myofibroblast and fibrogenesis by airway epithelial shedding. *Am J Respir Cell Mol Biol* 2001;24:1–11.
- Jester JV, Huang J, Fisher S, et al. Myofibroblast differentiation of normal human keratocytes and hTERT, extended-life human corneal fibroblasts. *Invest Ophthalmol Vis Sci* 2003;44:1850–1858.
- Kakudo N, Kushida S, Suzuki K, Ogura T, Notodihardjo PV, Hara T, Kusumoto K. Effects of transforming growth factor-beta1 on cell motility, collagen gel contraction, myofibroblastic differentiation, and extracellular matrix expression of human adipose-derived stem cell. *Hum Cell* 2012;25:87–95.
- Caja L, Dituri F, Mancarella S, et al. TGF- β and the tissue microenvironment: relevance in fibrosis and cancer. *Int J Mol Sci* 2018;19:1–24.
- Hansen JK, Theibaull SL. Current understanding and review of the literature: vocal fold scarring. *J Voice* 2006;20:110–120.
- Melkane AE, Matar NE, Haddad AC, et al. Management of postintubation tracheal stenosis: appropriate indications make outcome differences. *Respiration* 2010;79:395–401.
- Hosjoe DK, Franklin SW, Aarstad RF, Day TA, Stucker FJ. Posterior glottic stenosis mechanism and surgical management. *Laryngoscope* 1997;107:675–679.
- Colak N, Nazli Y, Tasoglu I, et al. The effect of Mitomycin-C in reducing pericardial adhesion after cardiac surgery in rabbits. *Can J Cardiol* 2013;29:712–717.
- Schoenberg ED, Blake DA, Swann FB, et al. Effect of two novel sustained-release drug delivery systems on bleb fibrosis: an in vivo glaucoma drainage device study in a rabbit model. *Transl Vis Sci Technol* 2015;4:4.
- Meyenberg A, Goldblum D, Zingg JM, et al. Tocotrienol inhibits proliferation of human Tenon's fibroblasts in vitro: a comparative study with

