

Investigations into the dynamic uptake of extracellular vesicles and their role in breast cancer metastasis after chemotherapy

Paschalia Pantazi

A thesis submitted in partial fulfilment of the requirements of Oxford Brookes University
for the degree of Doctor of Philosophy

March 2019

Author's Declaration

I, Paschalia Pantazi, declare that this thesis titled 'Investigations into the dynamic uptake of extracellular vesicles and their role in breast cancer metastasis after chemotherapy' and submitted for assessment is an original work of my own. Any works of other authors used within this thesis or the work presented in it are properly acknowledged. A list of the references employed is included.

Abstract

Extracellular vesicles (EVs) are small lipid bound spherical structures released by almost all types of cells. They are mediators of intercellular communication, however, the exact mechanisms of EV-cell interactions and uptake have not been fully characterised. EVs play key roles in cancer including all the stages of metastasis. Breast cancer is amongst the most common types of cancer for women and the primary cause of cancer related death. The high mortality rates are due to the metastatic spread of cancer cells and tumour recurrence after therapy. Studies have proposed chemotherapy to be a double-edged sword, which in some cases can promote metastasis. Here, it was hypothesised that EVs from chemotherapy treated cells were able to induce metastasis-related capacities to naïve cells. The aims of this project were: a) to explore the dynamics and specificity of EV uptake by recipient cells, and b) to investigate the effect of chemotherapy induced intercellular communication via EVs on breast cancer metastasis.

To study EV uptake, different methods of EV labelling were tested. It was observed that PKH26 labelling may result in false-positive signals due to micelle formation. EV labelling with CFSE also produced non-specific effects, while dual labelling with both PKH26 and CFSE resulted in EV populations with heterogeneous labelling. EVs bearing a fluorescently tagged EV marker (mEmerald-CD81) were preferential for experiments investigating EV dynamics, as labelling appeared to be more specific than the dyes previously tested. Using this type of labelled EVs, it was shown that EV uptake differs across cell lines and the EV origin affects docking on the plasma membrane. Using a range of uptake inhibitors, it was observed that cholesterol may be involved in early EV-plasma membrane interactions, thus affecting EV uptake. Single molecule tracking revealed two patterns of such interactions: short and fast, and long and slow. To study the effect of EVs after chemotherapy, docetaxel and mitomycin C, two anti-cancer agents used for the treatment of breast cancer, were employed. Chemotherapy-treated cells released significantly higher numbers of EVs compared to non-treated cells. EVs from chemotherapy-treated cells did not induce any pro-metastatic effects (increased motility and invasiveness, EMT, and ability to adhere to endothelial cells), although changes in the glycosylation patterns of recipient cells were observed.

Overall, these results: highlight the variability amongst different EV labelling methods; suggest a role for cholesterol in early EV-membrane interactions and specificity of uptake; and indicate that chemotherapy-derived EVs may confer glycosylation changes but not pro-metastatic effects in recipient cells.

Σύνοψη

Εξωκυτταρικά σωματίδια (ΕΣ) ονομάζονται οι μικρές λιπιδικές σφαιρικές δομές εκκρινόμενες από όλους τους τύπους κυττάρων. Είναι μεσολαβητές της διακυτταρικής επικοινωνίας, ωστόσο, οι μηχανισμοί αλληλεπίδρασης ΕΣ-κυττάρων δεν έχουν χαρακτηριστεί πλήρως. Τα ΕΣ παίζουν σημαντικό ρόλο στον καρκίνο και την μετάσταση. Ο καρκίνος του μαστού είναι ένας από τους συνηθέστερους τύπους καρκίνου στις γυναίκες. Τα υψηλά ποσοστά θνησιμότητας οφείλονται στη μεταστατική εξάπλωση των καρκινικών κυττάρων και στην επανεμφάνιση όγκων μετά τη θεραπεία. Μελέτες έχουν δείξει ότι η χημειοθεραπεία μπορεί να αποδειχθεί δίκικοπο μαχαίρι, και σε κάποιες περιπτώσεις να ευνοεί τη μετάσταση. Υποθέσαμε, λοιπόν, ότι ΕΣ απο κύτταρα που υποβλήθηκαν σε χημειοθεραπεία είναι ικανά να προάγουν μετάσταση. Οι στόχοι αυτής της μελέτης ήταν: α) η διερεύνηση των τρόπων επικοινωνίας μεταξύ ΕΣ και κυττάρων, και β) η διερεύνηση της επίδρασης της προκαλούμενης από χημειοθεραπεία διακυτταρικής επικοινωνίας, μέσω ΕΣ, στην μετάσταση του καρκίνου του μαστού.

Για να μελετηθεί η πρόσληψη ΕΣ απο κύτταρα-στόχους, δοκιμάστηκαν διαφορετικές μέθοδοι επισημάνσεως. Παρατηρήθηκε ότι η χρώση με PKH26 μπορεί να οδηγήσει σε ψευδώς θετικά αποτελέσματα λόγω σχηματισμού μικκυλίων από την ίδια τη χρωστική. Η επισημάνση των ΕΣ με CFSE παρήγαγε επίσης μη ειδικά αποτελέσματα, ενώ η διπλή χρώση με PKH26 και CFSE παρήγαγε πληθυσμούς ΕΣ με ετερογενή επισημάνση. ΕΣ επισημασμένα με φθορίζουσα πρωτεΐνη (mEmerald-CD81) προτιμήθηκαν για τα παραπέρα πειράματα. Δείξαμε ότι η πρόσληψη ΕΣ διαφέρει μεταξύ των κυτταρικών τύπων και η κυτταρική προέλευση των ΕΣ επηρεάζει την πρόσδεσή τους στη μεμβράνη των κυττάρων-στόχων. Χρησιμοποιώντας μια σειρά από αναστολείς πρόσληψης ΕΣ, παρατηρήθηκε ότι η χοληστερόλη μπορεί να εμπλέκεται στις πρώιμες αλληλεπιδράσεις ΕΣ-μεμβράνης, επηρεάζοντας έτσι την πρόσληψή τους. Ανακαλύψαμε δύο ειδών αλληλεπιδράσεις: σύντομες και μεγαλύτερης διάρκειας. Για τη μελέτη των ΕΣ μετά τη χημειοθεραπεία, χρησιμοποιήθηκαν δυο αντικαρκινικά φάρμακα, docetaxel και mitomycin C. Τα κύτταρα που υποβλήθηκαν σε χημειοθεραπεία απελευθέρωσαν υψηλότερα ποσοστά ΕΣ σε σύγκριση με τα μη υποβληθέντα σε αγωγή κύτταρα. Τα ΕΣ από κύτταρα που υποβλήθηκαν σε χημειοθεραπεία δεν προκάλεσαν προ-μεταστατικές ιδιότητες, αν και παρατηρήθηκαν αλλαγές στα πρότυπα γλυκοζυλίωσης των κυττάρων-στόχων.

Συνολικά, τα αποτελέσματα αυτά: υπογραμμίζουν την ανομοιογένεια μεταξύ των διαφορετικών μεθόδων επισημάνσεως των ΕΣ, υποδηλώνουν τη σημαντικότητα της χοληστερόλης στις πρώιμες αλληλεπιδράσεις των ΕΣ με την πλασματική μεμβράνη και την ειδικότητα στην περαιτέρω πρόσληψή τους, και υποδεικνύουν ότι τα προερχόμενα από χημειοθεραπεία ΕΣ μπορούν να προκαλέσουν αλλαγές στη γλυκοζυλίωση αλλά όχι προ-μεταστατικές ιδιότητες στα κύτταρα-στόχους.

Acknowledgements

I would like to express my gratitude to Oxford Brookes University and Prof Nigel Groome for providing generous funding for my research project. I am also grateful to my supervisors Prof Susan Brooks, Prof Dave Carter and Prof John Runions, for their continuous guidance and support in accomplishing this thesis. A big thank you to past and present members of the #carterpinklab for all their technical and emotional support, they made this experience fun. I would also like to thank all the collaborators mentioned in this thesis, Dr Lakis Liloglou, Dr Justin Hean and especially Dr Jorge Bernardino de la Serna, for sharing their expertise, resources, and facilities.

Thank you to my family and particularly my parents for their love and patience, my sister for her support during the first months of adjustment to the PhD life, my brother for helping with image analysis during his summer break, and my grandma for the sleepless hours she spent with me while I was writing this thesis. I would also like to thank all my friends from the UK (special mention to Amelia and Raquel) and Greece for their kindness and support. And a special thank you to Bianca, for sharing this PhD journey with me from day one.

Ευχαριστίες

Θα ήθελα πρωταρχικά, να ευχαριστήσω το Πανεπιστήμιο Oxford Brookes και τον Prof Nigel Groome για την παροχή γενναιόδωρης χρηματοδότησης για το ερευνητικό μου έργο. Είμαι επίσης ευγνώμων στους προϊστάμενους καθηγητές μου Prof Susan Brooks, Prof Dave Carter, και Prof John Runions, για τη συνεχή καθοδήγηση και υποστήριξή τους στην υλοποίηση αυτής της διατριβής. Ένα μεγάλο ευχαριστώ σε πρώην και νυν μέλη του #carterpinklab για όλη την τεχνική και ψυχολογική υποστήριξη· έκαναν αυτή την εμπειρία διασκέδαση! Θα ήθελα επίσης να ευχαριστήσω όλους τους συνεργάτες που αναφέρονται στην παρούσα εργασία, Dr Lakis Liloglou, Dr Justin Hean και ιδιαίτερα τον Dr Jorge Bernardino de la Serna, που μοιράστηκαν μαζί μου την εμπειρία τους, πόρους, και εγκαταστάσεις.

Ευχαριστώ την οικογένειά μου και ειδικά τους γονείς μου για την αγάπη και υπομονή τους, την αδερφή μου για την υποστήρξή της κατά τους πρώτους μήνες της προσαρμογής μου στην Οξφόρδη, τον αδερφό μου για την βοήθειά του με την ανάλυση των εικόνων κατά τη διάρκεια των διακοπών του, και τη γιαγιά μου για τις ατέλειωτες ώρες που έμεινε ξάγρυπνη κρατώντας μου συντροφιά κατά το γράψιμο αυτής της διατριβής ειδικά, και καθ'όλης της διάρκειας των σπουδών μου γενικά. Θα ήθελα επίσης να ευχαριστήσω όλους τους φίλους μου από την Αγγλία (ειδικά την Amelia και την Raquel) και την Ελλάδα για την καλοσύνη και την υποστήριξη που μου έδειξαν. Ιδιαίτερο ευχαριστώ στην Bianca που μοιράστηκε αυτό το ταξίδι του διδακτορικού μαζί μου από την πρώτη μέρα.

For grandma

Για τη γιαγιά

Table of Contents

Abstract.....	iii
Σύνοψη.....	iv
Acknowledgements.....	v
Ευχαριστίες.....	vi
Table of Figures.....	xiii
Table of tables.....	xv
Abbreviations.....	xvii
1 Introduction	3
1.1 Extracellular vesicles (EVs)	3
1.1.1 Definition and classification.....	3
1.1.2 EV biogenesis, cargo and release	4
1.1.3 EV uptake	6
1.2 Breast cancer	8
1.2.1 Incidence and mortality	8
1.2.2 Subtypes and treatment schemes	9
1.3 Metastasis.....	10
1.3.1 Metastasis driven by chemotherapy.....	12
1.3.2 The HPA as a marker of aggressive cancer	12
1.4 The role of EVs in cancer metastasis.....	13
1.5 Project aims and objectives	16
2 Materials and methods.....	21
2.1 Cell culture	21
2.1.1 Cell culture routine procedures	21
2.1.2 Production of stably transfected cell lines.....	24
2.2 EV preparations.....	26
2.2.1 EV isolation.....	26
2.2.2 EV characterisation	27
2.3 Drug treatments.....	29
2.3.1 EV uptake inhibitors.....	29
2.3.2 Chemotherapeutic agents.....	29
2.4 Gene expression.....	30
2.4.1 RNA extraction	30
2.4.2 DNase I treatment and reverse transcription	30
2.4.3 Real-time qPCR.....	31
2.5 Statistics and reproducibility.....	33
3 Optimisation of EV labelling.....	37

3.1 Background.....	37
3.1.1 PKH26	37
3.1.2 CFSE	38
3.1.3 mEmerald-CD81.....	38
3.2 Aim and objectives of chapter 3.....	39
3.3 Methods.....	40
3.3.1 EV labelling using PKH26	40
3.3.2 EV labelling using CFSE	41
3.3.3 EV labelling using a combination of PKH26 and CFSE	42
3.3.4 Labelling of EVs using a fluorescently tagged EV marker	42
3.4 Results	43
3.4.1 PKH26 labelled EVs are internalised by recipient cells.....	43
3.4.2 Use of BSA during PKH26 EV staining affects background fluorescence.....	44
3.4.3 Uptake of CFSE labelled EVs might be masked by dye carry-over	45
3.4.4 CFSE and PKH26 dual labelling of EVs does not increase specificity of uptake.....	46
3.4.5 Labelling diversity in EVs produced by cells stained with CFSE and PKH26	47
3.4.6 mEmerald-CD81 EVs are favourable for EV uptake studies	48
3.5 Discussion	50
3.6 Key findings.....	52
4 Investigations into the dynamic uptake of EVs	57
4.1 Background.....	57
4.2 Aim and objectives of chapter 4.....	59
4.3 Methods.....	60
4.3.1 Experimental setting.....	60
4.3.2 Image analysis.....	60
4.4 Results	62
4.4.1 Uptake depends on the recipient cell type	62
4.4.2 EV origin affects docking on the membrane of recipient cells.....	64
4.4.3. Dynasore and cholesterol oxidase treatment affects EV uptake in a variable manner depending on the cell and EV type.....	64
4.4.4 Lipids may be involved in EV-membrane interactions	65
4.4.5 Cholesterol on the membrane of target cells is involved in the early interactions with EVs	67
4.5 Discussion	70
4.6 Key findings.....	73
5 The effect of chemotherapy-induced EVs on breast cancer metastasis	77
5.1 Background.....	77
5.1.3 Methods to evaluate the different steps of the metastatic cascade <i>in vitro</i>	77
5.2 Aim and objectives of chapter 5.....	79

5.3 Methods.....	80
5.3.1 Viability / proliferation assay.....	80
5.3.2 NTA.....	81
5.3.3 Real-time qPCR for epithelial and mesenchymal marker expression.....	81
5.3.4 Matrigel® invasion assay.....	83
5.3.5 Cell migration / 'scratch' assay	85
5.3.6 Static adhesion assay	85
5.3.7 HPA labelling	86
5.4 Results.....	88
5.3.1 Calculating the sensitivity of T47D cells to docetaxel and mitomycin C.....	88
5.3.2 Characterising the model to study EVs from chemotherapy-treated cells	89
5.3.3 Chemotherapy-derived EVs do not affect the expression of EMT markers	91
5.3.4 Chemotherapy-derived EVs do not affect the migration rate of recipient cells.....	92
5.3.5 Chemotherapy-derived EVs do not increase invasiveness of recipient cells.....	93
5.3.6 Mitomycin C EVs decrease the ability of recipient cells to adhere to endothelial cells	94
5.3.7 Chemotherapy EVs alter HPA labelling levels	95
5.5 Discussion.....	97
5.6 Key findings.....	102
6 Final discussion	105
6.1 EV labelling.....	105
6.2 EV docking and uptake.....	108
6.3 The role of EVs in breast cancer metastasis after chemotherapy	110
6.4 Future directions.....	112
6.5 Concluding remarks	114
7 Appendices.....	117
Appendix 1: Supplementary material for chapter 2	117
Appendix 2: Supplementary material for chapter 4	119
Appendix 3: Supplementary material for chapter 5	122
8 References	125

Table of Figures

Figure 1.1 Subpopulations of extracellular vesicles.....	3
Figure 1.2 The metastatic cascade.....	11
Figure 2.1 Schematic representation of the mEmerald-CD81-10 plasmid used for stable transfection of T47D and hTERT-HME1 cells.	24
Figure 3.1 Schematic representation of the EV labelling methods used in this project.....	39
Figure 3.2 Schematic representation of the experimental procedure followed to assess the effect of BSA during PKH26 labelling of EVs.....	41
Figure 3.3 Uptake of PKH26 labelled EVs.....	44
Figure 3.4 The effect of BSA on PKH26 labelling of EVs.....	45
Figure 3.5 Confocal live cell imaging of CFSE labelled EV uptake.	46
Figure 3.6 Dual labelling of EVs using PKH26 and CFSE.	47
Figure 3.7 Indirect dual labelling of EVs.....	48
Figure 3.8 mEmerald-CD81 labelled EVs.....	49
Figure 4.1 EV counting and tracking.	62
Figure 4.2 Uptake of EVs by different cell types.	63
Figure 4.3 Uptake of different EV types.....	64
Figure 4.4 The effect of amiloride, dynasore and cholesterol oxidase on membrane EV numbers.	65
Figure 4.5 Increment of membrane EV change between early and late time-points.	67
Figure 4.6 Tracking of EVs on the plasma membrane of target cells.	69
Figure 5.1 Schematic representation of the viability / proliferation experiments.....	81
Figure 5.2 Optimization of multiplexed qPCR for the expression analysis of EMT markers.	82
Figure 5.3 Schematic representation of the Matrigel® invasion assay.....	84
Figure 5.4 Schematic representation of the HPA Alexa Fluor® 647 conjugate used in HPA labelling experiments.	87
Figure 5.5 Dose-response of T47D cells to docetaxel and mitomycin C.....	89
Figure 5.6 Overview of the experimental procedure to study the effect of chemotherapy-derived EVs.....	90
Figure 5.7 EVs from docetaxel and mitomycin C treated cells.	91
Figure 5.8 The effect of chemotherapy-derived EVs on EMT markers.....	92
Figure 5.9 The effect of chemotherapy-derived EVs on cell migration.	93
Figure 5.10 The effect of chemotherapy-derived EVs on cell invasion.	94
Figure 5.11 The effect of chemotherapy-derived EVs on the cells ability to adhere to the endothelial monolayer.....	95
Figure 5.12 The effect of chemotherapy-derived EVs on HPA labelling.....	96
Figure 7.1 Mycoplasma testing.....	117

Figure 7.2 Estimating the optimal concentration of the selective antibiotic.	118
Figure 7.3 Live cell confocal time lapse imaging of EV uptake.	119
Figure 7.4 Proportion of EVs on the membrane and in the cytoplasm over time.	120
Figure 7.5 Raw numbers of EVs on the membrane and in the cytoplasm.	121
Figure 7.6 EV release by chemotherapy-treated and control cells.	122
Figure 7.7 The effect of chemotherapy on EMT markers.....	122

Table of tables

Table 2.1 Characteristics of all the cells used in this work and details of growth conditions.	21
Table 2.2 Transfection mix components and quantities.....	26
Table 2.3 Components and quantities needed for the preparation of protein gels.	28
Table 2.4 Antibodies used in this study for the characterisation of EVs by western blot.	28
Table 2.5 DNase treatment reaction components and quantities.	31
Table 2.6 Reverse transcription components, quantities and thermal profile.....	31
Table 2.7 Primers, probes and assays used for expression analysis with real-time qPCR.....	32
Table 2.8 Real-time qPCR components, quantities and thermal profile.	33
Table 3.1 Published methods for the fluorescent labelling of EVs.	37
Table 7.1 Authentication of the cell lines used in this project.	117

Abbreviations

AKT	Protein kinase B
(c)DNA	(complementary) Deoxyribonucleic acid
(E)GFP	(Enhanced) green fluorescent protein
(m/r/t)RNA	(messenger/ribosomal/transfer) Ribonucleic acid
(q)PCR	(quantitative) Polymerase chain reaction
ACTB	Actin beta
ALIX	Alpha-1,3-mannosyltransferase-interacting protein X
Bcl-2	B-cell lymphoma 2 protein
<i>BRCA1/2</i>	Breast cancer type 1/2 susceptibility gene
BSA	Bovine serum albumin
CD63	Cluster of differentiation 63
CD81	Cluster of differentiation 81
CDH1	Cadherin-1
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CpG	Cytosine-phosphate-guanine
Cq	Quantification cycle
CTCF	Corrected total cell fluorescence
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxyribonucleotide triphosphate
ECL	Enhanced chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
EGF(R (vIII))	Epidermal growth factor (receptor (variant III))
EMT	Epithelial to mesenchymal transition
ESCRT	Endosomal sorting complex responsible for transport
EV	Extracellular vesicle
FasL	Fas-ligand
FBS	Foetal bovine serum
Gal	Galactosamine
GalNac	N-acetyl-galactosamine
GlcNac	N-acetyl-glucosamine
GP96	Glycoprotein-96
GTPase	Guanosine triphosphate hydrolase
HER2	Human epidermal growth factor receptor 2
hFGF-B	Human fibroblast-derived growth factor-B
HIV	Human immunodeficiency virus
HME1 ^{mCD81}	mEmerald-CD81 hTERT-HME1
HPA	<i>Helix pomatia</i> agglutinin
IC50	Half maximal inhibitory concentration
ILV	Intraluminal vesicle
ITGb4	Integrin subunit beta 4
MBG	Minor groove binder
MET	Mesenchymal to epithelial transition
MHC-I/II	Major histocompatibility complex I/II

MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVB	Multivesicular body
NFQ	Non fluorescent quencher
NTA	Nanoparticle tracking analysis
PALM	Photo-activated localization microscopy
Palm-	Palmitoylated
PBS	Phosphate buffered saline
PDK	Phosphoinositide-dependent kinase-1
R3-IGF1	Recombinant analogue of insulin growth factor
RAB	Ras-associated binding protein
RFP	Red fluorescent protein
RICS	Raster imaging correlation spectroscopy
RPM	Revolutions per minute
RPMI	Roswell park memorial institute medium
SDS-PAGE	Discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SNARE	Soluble N-ethylmaleimide sensitive fusion protein attachment receptor protein
STORM	Stochastic optical reconstruction microscopy
T47D ^{mCD81}	mEmerald-CD81 T47D
TIRF	Total internal reflection fluorescence microscopy
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TSG1	Tumor suppressor TSG1
uPA	Urokinase-type plasminogen activator
VEGF(R)	Vascular endothelial growth factor (receptor)

Measurement units

v/v	volume per volume
w/v	weight per volume
bp	basepair(s)
°C	Degrees Celcius
l	litre(s)
m	meter(s)
M	Molar
h	hour(s)
g	gram
G	Gravitational constant
min	minute(s)
sec	second(s)
Da	Dalton(s)

Metric prefixes

k	kilo; 10 ³
c	centi; 10 ⁻²

m	milli; 10^{-3}
μ	micro; 10^{-6}
n	nano; 10^{-9}

Introduction

Chapter 1

1 Introduction

1.1 Extracellular vesicles (EVs)

1.1.1 Definition and classification

Extracellular vesicles (EVs) are small membrane bound spherical structures, which carry and transport various types of biomolecules. They represent a means of intercellular communication, conserved among species – from prokaryotes to higher eukaryotes (Colombo *et al.*, 2014). In normal physiology but also in pathological conditions, EVs serve as multimolecular information carriers for near or distant cells (Yuana *et al.*, 2013). Their long distance transfer is reflected by their existence in body fluids such as blood (Caby *et al.*, 2005), urine (Pisitkun *et al.*, 2004), malignant effusions (Andre *et al.*, 2002) etc.

EVs are divided in three main categories based on their biogenesis: a) exosomes, b) microvesicles and c) apoptotic bodies (Figure 1.1). Exosomes derive from multivesicular endosomes inside the cell and their diameter ranges from 40 to 150 nm. Microvesicles arise from the cell membrane and are 50 nm to 1 μ m in diameter. Finally, apoptotic bodies are released by cells which undergo apoptosis and their size is evidently larger (500 – 2000 nm)(El-Andaloussi *et al.*, 2013). Because of the overlapping sizes of vesicles, the size-dependent methods to isolate them and the lack of specific EV markers, distinct populations of EVs are hard to obtain. The isolation methods used in this project allow the enrichment of small vesicles bellow 200 nm in diameter, which will be referred to as EVs throughout this thesis.

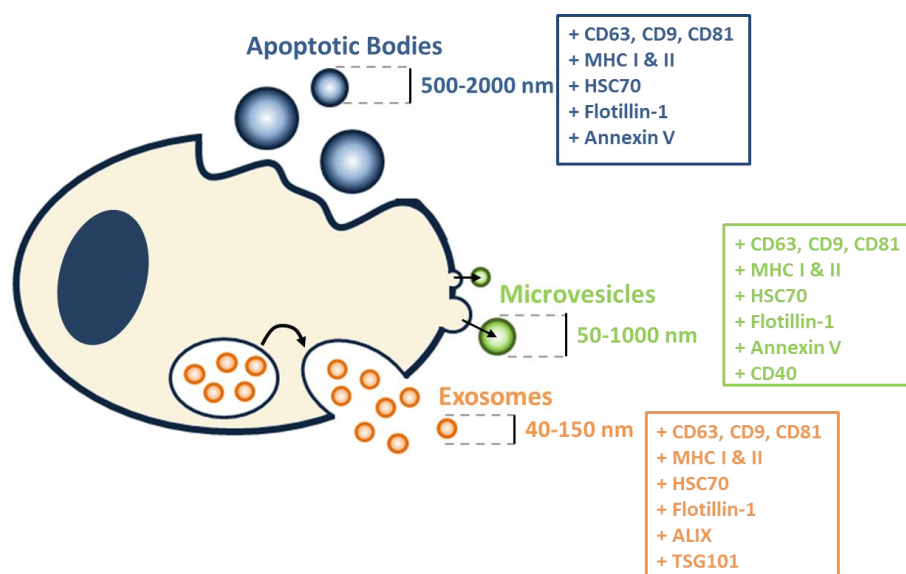


Figure 1.1 Subpopulations of extracellular vesicles.

Exosomes, microvesicles, apoptotic bodies, and some of their characteristic markers.

1.1.2 EV biogenesis, cargo and release

Biogenesis

Exosomes and microvesicles have distinct pathways of biogenesis. Exosomes are of endosomal origin, formed by invagination of the limiting endosomal membrane into intraluminal vesicles (ILVs), which give rise to multivesicular bodies (MVB) that fuse with the plasma membrane to release ILVs/exosomes into the extracellular environment (Thery *et al.*, 2002; Stoorvogel *et al.*, 1991). The mechanisms underlying exosome formation are not fully understood. The endosomal sorting complex responsible for transport (ESCRT) (Colombo *et al.*, 2013), lipids (e.g. ceramide) (Trajkovic *et al.*, 2008), and specific tetraspanins (CD63 and CD81) (van Niel *et al.*, 2011) appear to participate in exosome biogenesis and sorting of their cargo. These effectors act independently or reciprocally, depending on the cell type, EV type and cargo loaded (Lo Cicero *et al.*, 2015). Microvesicles derive from the outward budding of the plasma membrane (Raposo and Stoorvogel, 2013). Despite the distinct biogenesis pathways, many mechanistic elements involved in membrane-trafficking processes are common in exosome and microvesicle generation (Colombo *et al.*, 2014).

Cargo

EVs carry various functional molecules including proteins, lipids and nucleic acids. Their content can be varied and it does not purely reflect the content of the parental cell (Villarroya-Beltri *et al.*, 2014). On the contrary, the cargo is sorted in the vesicles in a coordinated way that can be either ESCRT-dependent or ESCRT-independent (Kowal *et al.*, 2014). Specific lipid structures and proteins like tetraspanins and chaperones are also involved in EV cargo loading (Perez-Hernandez *et al.*, 2013; Geminard *et al.*, 2004).

Proteins are present both on the surface and in the lumen of EVs (Graner *et al.*, 2009), and have been analysed with various methods such as mass spectrometry, western blot, flow cytometry and others (Haraszti *et al.*, 2016; Suarez *et al.*, 2017). Specific proteins are enriched in the different types of EVs and therefore used as EV markers. Extracellular vesicles usually contain multivesicular body markers like ALIX and TSG101, lipid raft associated proteins such as flotilin and clathrin, cell membrane proteins such as the tetraspanins CD81, CD9 and CD63 and the major histocompatibility complex I and II (MHC-I/II), and in a lesser extend endoplasmic reticulum and mitochondrial associated proteins such as GP96 and mitofilin respectively, (Thery *et al.*, 2006; Kowal *et al.*, 2016).

Lipids are also found in EVs. EV membrane composition is different from the plasma membrane of the parental cells. Lipids found in EVs include cholesterol and sphingomyelin that provide rigidity and support. Ceramide is also found in EVs and has been associated with the biogenesis and release of vesicles (Skotland *et al.*, 2017).

Different types of RNA molecules exist in EVs (Perez-Boza *et al.*, 2018; Fabbri *et al.*, 2012). Up to the time of this thesis, mRNA, microRNA, long non-coding RNA, rRNA, tRNA, vault- and Y-RNA have been found in EVs (Yanez-Mo *et al.*, 2015). Several non-coding RNAs have been reported to confer drug resistance (Chen *et al.*, 2014b; Xiao *et al.*, 2014) or aggressive phenotype (Donnarumma *et al.*, 2017) to intact recipient cells. This sort of horizontal transfer seems to be common in EV-mediated intercellular communication.

Double stranded DNA has also been found in EVs from plasma or cultured cells. Studies have shown that DNA from EVs can be passed on to recipient cells and localise to the nucleus, where it is transcribed (Cai *et al.*, 2013). It has been reported that EV DNA from tumour cells can trigger the activation of specific pathways in recipient dendritic cells, thus modulating tumour immunity (Kitai *et al.*, 2017). Furthermore, Al-Nedawi *et al.* (2008) showed that astrocytoma cells overexpressing the oncogenic epidermal growth factor receptor variant III (EGFRvIII) are able to enhance aggressiveness of glioma cells by transferring EGFRvIII through microvesicles.

Microarray studies have shown that EVs bear highly glycosylated epitopes that in many cases differ from those of the parental cell (Batista *et al.*, 2011). Moreover, different types of EVs contain moieties with different glycosylation patterns that might have a role in their uptake by target cells (Escrevente *et al.*, 2011). EVs may also carry lectins, proteins that recognise and interact with carbohydrate moieties. Welton *et al.* (2010) showed that EVs from bladder cancer cells contain galectin-3, a lectin that is involved in cell-cell adhesion, cell-matrix interactions, metastasis, etc. (Dumic *et al.*, 2006). The knowledge for the existence of this lectin in EVs arose from proteomics analyses of vesicular contents therefore the role of lectins in EVs has not been elucidated yet.

Release

Microvesicles pinch off the plasma membrane into the extracellular milieu (Raposo and Stoorvogel, 2013). On the other hand, exosomes that derive from intracellular membranes have a different secretion route, involving the fusion of the MVBs with the plasma membrane that leads to the release of ILVs/exosomes to the extracellular space. Members of the RAB GTPase family of proteins, such as RAB11, RAB27, and RAB35 have been implicated in transfer of MVBs to the plasma membrane and subsequent exosome secretion (Kowal *et al.*, 2014; Hsu *et al.*,

2010). Soluble N-ethylmaleimide sensitive fusion protein attachment receptor proteins (SNAREs) assist the final step of exosome release - the fusion of MVBs with the cell membrane (Jahn and Scheller, 2006).

1.1.3 EV uptake

The way that EVs are taken up by recipient cells is still not entirely understood. Internalization of EVs can be studied directly, by monitoring labelled EVs using microscopy, or indirectly, by assessing the changes they confer to recipient cells. So far, three mechanisms to describe EV communication with target cells have been suggested: endocytosis, cell surface membrane fusion and soluble/juxtacrine signalling (Mulcahy *et al.*, 2014; McKelvey *et al.*, 2015).

Endocytosis

Endocytosis is a broad term that covers a plethora of distinct endocytic routes (Hupalowska and Miaczynska, 2012). EVs can be taken up by target cells via different endocytic pathways. During clathrin-mediated endocytosis, EVs interact with specific clathrin-rich domains on the cell membrane called clathrin pits and are then internalised. Subsequently, the clathrin coat is dismantled and the internalised particle fuses with the appropriate cell compartment/endosome (Kirchhausen, 2000). Inhibition of the clathrin-mediated endocytosis pathway has been shown to lower the levels of EV uptake in ovarian cancer cells (Escrevente *et al.*, 2011). Moreover, several studies have shown that inhibition of crucial for the clathrin-mediated endocytic route factors dramatically decreased EV uptake, highlighting the importance of this type of endocytosis in EV uptake (Tian *et al.*, 2014; Barres *et al.*, 2010).

EVs can be also taken up by recipient cells via caveolin-dependent endocytosis. Caveolae are a special type of glycolipid rafts on the plasma membrane and, like clathrin pits, remodel the plasma membrane in a unique way in order to internalise EVs and viruses (Anderson, 1998; Nabi and Le, 2003). There are controversial data on caveolin-dependent endocytosis of EVs. Blocking Dynamin2, or other important players of the caveolin-dependent endocytic route, inhibits EV uptake in several studies (Newton *et al.*, 2006; Nanbo *et al.*, 2013; Inder *et al.*, 2014). On the other hand, suppression of caveolin-1 protein expression, showed either no effect on EV uptake levels (Tian *et al.*, 2014) or increased internalisation of EVs (Svensson *et al.*, 2013). These results suggest that multiple mechanisms might be responsible for the uptake of EVs and further research is needed to elucidate the role of specific endocytic pathways like the caveolin-dependent endocytosis.

Macropinocytosis is an actin-dependent form of endocytosis and is clathrin-independent. It is portrayed as the formation of surface lipid-bilayer ruffles that fuse with the membrane creating irregular shaped macropinosomes into the cytosol (Lim and Gleeson, 2011). A study by Fitzner *et al.* (2011) shows that EVs from oligodendrocytes can be internalised by microglial cells through macropinocytosis, and using different inhibitors of the macropinocytosis pathway, this group managed to limit EV uptake. A more recent study has shown that delivery of miR-21 (cancer-related microRNA) to recipient cells is achieved via macropinocytosis of tumour-derived EVs (Tian *et al.*, 2014). On the contrary, Nanbo *et al.* (2013) using a macropinocytosis inhibitor, showed that micropinocytosis was not involved in the uptake of Epstein Barr virus positive Hodgkin lymphoma EVs by nasopharyngeal carcinoma cells.

Another mechanism of EV uptake is through phagocytosis. Phagocytosis can be performed both by professional immune cells like macrophages but also by non-professional cells (all nucleated body cells). Feng *et al.* (2010) demonstrated that EVs can be effectively internalised by phagocytic cells, but the levels of uptake drop for non-phagocytic cells. Inhibition of Dynamin2 (phagocytosis regulator) leads to exceptionally low levels of EV uptake (Feng *et al.*, 2010).

Subdomains of the cell membrane with high amounts of cholesterol and sphingolipids, called lipid rafts, control protein trafficking by adjusting the membrane fluidity/fluency (Nicolson, 2015). Flotilins (EV marker proteins) have been shown to bind GPI-anchored proteins on the lipid rafts during internalisation of EVs (Glebov *et al.*, 2006; Frick *et al.*, 2007). It has been demonstrated that lipid-reducing agents can interrupt EV uptake (Montecalvo *et al.*, 2012; Svensson *et al.*, 2013; Koumangoye *et al.*, 2011).

Cell surface membrane fusion

Vesicles can dock to the membrane of the recipient cell and then fuse with it, delivering their content into the cytoplasm. This fusion can be mediated by proteins or lipids on the membrane, but unlike endocytic pathways, it has not been extensively studied. Parolini *et al.* (2009) showed that melanoma tumour-derived EVs fuse easier with primary cells from metastatic lesions than with normal primary melanoma cells in a pH dependent manner. Tetraspanins seem to be important for many stages of EV biogenesis, release and uptake. Inhibition of CD81 and CD9 (EV tetraspanin markers) leads to decreased levels of EV uptake (Morelli *et al.*, 2004). Integrins and immunoglobulins (Morelli *et al.*, 2004; Hwang *et al.*, 2003), glycoproteins (Christianson *et al.*, 2013) and lectins (Naslund *et al.*, 2014) have been associated with EV-membrane fusion events.

Juxtacrine signalling

Juxtacrine signalling is the contact-dependent signalling that allows signal transduction through diffusion or via the formation of ‘tunnels’ where the signalling molecules can pass through. This type of signalling is more prevalent in apoptotic bodies and involves the cleavage of FasL and TRAIL that then act as a soluble effector that can bind to target cells (Schneider and Tschopp, 2000). EVs from cancer cells can induce apoptosis in cells of the immune system in this manner (Wieckowski *et al.*, 2009).

Heterogeneity in EV uptake

Several mechanisms of EV-mediated communication have been proposed. There is little agreement in the literature as to which type of EV uptake is prevalent, however, this variability among studies might be reflective of the heterogeneity in both the EV and cell types employed. It has been suggested that multiple mechanisms might act simultaneously (Mulcahy *et al.*, 2014). Moreover, different endocytic pathways share common regulators. For example, Dynamin2 is involved in both caveolin-dependent endocytosis and phagocytosis (Hinshaw, 2000; Gold *et al.*, 1999). Therefore, to identify pathways involved in EV uptake, a careful selection of inhibitors should be made. Furthermore, undiscovered pathways and mechanisms of EV uptake might exist.

One of the reasons for the variability observed might be technical and reflect the different experimental settings. For example, different isolation methods can result in heterogeneous preparations of vesicles (Li *et al.*, 2017). Also, (where applicable) the EV labelling method might influence the outcome of the study (Puzar Dominkus *et al.*, 2018). Overall, more studies are needed to elucidate the mechanisms of EV uptake in the different biological settings.

1.2 Breast cancer

1.2.1 Incidence and mortality

Approximately 360,000 cases of cancer were diagnosed in 2015 in the UK (Cancer Research UK 2018). Breast cancer is the most common type of cancer in women in the western world and the second most common cancer (after lung cancer) worldwide (Hutchinson, 2010). Despite advances in early detection and treatment, breast cancer still accounts for one-sixth of cancer-related deaths according to European metrics (Ferlay *et al.*, 2013).

1.2.2 Subtypes and treatment schemes

The majority of breast cancers (approximately 95%) are carcinomas arising from transformed epithelial cells (Tomaskovic-Crook *et al.*, 2009), while the other 5% represent cancers that are caused by hereditary mutations in the *BRCA1* and *BRCA2* genes (Malone *et al.*, 1998). Environmental factors such as oestrogens appear to promote the onset of the disease (Hilakivi-Clarke, 2000). Breast cancer risk increases with age, as mutations and gene defects accumulate. Almost a third of all breast cancer cases each year in the UK are attributed to major lifestyle and other risk factors (Cancer Research UK 2018).

Based on expression profiling, three main types of breast cancer can be defined: hormone receptor positive; human epidermal growth factor receptor 2 (HER2) positive; and triple-negative (Sorlie *et al.*, 2001). Almost 70% of breast cancers express oestrogen and progesterone receptors and therefore are considered hormone-receptor positive (Dunnwald *et al.*, 2007). Less than one third of breast cancers overexpress HER2 (Dawood *et al.*, 2010). Triple-negative cancers are hormone receptor and HER2 negative, and account for 10 to 20% of all breast cancers (Dawood, 2010).

The different subtypes of breast cancer require different treatment approaches (Senkus *et al.*, 2013). For example, cancers that express hormone receptors are usually found in older women and have better prognosis than the other two types (Senkus *et al.*, 2013). These cancers are primarily treated with endocrine therapy, which counteracts the stimulatory effects of hormones on cancer cell growth. Chemotherapy might also be administered to these patients usually in combination with endocrine therapy (e.g. tamoxifen) (Dunnwald *et al.*, 2007; Senkus *et al.*, 2013). Targeted drugs have been developed against key modulators of tumour growth and survival such as the mammalian target of rapamycin (mTOR) (Baselga *et al.*, 2009) and HER2 (Slamon *et al.*, 2001). Triple-negative cancers are more prevalent in younger women and are associated with poor prognosis (Dawood, 2010). Conventional chemotherapy is used for triple-negative breast cancers, for cancers that have acquired resistance and do not respond to targeted therapy, or in combination with target-based treatments (Chavez *et al.*, 2010; Brenton *et al.*, 2005). Docetaxel and mitomycin C are two chemotherapeutic agents broadly used in the clinic for the treatment of breast cancer (Pacheca-Palomar, 2009 ; Esteva, 2002).

Docetaxel

Docetaxel is a second generation taxane originally isolated from the European yew tree. It is an antitumour agent commonly used for the treatment of locally advanced or metastatic breast cancer. It is highly effective against tumours that are resistant to other agents such as

anthracyclines (Esteve, 2002). Docetaxel belongs to the drug class taxanes. It binds to and stabilises microtubules and at the same time prevents their depolymerisation. This halts mitosis, eventually leading to apoptosis (Eisenhauer and Vermorken, 1998). Docetaxel also promotes phosphorylation and further inhibition of the anti-apoptotic Bcl-2 oncoprotein, thus encouraging apoptosis (Kraus *et al.*, 2003).

Mitomycin C

Mitomycin C is an antitumour antibiotic, originally found in cultures of *Streptomyces caespitosus* (Szybalski and Iyer, 1964), broadly used for the treatment of oesophageal, anal, breast and bladder cancer. It is usually administered as a palliative therapy in patients with metastatic breast cancer and frequently in combination with other chemotherapeutic agents. Its cytotoxic properties are due to its ability to cause DNA inter- and intra-strand crosslinks at cytosine-phosphate-guanine (CpG) sites, thus affecting DNA replication and other vital for the cell processes, eventually leading to apoptosis (Warren *et al.*, 2001).

1.3 Metastasis

Metastasis is a complex process that can be divided into sequential steps (Figure 1.2) (Brooks *et al.*, 2010; Nicolson, 2015). At the primary tumour site, low oxygen levels lead to the formation of new blood vessels, the nutrient and oxygen suppliers that also serve as a pathway to other sites in the body (Bergers and Benjamin, 2003). The next step in the metastatic cascade is the dissemination of cells from the primary tumour. This process requires abrogation of cell-cell and cell-basement membrane adhesion that occurs through changes in the expression of adhesion molecules such as integrins, cadherins, and catenins (Byers *et al.*, 1995). Epithelial to mesenchymal transition (EMT) allows cancer cells to acquire a more motile phenotype, by losing their polarity, enabling them to travel more effectively. As they acquire a more mesenchymal phenotype, tumour cells become resistant to anoikis, where epithelial cells that lose anchorage dependency are normally subjected to programmed cell death (Frisch *et al.*, 2013). Tumour cells with increased motility can degrade the extracellular matrix by releasing metalloproteinases (Savagner, 2001). Subsequently, the cells degrade and invade the local basement membrane and enter neighbouring tissues. Cell protrusions, such as invadopodia aid motility of tumour cells (Friedl and Wolf, 2010). Then, cells break through the blood vessel walls (intravasation) and move into the circulation. For the attachment to the endothelium, the role of specific adhesion molecules expressed by both the tumour cells and endothelial cells is vital. Three mechanisms have been proposed according to which, tumour cells permeabilise the endothelium; a) abrogation of cell-cell junctions, b) passage through/within an endothelial cell, and c) use of

reactive oxygen species to produce a permeant perforation in the endothelium (Tremblay *et al.*, 2008). In the circulation, tumour cells need to survive blood flow and escape immune attack until they find the capillary bed of the target organ site. Then, cells exit the blood vessel (using the same mechanisms as described above) and invade the local tissue to form a secondary tumour provided that the environment is suitable (Chambers *et al.*, 2002). A reversal of EMT may occur, where cells undergo mesenchymal to epithelial transition (MET), a process resulting in the initial epithelial cell phenotype (Yao *et al.*, 2011). Tumour cells often also utilise the lymphatic system to travel to distant sites, especially to neighbouring lymph nodes. However, it is not clear how the cells enter the lymphatic vessels and whether this process is tightly regulated (Pang *et al.*, 2016).

Metastasis is a milestone in cancer progression that dramatically raises the risk of death in cancer patients. More than 90% of cancer-related deaths are due to metastases, which are often resistant to therapy (Weigelt *et al.*, 2015). Metastasis can be halted if one of the sequential steps of the cascade illustrated in Figure 1.2 is not successfully completed (Poste and Fidler, 1980). Despite the great amount of research on the mechanisms of metastasis, there are still no established ways to block it.

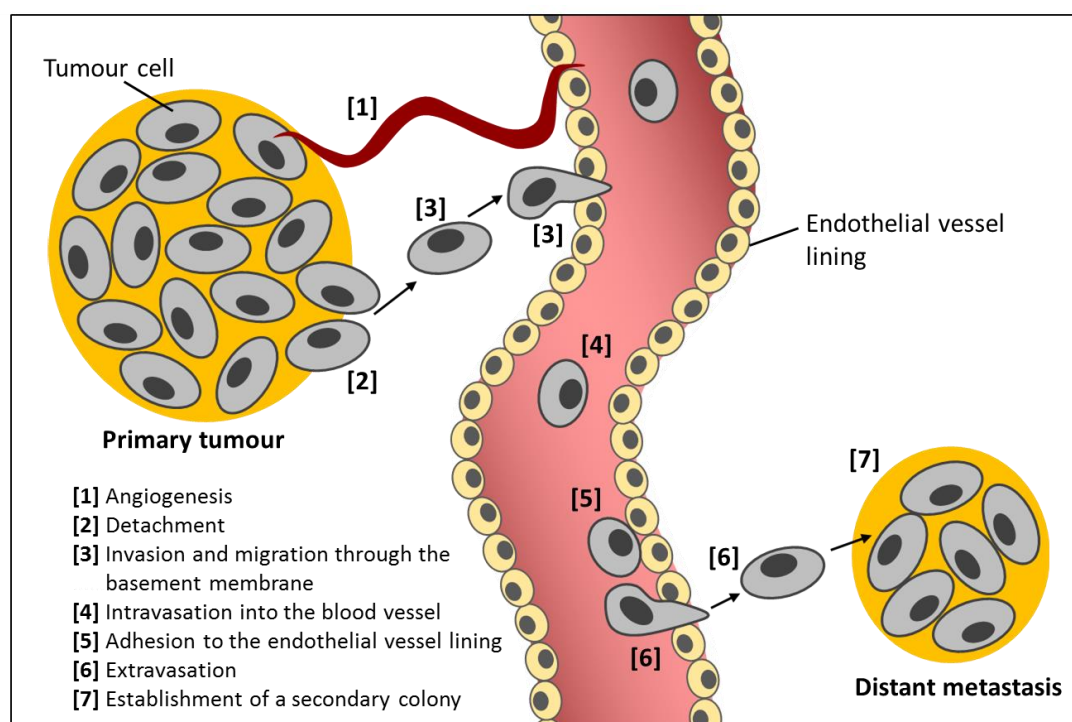


Figure 1.2 The metastatic cascade.

The distinct 'steps' that must be completed for cancer spread to occur. (1) Angiogenesis, development of a tumour blood supply. (2) Disaggregation of cancer cells from the primary tumour mass, followed by (3) invasion of the surrounding tissue. (4) Intravasation into the vasculature and (5) haematogenous dissemination. (6) Cancer cells can adhere to the vascular endothelium and (7) extravasate into the local tissue to form secondary tumour foci.

1.3.1 Metastasis driven by chemotherapy

Accumulating evidence shows that chemotherapy, under certain circumstances, may facilitate the metastatic spread of tumour cells, especially when administered pre-operatively (Karagiannis *et al.*, 2018). The cytotoxicity, tissue damage, hypoxic stress, and long wound healing-like processes caused by chemotherapy initiate pro-angiogenic and pro-metastatic effects, further contributing to metastatic dissemination (Volk-Draper *et al.*, 2014; Ratajczak *et al.*, 2013; Chang and Erler, 2014). Chemotherapy can also stimulate blood platelets to release microvesicles carrying adhesion receptors that can coat the circulating tumour cells, hence protect them from shear forces and allow extravasation and formation of secondary tumour (Wysoczynski and Ratajczak, 2009; Nierodzik *et al.*, 1991; McCarty *et al.*, 2000). Apart from the systemic reaction supporting metastasis, chemotherapy can also favour metastasis by altering intrinsic characteristics of tumour cells. For example, paclitaxel, a taxane based chemotherapeutic agent, has been reported to induce EMT in breast carcinoma indicated by upregulation of vimentin and downregulation of E-cadherin (Ren *et al.*, 2015). Different mechanisms of tumour metastasis have been attributed to different chemotherapeutic agents. For example, cisplatin and paclitaxel support spread of the disease by upregulating the VEGF receptor 1 in endothelial cells (Daenen *et al.*, 2011). An *in vivo* study showed that paclitaxel and carboplatin sped up lung metastasis in breast cancer-bearing mice, while doxorubicin and fluorouracil did not. The same study reported that paclitaxel and carboplatin caused these effects by inducing the production of cytokines and other angiogenic factors (Liu *et al.*, 2015). Finally, inflammation caused by chemotherapy (cisplatin, paclitaxel, fluorouracil and doxorubicin reviewed by Vyas *et al.* (2014)) can be one of the mechanisms responsible for metastasis.

1.3.2 The HPA as a marker of aggressive cancer

Lectins are proteins that bind non-covalently and reversibly to carbohydrates with high specificity and are ubiquitously present in nature. More specifically, a lectin is defined as a “protein or glycoprotein of non-immune origin, not an enzyme, that binds to carbohydrates and agglutinates cells” (Goldstein *et al.*, 1980). The *Helix pomatia* agglutinin (HPA) is a lectin with six carbohydrate binding sites that displays high specificity to N-acetyl-galactosamine (GalNAc) residues. Glycan structures with terminal GalNAc residues will be recognised by HPA. It also binds to N-acetyl-glucosamine (GlcNAc) and galactosamine (Gal) with lower specificity (Sanchez *et al.*, 2006).

HPA binding patterns have been used to predict outcome in various types of cancer including breast. HPA-positivity of tumour biopsies is well characterised and associated with metastatic

competence (Leathem and Brooks, 1987; Noguchi *et al.*, 1993; Brooks and Leathem, 1991). According to Brooks and Leathem (1991), breast cancer patients with HPA-negative tumours had worse disease prognosis compared with patients with HPA-positive tumours. Survival rates were lower for HPA-positive patients and there was a significant association between lymph node metastasis and HPA binding. However, both HPA positive and negative cell populations are found within tumours and interestingly, HPA positive tumours can result in both HPA positive and negative metastatic foci (Schumacher *et al.*, 1992; Brooks, 2000). Studies on cell lines have shown that aggressive cell lines exhibit higher amounts of HPA binding compared with less aggressive or non-malignant breast cells (Rambaruth *et al.*, 2012). Valentiner *et al.* (2005) transplanted cell lines exhibiting different levels of HPA binding into mice and tested their capacity to produce tumours. Cell lines with strong HPA-binding patterns were able to form primary and metastatic deposits in the mice, while cell lines with low HPA affinity did not engraft. *In vitro* studies have demonstrated that HPA-binding glycans were not involved in invasion (Brooks *et al.*, 2001), but were functionally involved in the cancer cells adhering to endothelium (Bapu *et al.*, 2016). Recent unpublished data obtained in our lab, suggest that cells show a plasticity regarding their HPA-binding by changing the synthesis of surface glycans during the metastatic cascade. The proposed model suggests that HPA-negative cells are able to detach from the primary tumour, invade the basement membrane, intravasate, and then, during haematogenous dissemination, alter their cell surface glycosylation in order to be able to adhere to vascular endothelium, before extravasating and migrating into tissues at a secondary site (Ellie Beaman, personal communication).

1.4 The role of EVs in cancer metastasis

EVs play important roles in cancer (Meehan and Vella, 2016) including metastasis (Green *et al.*, 2015; Chin and Wang, 2016; Suchorska and Lach, 2016). During this journey, cancer cells require the ancillary recruitment of other cells in the body to bypass the physical barriers (e.g. endothelium) or even to pave the way (pre-metastatic niche formation) towards the secondary site. EVs have emerged as pivotal players in all steps of the metastatic cascade (Meehan and Vella, 2016; Becker *et al.*, 2016)

Angiogenesis

Blood vessels are the nutrient and oxygen suppliers of an evolving tumour but also serve as a pathway to other sites in the body. The formation of new blood vessels is an intrinsic mechanism for tumour growth and progression, mediated by different cancer-derived signals (Bergers and Benjamin, 2003). A plethora of studies have shown that tumour-derived EVs induce angiogenesis

through different mechanisms (Lindoso *et al.*, 2015; Lopatina *et al.*, 2018; Lu *et al.*, 2018; Hsu *et al.*, 2017; Al-Nedawi *et al.*, 2009; Janowska-Wieczorek *et al.*, 2005; Dörsam *et al.*, 2018; Grange *et al.*, 2011). For instance, breast cancer cells release Annexin II containing EVs that initiate angiogenesis by stimulation of the tissue plasminogen activator (Maji *et al.*, 2017). Hypoxic tumour EV-derived miR-23a and miR-135b favour angiogenesis in lung and myeloid carcinomas respectively (Hsu *et al.*, 2017; Umezu *et al.*, 2014). Likewise, epidermoid carcinoma microvesicles harbour oncogenic EGFR that can stimulate an autocrine VEGFR-2 response in endothelial cells (Al-Nedawi *et al.*, 2009), promoting angiogenesis. Nevertheless, tumour EVs have been also shown to inhibit angiogenesis. Lu *et al.* (2018) recently showed that nasopharyngeal carcinoma EVs enclose miR-9 that reduces angiogenesis by directly targeting the heparin growth factor Midkine leading to inhibition of the PDK/AKT pathway. Similarly, four miRNAs (miR-15a, miR-18-1b, miR-320c, miR-874) enclosed in EVs from liver stem-like cells have been reported to inhibit angiogenesis by downregulating the expression of angiogenic surface receptors, growth factors, and the activity of the AKT signalling pathway (Lopatina *et al.*, 2018). Taken together, EVs from different types of cancer or even different cell types found within tumours can cause contradictory effects on angiogenesis.

Epithelial to mesenchymal transition (EMT)

Epithelial to mesenchymal transition is a dynamic process characterised by major changes in gene expression. Genes that determine the mesenchymal phenotype, such as vimentin and N-cadherin, are highly expressed, while the epithelial phenotype is suppressed (Moreno-Bueno *et al.*, 2008). Proteomics analyses performed by various groups have confirmed that EVs from metastatic cell lines are overloaded with proteins associated with the mesenchymal phenotype (e.g. vimentin, metalloproteases etc.) compared with EVs from non-metastatic or normal cells (Ji *et al.*, 2013; Jeppesen *et al.*, 2014). Functional experiments have shown that treatment of non-metastatic/normal cells with EVs from tumour cells that undergo EMT can induce the EMT phenotype in the recipient cells (Galindo-Hernandez *et al.*, 2014). Furthermore, cancer cells under hypoxic environment release EVs enriched in EMT linked molecules in comparison to EVs produced under normoxia (Ramteke *et al.*, 2015).

Invasion

To achieve the motility required in the different stages of metastasis, cells alter their expression profile to benefit extracellular protease activities, cadherin-mediated cell-cell adhesion, loss of cell polarity, and cytoskeletal arrangement (Yilmaz and Christofori, 2010). There is a cross-talk between tumour and stroma cells that is found to be mediated by EVs. Cancer cells can alter

tumour microenvironment to support tumour invasion (Webber *et al.*, 2010). Various classes of metalloproteinases, the enzymes that degrade the extracellular matrix, have been discovered within the lumen of EVs from tumour cells (Salamone *et al.*, 2006; Taraboletti *et al.*, 2002). EVs from tumour associated fibroblasts on the other hand, have been reported to stimulate pro-migratory effects in the recipient tumour cells (Luga and Wrana, 2013), revealing a bidirectional mode of tumour-stroma communication. Vesicles from malignant ascites of ovarian cancer patients were found to carry proteases, including matrix metalloproteinases (MMPs) and the urokinase-type plasminogen activator (uPA), able to induce invasion as tested by a Matrigel®-based assay. Higher EV numbers reflected higher tumour grade, however, the invasive potential of the EVs remained consistent among the tumour grades (Graves *et al.*, 2004).

Intravasation

Tumour cells need to enter the vasculature in order to travel to distant organs. During this process, tight junctions are impaired, so that the endothelium 'loosens up' to allow cancer cells to enter the circulation. It has been shown that breast cancer EVs can modulate blood vessel permeability by the release of miR-105, thus suppressing a natural defence against metastasis (Zhou *et al.*, 2014).

Surviving in circulation

Cancer cells encounter different types of immune cells while in the circulation. In order to survive, immune responses need to be suppressed. Tumour-derived EVs have been shown to modulate immune reaction in various ways (Whiteside, 2016). For example, EVs can directly transfer factors that can cause apoptosis to T cytotoxic cells (Kim *et al.*, 2005). Another way of suppressing immune responses is by engaging more myeloid-derived suppressor cells, thus altering the tumour environment (Pyzer *et al.*, 2016). A study involving melanoma patients revealed that tumour secreted microvesicles stimulate the differentiation of myeloid cells that could suppress T cell responses through TGF β signalling (Valenti *et al.*, 2006).

Extravasation

It is established that platelets chaperone tumour cells in circulation in order to secure survival. Platelets support the adhesion of the tumour cells to the endothelium via P-selectin (Gay and Felding-Habermann, 2011). Activated platelets secrete microvesicles and exosomes that carry P-selectin, amongst other adhesion molecules (Heijnen *et al.*, 1999). This study suggests that EVs from activated platelets may be involved in communication with other cells, such as endothelial

or immune cells. It is possible that these EVs chaperone tumour cells and aid their extravasation via P-selectin-mediated adhesion to the endothelium.

Secondary tumour formation

Alterations in the secondary organs before the arrival of cancer cells support the growth of the metastatic tumour. Metalloproteinases, bone marrow dendritic cells and soluble factors such as fibronectin are involved in the development of the pre-metastatic niche (Kaplan *et al.*, 2005). EVs have emerged as a new component of the tumour microenvironment, supporting the formation of the pre-metastatic niche and organotropic metastasis (Peinado *et al.*, 2012; Costa-Silva *et al.*, 2015; Deng *et al.*, 2017). Experiments with conditioned media from breast cancer cells showed that EVs from primary tumour can recruit specific types of immune cells, bone-marrow derived, to secondary organs in order to prepare the 'soil' for the secondary tumour formation (Sceneay *et al.*, 2012). Hoshino *et al.* (2015) showed that EVs from primary tumours were capable of 'directing' circulating tumour cells to the secondary site, using cells that were previously unable of organotropic metastasis. This meant that the secondary organ was primed well before the initial steps of metastasis. Moreover, it was found that integrins, such as ITG β 4, were responsible for the specific distribution of EVs in the body, enabling organ specific metastasis (Hoshino *et al.*, 2015). Another study that confirms the role of EVs in organotropic metastasis, showed that pancreatic cancer-derived EVs trigger TGF signalling in Kupffer cells (stellate liver macrophages) resulting in extracellular matrix remodelling. Subsequently, fibronectin accumulation and macrophage recruitment to the liver prime the 'soil' for liver metastasis (Costa-Silva *et al.*, 2015). Gastric cancer is also known to metastasise to the liver, a process that has been reported to be mediated by EGFR-containing EVs shedded by gastric tumour cells (Zhang *et al.*, 2017).

1.5 Project aims and objectives

EVs are mediators of intercellular communication, however studies show great diversity in the mechanisms of EV uptake, which might be due to the variability in the methods employed. Moreover, EVs have been reported to play key roles in all 'steps' of the metastatic cascade. Studies have proposed that chemotherapy may in some cases promote metastasis. Here, it was hypothesised that EVs from chemotherapy treated cells were able to induce metastasis-related properties to naïve cells, and that this may be associated with changes in glycosylation status. The aims of this project were: a) to explore the dynamics and specificity of EV uptake by recipient cells, and b) to investigate the effect of chemotherapy induced intercellular communication via EVs on breast cancer metastasis.

Objectives:

- To develop and optimise an EV labelling method to accurately measure EV uptake in recipient cells.
- To describe the mechanisms of uptake employed by different types of cells using labelled EVs and uptake inhibitors.
- To investigate whether EVs obtained from chemotherapy treated cells can induce a pro-metastatic phenotype to naïve cells *in vitro*.

Materials and methods

Chapter 2

2 Materials and methods

2.1 Cell culture

2.1.1 Cell culture routine procedures

Cell lines

All the cell lines used in this project are listed in Table 2.1 along with information about their origin, characteristics and culture details.

Table 2.1 Characteristics of all the cells used in this work and details of growth conditions.

Cell line	Type	Derived from	Complete culture medium
T47D	Human epithelial breast cancer cell line (ductal carcinoma)	Metastatic site: pleural effusion (Keydar <i>et al.</i> , 1979)	RPMI-1640 (Gibco™) + 10% FBS (Gibco™)
MCF7	Human epithelial breast cancer cell line (adenocarcinoma)	Metastatic site: pleural effusion (Soule <i>et al.</i> , 1973)	DMEM/F12 (Sigma-Aldrich®) + 10% FBS (Gibco™)
hTERT-HME1	Human epithelial breast non-cancer cell line - immortalised using human telomerase reverse transcriptase (hTERT)	Mammary epithelium of patient undergoing reduction mammoplasty surgery (no cancer history) (Van der Haegen and Shay, 1993)	DMEM/F12 (Sigma-Aldrich®) + 20 ng/mL EGF (PeproTech®) + 10 g/ml insulin (Sigma-Aldrich®) + 100 g/ml hydrocortisone (Sigma-Aldrich®) + 10% FBS (Gibco™)
HeLa	Human epithelial cervical cancer cell line (adenocarcinoma)	Primary cervical carcinoma (Jones <i>et al.</i> , 1971)	DMEM/F12 (Sigma-Aldrich®) + 10% FBS (Gibco™)
HUVEC	Human umbilical vein/vascular endothelial cells (primary cell line)	Umbilical vein/vascular endothelium (Hoshi and McKeenan, 1984)	EBM-2 (Lonza) + EGM™-2 Endothelial SingleQuots™ Kit (Lonza), which includes 0.4% hydrocortisone, 0.1% hFGF-B, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% EGF, 0.1% Gentamycin and amphotericin, 2% FBS

% concentrations are given as v/v.

RPMI, Roswell park memorial institute medium; DMEM, Dulbecco's modified Eagle's medium; FBS, foetal bovine serum; EGF, epidermal growth factor; hFGF-B, human fibroblast-derived growth factor-B; VEGF, vascular endothelial growth factor; R3-IGF-1, recombinant analogue of insulin growth factor.

Re-culturing from frozen stock

All cell lines were defrosted from liquid nitrogen in a 37°C water bath for 1 min. Once thawed, the cell suspension was transferred into a 50 ml centrifuge tube containing 20 ml cold phosphate buffered saline (PBS, Thermo Fisher Scientific) and spun down at 200 x G for 5 min. The supernatant was discarded and the pellet was resuspended in 14 ml warmed complete medium and transferred into a 75cm² cell culture flask, which was placed in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere.

Cell maintenance

When approximately 75% confluent, cells were passaged. Firstly, the medium from the flask was removed and the cells were washed with PBS. The appropriate amount of 0.5% w/v trypsin/EDTA (Gibco™) was added to cover the growth area of the flask (e.g. 2ml for 75 cm² flask, 5ml for 175cm² flask) and the flask was placed in a 37°C incubator until cells were dissociated (usually 2-3 min). Once the cells were detached, an equal volume of complete medium was added to the flask to inactivate the trypsin. The flask contents were transferred into a 50mL centrifuge tube (Falcon™) and spun down at 200 x G for 5 min. The supernatant was discarded and the pellet was resuspended in the appropriate medium and quantity, depending on the downstream application. To make cell stocks, cell pellets were resuspended in 1ml cell freezing medium (complete medium + 10% dimethyl sulfoxide (DMSO, Corning™)) and placed in cryogenic vials (Thermo Fisher Scientific). The vials were then placed in a Mr Frosty™ freezing container (Nalgene®) at -80°C for two days. This allowed the temperature to drop by one degree every minute. They were then transferred to liquid nitrogen for long-term storage.

Mycoplasma testing and short tandem repeat (STR) profiling

Mycoplasma is a common but serious cell line contamination. Mycoplasmas are small bacterial species that are not visible when imaged using light microscopy. They can alter the characteristics of the cells they infect, including cell growth, metabolism and morphology (Drexler and Uphoff, 2002). Therefore, it is essential that apart from adopting good laboratory practices, the cell lines need to be tested frequently. STR profiling is the analysis of repetitive sequences in the human genome which are highly variable among individuals and can be used for identification purposes. After an unanticipated report by Drexler *et al.* (1999) showing that 17 - 35% of the hematopoietic cell lines tested were cross-contaminated with other lines, the need to implement authentication of cells in the cell culture routine becomes clear.

For both mycoplasma testing and STR profiling, DNA was isolated from cultured cells. Briefly, cells were cultured for 2 - 3 days in a 25 cm² flask (no medium change) until approximately 60-80% confluent. The cells were detached from the flask using cell scrapers. Approximately 4 ml of cell suspension was transferred in centrifuge tubes and spun at 10,000 x G for 15 min to pellet the cells and any floating microorganisms, including mycoplasma. The supernatant was discarded and the pellet was resuspended in 200 µl PBS. The DNA was extracted using a PureLink® Genomic DNA Kit. Briefly, 20 µl Proteinase K (Invitrogen™, 20 mg/ml stock) and 20 µl RNase A (Invitrogen™, 20 mg/ml stock) were added to the samples, mixed, and incubated at room temperature for 2 min. After this time, 200 µl of PureLink™ genomic lysis/binding buffer was added and samples were incubated at 55°C for 10 min. The DNA was precipitated by adding 200 µl of 96% ethanol (Sigma-Aldrich®) and the lysate was transferred onto a PureLink™ spin column. The column was centrifuged at 10,000 x G for 1 min. The flow-through was discarded and the column was washed firstly with 500 µl of PureLink™ Buffer 1 and secondly with Buffer 2 followed by a dry-spin. The DNA was eluted in 50 µl PureLink™ genomic elution buffer. The concentration and purity of DNA were measured using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). All DNA samples were diluted to 50 ng/µl.

The cell lines used in this study were tested once at the beginning and again at the end of the project for mycoplasma contamination. The e-Myco™ Mycoplasma PCR Detection Kit (iNtRON) was used following the manufacturer's protocol. PCR products along with positive and negative controls provided with the kit were run on a 2% agarose gel with SafeView Nucleic acid stain (nbs biologicals) and mycoplasma presence was examined (Figure 7.1). Once tested for mycoplasma, at the beginning of this project, DNA was sent to the University of Liverpool, Institute of Translational Medicine for STR profiling in order to verify the identity of the cell lines used in this project. The GenePrint® 10 System (Promega) was used, following the manufacturer's instructions. Briefly, 5 ng of DNA were used in a PCR reaction to amplify 10 human genetic loci (TH01, TPOX, vWA, Amelogenin, CSF1PO, D16S539, D7S820, D13S317, D5S818 and D21S11). The products along with the allelic ladder were then mixed with a size standard and fragment analysis was performed using the 3130 Genetic Analyser (Applied Biosystems). The results from the genetic analyser were matched against the ATCC STR profile information of each cell line. All the cell lines used in this project were authenticated using this approach (Table 7.1).

Production of EV depleted medium

For cell maintenance, complete medium was used as in Table 2.1. For many experiments medium needed to be cleared of bovine EVs, therefore EV-depleted medium was used. The

difference between complete and EV-depleted medium is that the latter contains EV-depleted FBS rather than normal FBS. To prepare EV-depleted FBS, regular FBS was spun at 120,000 x G for 16 h at 4°C in a Beckman Coulter Optima XPN centrifuge using the SW31Ti rotor (Beckman Coulter). The supernatant was passed through 0.22 µm filters (Thermo Fisher Scientific) and was added to cell culture medium at a 10% concentration.

2.1.2 Production of stably transfected cell lines

For fluorescent EV labelling, T47D and hTERT-HME1 cells were stably transfected with the mEmerald-CD81 plasmid, which was kindly provided by Dr Justin Hean, University of Oxford, and is available from Addgene under the accession number #54031 (Figure 2.1).

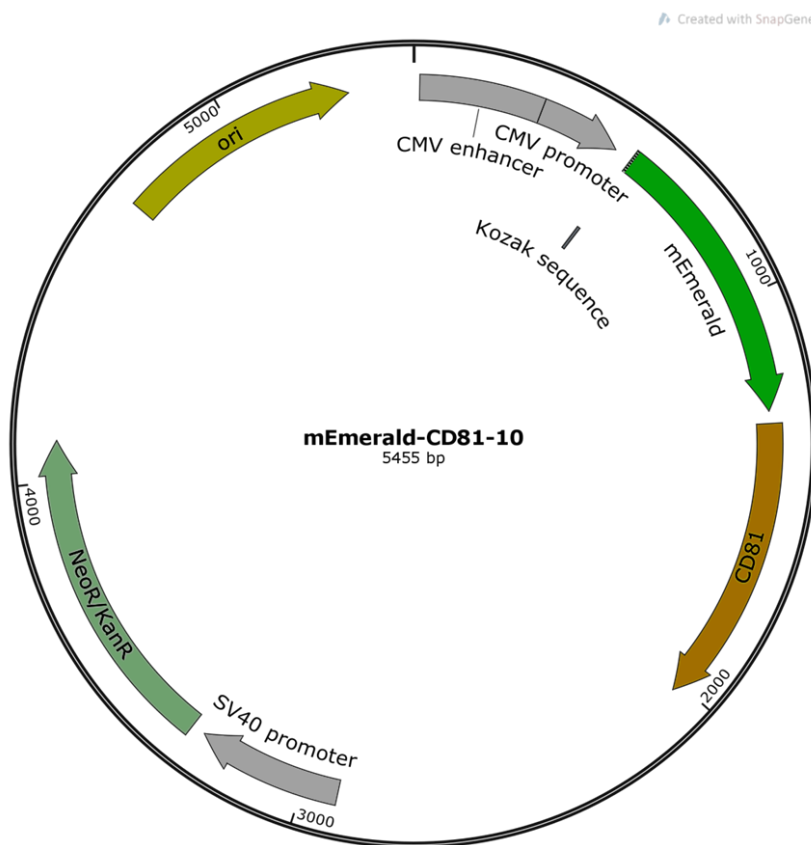


Figure 2.1 Schematic representation of the mEmerald-CD81-10 plasmid used for stable transfection of T47D and hTERT-HME1 cells.

The fusion protein of mEmerald and CD81 is under the regulation of the cytomegalovirus promoter. Resistance to kanamycin was used for positive selection of bacteria and resistance to neomycin was used for selection of positive mammalian clones. Map produced in SnapGene®.

Bacterial cultures

For bacterial transformations, 100 ng of plasmid was added to 100 µl of Dh5α competent cells (New England Biolabs) and incubated on ice for 30 min. The cells were heat shocked at 37°C for

20 sec and then immediately placed on ice for 2 min; 900 µl pre-warmed SOC medium (Corning™) was added in each vial and bacteria were incubated at 37°C for 1 h shaking at 225 RPM. Finally, 100 µl of cells were spread onto an LB agar plate containing 50 µg/ml kanamycin (also plated on an ampicillin-containing plate as a control) and incubated at 37°C for 16 h shaking at 225 RPM. Three colonies were randomly picked and further cultured in LB containing antibiotics for 16 h. Bacterial stocks were prepared by mixing 800 µl of culture with 200 µl of autoclaved 100% glycerol and stored at -80°C.

Plasmid isolation

Bacterial liquid cultures were prepared from glycerol stock (10 µl stock in 100 ml kanamycin containing LB) and incubated at 37°C for 16 h shaking at 225 RPM. Plasmid was extracted using a QIAGEN Plasmid Midi Kit. Briefly, bacterial cultures were centrifuged at 6,000 x G for 15 min at 4°C and the pellet was resuspended in 4 ml Buffer P1. Buffer P2 was added and the tube was mixed and incubated at room temperature for 5 min. Subsequently, 4 ml cold Buffer P3 was added and the mixture was incubated on ice for 15 min. The lysate was centrifuged at 15,000 x G for 35 min at 4°C, the pellet was discarded, and the supernatant was centrifuged again at the same speed and temperature for 18 min. A QIAGEN-tip column was equilibrated with 4 ml Buffer QBT. The supernatant from the last centrifugation was applied on the column and allowed to pass through by gravity flow. The column was washed twice with 10 ml Buffer QC and the DNA was eluted using 5 ml Buffer QF. The DNA was then precipitated with 3.5 ml (0.7 volumes) of isopropanol (Sigma-Aldrich®) and centrifuged at 15,000 x G for 30 min at 4°C. The supernatant was discarded and the pellet was washed with 2 ml 70% ethanol and centrifuged again at 15,000 x G for 10 min. The supernatant was discarded; the pellet was air-dried for 10 min and then resuspended in 50 µl Buffer TE. The plasmid DNA concentration and purity were measured using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

Mammalian cell transfection

The goal here was to develop cell lines stably transfected with mEmerald-CD81 vector in order to produce fluorescent EVs on a large scale.

Cells were cultured in 10 cm² cell culture dishes (Greiner Bio-One) until they reached 50% confluence. The transfection mix in Table 2.2 was prepared and let stand for 8 min at room temperature. The cells were washed with PBS and 10 ml complete medium containing 5% (instead of 10% FBS, to slow growth rate) was added to the cells along with the transfection mix for 48 h. After this time, the transfection efficiency was checked using a cell culture microscope (Primovert iLED with Epi-Fluorescence 470 nm and LD 20x Ph1 objective (Zeiss)). Ideal

transfection efficiency at this point should be low so that single colonies could be picked later on. By eye, approximately 10 – 30 cells were transfected in each plate. The cells were washed with PBS, the medium was refreshed, and the selective antibiotic, geneticin, (Gibco™) was added at a concentration of 800 µg/ml. Geneticin is lethal for the cells that have not received the vector and therefore are not resistant to neomycin. This concentration of antibiotic was selected as it proved sufficient to kill the un-transfected host cells after approximately 7 days (Figure 7.2). Medium with antibiotic was replaced every two days for a period of approximately two weeks until single transfected colonies were visible. To pick single colonies, the plates were washed with PBS and cloning rings (Sigma-Aldrich®) were placed around each colony (10 - 20 colonies were selected for each cell line). Then, 50 µl of 0.5% w/v trypsin/EDTA was added in each cloning ring until cells dissociated. An equal volume of FBS was added in the cloning rings and the cell suspension was transferred into 25cm² flasks containing complete medium with 800 µg/ml geneticin. The brightest and most homogeneous colonies were selected and cultured further in complete medium with 800 µg/ml geneticin for one month. After that time, cells were cultured in complete medium without antibiotic and stocks were made. The cells maintained the same fluorescence intensity and transfection levels throughout the course of this project, as observed using the tissue culture fluorescence microscope during routine culturing.

Table 2.2 Transfection mix components and quantities.

Component	Quantity per transfection (10cm ² dish)
Complete medium	384 µl
Plasmid (4 µg/µl)	1 µl
FuGENE® HD Transfection Reagent	15 µl

2.2 EV preparations

2.2.1 EV isolation

When cells reached 50 - 60 % confluence, complete medium was changed to EV depleted medium. After 48 h, conditioned medium was collected and EVs were isolated by serial centrifugation: Conditioned medium was spun firstly at 300 x G for 5 min (Megafuge 16 Benchtop Centrifuge, Thermo/Hereaus) to remove cells, and then at 16,500 x G for 20 min at 4°C (Avanti JXN-26, High-Speed Centrifuge, JA14.50 Tapered Rotor - Beckman Coulter) to discard cell debris and larger EVs. Conditioned medium was then passed through 0.01% bovine serum albumin (BSA, Sigma-Aldrich®) blocked 0.22 µm filters to remove remaining cell debris and larger vesicles. EVs were pelleted with ultracentrifugation at 120,000 x G for 90 min at 4°C (Beckman Coulter Optima XPN centrifuge, SW31Ti rotor - Beckman Coulter). The pellet was resuspended in

PBS and centrifuged again at 120,000 x G for 90 min at 4°C to reduce the level of contaminating proteins. The EV pellet was resuspended in 100 µl PBS and kept at 4°C for 3 days or -80°C for long-term storage. Alternatively, the EV pellet was resuspended directly in 100 µl RIPA buffer for protein extraction (section 2.2.2) or 50 µl PBS for labelling using the PKH26 labelling kit (section 3.3.1).

2.2.2 EV characterisation

Nanoparticle tracking analysis (NTA)

NTA allows fast detection of size and concentration of vesicles ranging from 10 nm to 1 µm, based on their Brownian movement. Briefly, a laser light scattering microscope illuminates and monitors the scattered light from each individual particle in suspension, creating concentration calculations and particle size distributions. Here, the ZetaView 110 instrument supplied with a 488 laser and the accompanying software (Particle Metrix, GmbH) was used to determine the concentration of EV preparations. Firstly, the instrument was calibrated with known concentrations of 100 nm polystyrene beads. Samples were diluted 1:1000 or 1:10000 in PBS, depending on the amount of EVs in each preparation; 0.5 sec videos were recorded and analysed at 5 - 11 positions, and size and concentration of EVs were calculated.

Transmission electron microscopy (TEM)

Each EV sample (in PBS) was mixed with an equal volume of 4 % PFA, pH 6.9 (Sigma-Aldrich®), and allowed to fix on ice for 15 min. A droplet of each EV sample was placed for 45 min on formvar/ carbon coated 300 mesh nickel grids (Agar Scientific), previously discharged in 0.01% Alcian blue solution (Sigma-Aldrich®) for 30 sec. After this time, each grid was washed three times with ultra-pure water.

For immunogold labelling, the grids were blocked with 0.1% BSA for 30 min and then incubated with a mouse monoclonal anti-CD81 primary antibody raised in mouse (Abcam (ab79559), 1:200 in 0.1% BSA) for 40 min. The grids were then washed three times with PBS and incubated with 10 nm gold bead-conjugated secondary rabbit anti-mouse IgG antibody (Abcam (ab39619), 1:200 in 0.1% BSA) for 20 min. For negative staining, 2% uranyl acetate (Sigma-Aldrich®) droplets were placed on the grids for 10 min. Then, the grids were washed three times with ultra-pure water and allowed to air-dry. Finally, the grids were washed twice with PBS and once with ultra-pure water and let air-dry. EVs on grids were visualised using a Hitachi H7650 electron microscope at 100 kV at 10,000 to 40,000x magnification.

Western blot

Cells and EVs were lysed in 1x RIPA buffer (Merck) supplemented with Halt™ protease inhibitor cocktail (Thermo Fisher Scientific) and protein concentration was measured using the bicinchoninic acid assay kit (Thermo Fisher Scientific). Proteins were separated based on their molecular weight by discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) consisting of two separate stacking and resolving gels cast together (Table 2.3). Protein samples (4 µg per sample) were mixed with 4x Laemmli loading buffer (BioRad), heated for 10 min at 95°C and loaded onto the gel. Electrophoresis was performed in 1x Tris-glycine SDS buffer (BioRad) at 100 Volts for 1 h. Proteins were then transferred to polyvinylidene difluoride membranes using a semi-dry transfer system (TransBlot® Turbo™ Transfer Starter System, BioRad). at 10 Volts for 10 min. The membranes were subsequently blocked in 5% milk powder in Tris-buffered saline with Tween 20 (TBST, Sigma-Aldrich®) for 1 h at room temperature and then incubated with primary antibodies (Table 2.4) overnight at 4°C. Next day, the membranes were washed three times with TBST and incubated with the secondary antibody (Table 2.4) for 1h at room temperature. Membranes were then washed three times with TBST and protein bands were visualized using the Pierce™ enhanced chemiluminescence (ECL) Western Blotting Substrate kit (Thermo Fisher Scientific) and the ChemiDoc™ Imaging System (BioRad).

Table 2.3 Components and quantities needed for the preparation of protein gels.

10% Resolving Gel		5% Stacking Gel	
Distilled water	5.9 ml	Distilled water	3.4 ml
1 M Tris (pH 8.8)	3.8 ml	1 M Tris (pH 6.8)	630 µl
Acrylamide/bisacrylamide (30%)	5 ml	Acrylamide/bisacrylamide (30%)	830 µl
Ammonium persulfate (10% w/v)	150 µl	Ammonium persulfate (10% w/v)	50 µl
Sodium dodecyl sulfate (10%)	150 µl	Sodium dodecyl sulfate (10%)	50 µl
Tetramethylethylenediamine	6 µl	Tetramethylethylenediamine	5 µl

Table 2.4 Antibodies used in this study for the characterisation of EVs by western blot.

	Target	Host species	Dilution (in 5% milk in TBST)	Supplier / catalogue number
Primary	Anti-human Ig CD81	Mouse	1:1000	Abcam (ab79559)
Secondary	Anti-mouse IgG (conjugated to horseradish peroxidase)	Rabbit	1:2000	Abcam (ab6728)

2.3 Drug treatments

2.3.1 EV uptake inhibitors

To inhibit different pathways of EV uptake (5-(N-ethyl-N-isopropyl) amiloride (Sigma-Aldrich®), dynasore (dynasore hydrate - Sigma-Aldrich®), and cholesterol oxidase (from *Streptomyces sp.* Sigma-Aldrich®) were used. Amiloride blocks macropinocytosis, Na^+/H^+ exchange and decreases cytosolic pH (Koivusalo *et al.*, 2010). Dynasore is a dynamin inhibitor that prevents endocytosis, and also reduces labile cholesterol in the plasma membrane, thus disrupting lipid raft organization (Preta *et al.*, 2015a). Cholesterol oxidase is an enzyme which catalyses the dehydrogenation of cholesterol, therefore altering the cholesterol on the plasma membrane and disrupting lipid rafts (Castro *et al.*, 2013).

Amiloride

Amiloride was dissolved in DMSO at a stock concentration of 108 mM and stored at -20°C. It was used at 50 μM for T47D and HeLa cells and at 25 μM for hTERT-HME1 cells, diluted in EV depleted medium. DMSO concentration in the final sample was less than 0.05%. Amiloride was added to the cells 30 min prior to EV administration and was present throughout the course of the experiment.

Dynasore

Dynasore was dissolved in DMSO at a stock concentration of 31 mM and stored at -20°C. It was used at 50 μM for T47D and HeLa cells and at 25 μM for hTERT-HME1 cells, diluted in EV depleted medium. DMSO concentration in the final sample was less than 0.05%. Dynasore was added to the cells 30 min prior to EV administration and was present throughout the course of the experiment.

Cholesterol oxidase

Cholesterol oxidase was dissolved in PBS at a stock concentration of 200 Units/ml and stored at -20°C. It was used at 2 Units/ml for all cell lines, diluted in EV depleted medium. Cholesterol oxidase was added to the cells 4 h prior to EV administration and was present throughout the course of the experiment.

2.3.2 Chemotherapeutic agents

Two chemotherapeutic agents were employed in this study, docetaxel (Sigma-Aldrich®) and mitomycin C (Sigma-Aldrich®). Docetaxel is a taxane-based anti-cancer drug used as first-line

chemotherapy in the clinic for primary and advanced breast cancer (Crown *et al.*, 2004). Mitomycin C is a DNA cross-linking molecule often used in combination with other anti-cancer drugs in patients with metastatic breast cancer (Pacheca-Palomar, 2009).

Docetaxel

Docetaxel was dissolved in DMSO at a stock concentration of 12.4 mM and stored at -20°C. It was added to the cells at concentrations ranging from 1 nM to 50 µM for 24 h.

Mitomycin C

Mitomycin C was dissolved in ultra-pure water at a stock concentration of 1.5 mM and stored at -20°C. It was added to the cells at concentrations ranging from 10 nM to 50 µM for 24 h.

2.4 Gene expression

2.4.1 RNA extraction

For RNA extraction the mRNeasy kit (Qiagen) was used according to the manufacturer's instructions. Briefly, cells in 6-well plates were washed with PBS, 350 µl RLT buffer was added per well and cells were disrupted using cell scrapers. The lysate was mixed with an equal volume of 70% ethanol, transferred onto an RNeasy spin column and spun at 10,000 x G for 1 min. Columns were then washed once with 700 µl Buffer RW1 and twice with 500 µl Buffer RPE followed by a dry-spin. RNA was eluted in 30 µl of nuclease-free water. The concentration and purity of RNA were measured using the NanoDrop™ One Microvolume UV-Vis Spectrophotometer. All RNA samples were normalised to 100 ng/µl.

2.4.2 DNase I treatment and reverse transcription

DNase I treatment

DNase I is an endonuclease that digests double- and single-stranded DNA. Even though qPCR primers were designed to only bind and amplify intronless DNA (cDNA), it is good laboratory practise to remove any contaminant genomic DNA that might be carried over in the RNA preparations. Here, the AMPD1-1KT DNase I kit (Sigma-Aldrich®) was used according to the manufacturer's instructions.

Samples were prepared in 8-strip wells as described in Table 2.5. The mix was incubated at room temperature for 15 min and then 1 µl of Stop solution was added. Samples were then denatured

at 70°C for 10 min and immediately placed on ice for 2 min. Reverse transcription followed directly after DNase I treatment.

Table 2.5 DNase treatment reaction components and quantities.

Component	Quantity per sample
10 x Reaction buffer	1 µl
DNase I, amplification grade, 1 unit/µl	1 µl
Nuclease-free water	2 µl
RNA (100 ng/µl)	6 µl
Total volume	10 µl

Reverse transcription

Reverse transcription is the synthesis of single-stranded complementary DNA (cDNA) using single-stranded RNA as a template, mediated by the enzyme reverse transcriptase. There are three main RNA priming strategies: a) random oligomers, b) oligo (dT), c) specific primers. Both oligo (dT) and random oligomers allow many different targets to be studied from the same cDNA pool, while specific primers reverse transcribe a specific target. In this project, random hexamers were used for reverse transcription.

A High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used according to the manufacturer's protocol. The mix in Table 2.6 was prepared and added in each RNA sample from the previous step (DNase I treatment) and the thermal profile in the same table was performed. After completion of cDNA synthesis, 80 µl of nuclease free water was added in each sample.

Table 2.6 Reverse transcription components, quantities and thermal profile.

Component	Quantity per sample	Step	Time	Temperature
25x dNTP mix (100mM)	0.8 µl	Hold	10 min	25 °C
MultiScribe™ reverse transcriptase, (50 U/µL)	1 µl	Hold	2 h	37 °C
10x Reverse transcription buffer	2 µl	Hold	5 min	85 °C
10x Random primers	2 µl	Hold	∞	4 °C
Nuclease-free water	4.2 µl			
Total volume	10 µl			

2.4.3 Real-time qPCR

The real-time qPCR method allows detection of PCR amplification during early phases of the reaction (Arya *et al.*, 2005). Accumulation of the amplicon is detected during the reaction and data are measured at the exponential phase. Hydrolysis assays (TaqMan or 5' nuclease assays)

utilize a set of primers, a probe, polymerase with exonuclease activity, dNTPs and DNA template. The probe consists of two types of fluorophores. While the probe is intact, the quencher is close to, and reduces the fluorescence from, the reporter fluorophore. The polymerase then amplifies the product and removes (5' exonuclease activity) the TaqMan probe from the template DNA. This separates the quencher from the reporter and allows the reporter to give off its energy. The emitted light is captured by a camera. The more times the denaturing and annealing takes place, the more opportunities there are for the TaqMan probe to bind and, in turn, the more emitted light is detected. In addition, TaqMan probes allow multiple genes to be measured at the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to different probes. Multiplex PCR allows internal controls to be co-amplified in a single tube minimising pipetting errors (Arya *et al.*, 2005).

Primers and probe for ACTB were designed using the OLIGO 7 software (Molecular Biology Insights) and purchased from Sigma-Aldrich®, while off the shelf assays for CDH1 and vimentin were purchased from Thermo Fisher Scientific (Table 2.7).

Table 2.7 Primers, probes and assays used for expression analysis with real-time qPCR.

Primer/Probe	Sequence 5'→3'	Dye	Annealing temperature
ACTB - Forward primer	GGCACCAGCACAATGAAG	none	58.7 °C
ACTB - Reverse primer	CATACTCCTGCTTGCTGATCCA	none	58.9 °C
ACTB - Probe	CTCCTCCTGAGCGCAAGTACTCCGTG	5' HEX-3' NFQ	68.8 °C
Assay (20x)	Part Number	Dye	Annealing temperature
CDH1	Hs01023895_m1	5' FAM-3' MBG	60 °C
Vimentin	Hs00958111_m1	5' FAM-3' MBG	60 °C

HEX and FAM are both fluorescent dyes. NFQ and MBG are both fluorescent quenchers.

NFQ, Non fluorescent quencher; MBG, minor groove binder.

A master mix of the reagents detailed in Table 2.8 was prepared for the required number of reactions plus 10% to allow for pipetting errors. 15 µl reactions were run in duplicate for each sample. The plate was spun down using a microplate centrifuge (VWR) to remove any air bubbles and then placed in a CFX96 Touch™ thermal-cycler (Bio-Rad) and the thermal profile in Table 2.8 was performed.

Table 2.8 Real-time qPCR components, quantities and thermal profile.

Component	Quantity per sample	Step	Time	Temperature
SsoAdvanced™ Universal Probes Supermix (2x) (Bio-Rad)	7.5 µl	Hold	30 sec	95°C
20x CDH1 or vimentin assay	0.75 µl	40 cycles	15 sec	95°C
20x ACTB assay*	0.75 µl		30 sec	60°C [†]
ddH ₂ O	3 µl			
cDNA	3 µl			
Total volume	15 µl			

*20x ACTB assay consisted of 15 µM of each ACTB primer and 5 µM ACTB probe in water

[†]Measurements were taken at this stage

Data analysis

The $\Delta\Delta C_q$ method was used to analyse all expression experiments. With this method, C_q (quantification cycle) for the gene of interest (GOI) in both the test sample and calibrator sample are adjusted in relation to a normaliser (norm) gene C_q from the same two samples. The resulting $\Delta\Delta C_q$ (Δ represents the difference) value is incorporated to determine the fold difference in expression. Here, the genes of interest are CDH1 and vimentin, normaliser is the housekeeping gene ACTB and as a calibrator the same RNA/cDNA sample was used in every run so that different runs (from three independent experiments) could be compared and combined.

$$\text{Fold difference} = 2^{-\Delta\Delta C_q}$$

$$\Delta C_q \text{ sample} - \Delta C_q \text{ calibrator} = \Delta\Delta C_q$$

$$C_q \text{ GOI}^{\text{sample}} - C_q \text{ norm}^{\text{sample}} = \Delta C_q \text{ sample}$$

$$C_q \text{ GOI}^{\text{calibrator}} - C_q \text{ norm}^{\text{calibrator}} = \Delta C_q \text{ calibrator}$$

2.5 Statistics and reproducibility

The SPSS (versions 22 and 25) statistical package (IBM) was used for statistical analysis. The Shapiro-Wilk test was used to analyse for normal distribution of data and parametric or non-parametric statistical tests for each figure were justified, as appropriate. Sample sizes were chosen with adequate power based on the literature and previous studies, in general without using statistical methods to predetermine sample size. No samples or data points were arbitrarily excluded from statistical analysis.

Optimisation of EV labelling

Chapter 3

3 Optimisation of EV labelling

3.1 Background

The field of extracellular vesicles is evolving rapidly. The need to visualise EVs and understand the mechanisms of their uptake by recipient cells has led to the development of several EV labelling protocols. Labelling methods include the use of generic fluorescent dyes to stain the EV membrane or cargo, fusion of fluorescent proteins with EV markers, and other more complex technologies that utilise palmitoylated or pH-sensitive fluorophores (listed in Table 3.1). In this project, different EV labelling methods were tested including PKH26 and carboxyfluorescein succinimidyl ester (CFSE) staining as well as tagging of the EV marker CD81 with a green fluorescent protein.

Table 3.1 Published methods for the fluorescent labelling of EVs.

	Marker	Mechanism / Protocol	Publications
Fluorescent dyes	PKH26, PKH67, Di-O,I,D,A,R, Rhodamine B	Lipophilic dyes, non-covalent embedment in the plasma membrane	(Banizs <i>et al.</i> , 2018; Hoshino <i>et al.</i> , 2015; Kim <i>et al.</i> , 2016)
	CFSE, Calcein AM, acridine orange	Membrane permeable compounds, fluorescent after cleavage by esterases, bind on cargo proteins or RNA	(Gray <i>et al.</i> , 2015; Grimolizzi <i>et al.</i> , 2017; Pospichalova <i>et al.</i> , 2015)
	Di-8-ANEPPS	Fast-response probe, highly fluorescent after binding to the membrane's lipophilic environment	(Stoner <i>et al.</i> , 2016)
	C5-maleimide conjugated to a fluorophore	Chemical compound conjugated to a fluorescent dye (e.g. Alexa-488), binds to thiol groups on EV proteins	(Roberts-Dalton <i>et al.</i> , 2017)
Fluorescent proteins	GFP-, RFP- tagged EV markers	Overexpression of the fusion EV marker – fluorescent protein in donor cells	(Heusermann <i>et al.</i> , 2016)
	pH-sensitive GFP - tagged EV markers	Overexpression of the fusion EV marker – pH-sensitive fluorescent protein in donor cells	(Verweij <i>et al.</i> , 2018)
	PalmGFP, PalmtmTomato	Overexpression of the fusion of a palmitoylation signal with a fluorescent protein in donor cells, attachment of the fluorophore to the membrane due to palmitoylation	(Lai <i>et al.</i> , 2015)

3.1.1 PKH26

Lipid dyes such as PKH26 are the most frequently used in uptake experiments. PKH26 is a red fluorescent dye bearing an aliphatic chain that intercalates with, and therefore marks, the

plasma membrane (Figure 3.1). It is a generic dye traditionally used to label whole cells, but with minor modifications in the protocol, numerous studies have used it to stain EVs (Bilen *et al.*, 2017; Franzen *et al.*, 2014; Riches *et al.*, 2014; Hoshino *et al.*, 2015). EVs labelled with PKH26 have been used to visualise their internalisation by recipient cells: as a proof of concept in studies on EV-mediated effects, but also as a means of describing EV uptake as a process. For example, Singh *et al.* (2016), used PKH26 labelled EVs to demonstrate that they are incorporated by the recipient cells, and therefore support their findings that EVs from tumour cells promote a migratory phenotype in non-tumourigenic cells, while Franzen *et al.* (2014), used PKH26 labelled EVs to study the mechanisms of EV uptake by bladder cancer cells. Overall, PKH26 is widely used because it provides an easy and fast way to label EVs and it is highly and stably fluorescent.

3.1.2 CFSE

CFSE labelling is another generic method to label EVs that primarily depends on esterase activity inside the cells or vesicles. CFSE is a non-fluorescent cell-permeable molecule, which is hydrolysed by esterases that cleave its diacetate groups when it enters the cell (or the EVs in this case), activating it to fluoresce green. Active CFSE is then bound to the amino ends of proteins, labelling the contents of the cells or EV cargo (Gray *et al.*, 2015) (Figure 3.1). Various studies have used CFSE to monitor EV uptake with flow cytometry (Pospichalova *et al.*, 2015) or microscopy (Czernek *et al.*, 2015). There are two main protocols for labelling EVs with CFSE; direct staining of the isolated EVs (Aliotta *et al.*, 2015) and staining of the donor cells prior to EV collection (Pospichalova *et al.*, 2015).

3.1.3 mEmerald-CD81

One way of labelling EVs without utilising dyes is by fluorescently tagging EV markers. EVs are enriched in particular membrane-bound proteins such as CD81 (Kowal *et al.*, 2016). This is a tetraspanin often used along with other EV enriched proteins to identify and characterise EV populations. Several studies have used fluorescently tagged EV markers to investigate EV biogenesis and uptake dynamics (Heusermann *et al.*, 2016; Cossetti *et al.*, 2014). For the work described in this thesis, CD81 was fused with the mEmerald fluorescent protein and EVs were labelled by stable transfection of T47D and hTERT-HME1 cells with mEmerald-CD81 (Figure 3.1). The fluorescent protein mEmerald is a brighter and more stable derivative of the green fluorescent protein (GFP), allowing for longer exposure and improved imaging quality (Tsien, 1998).

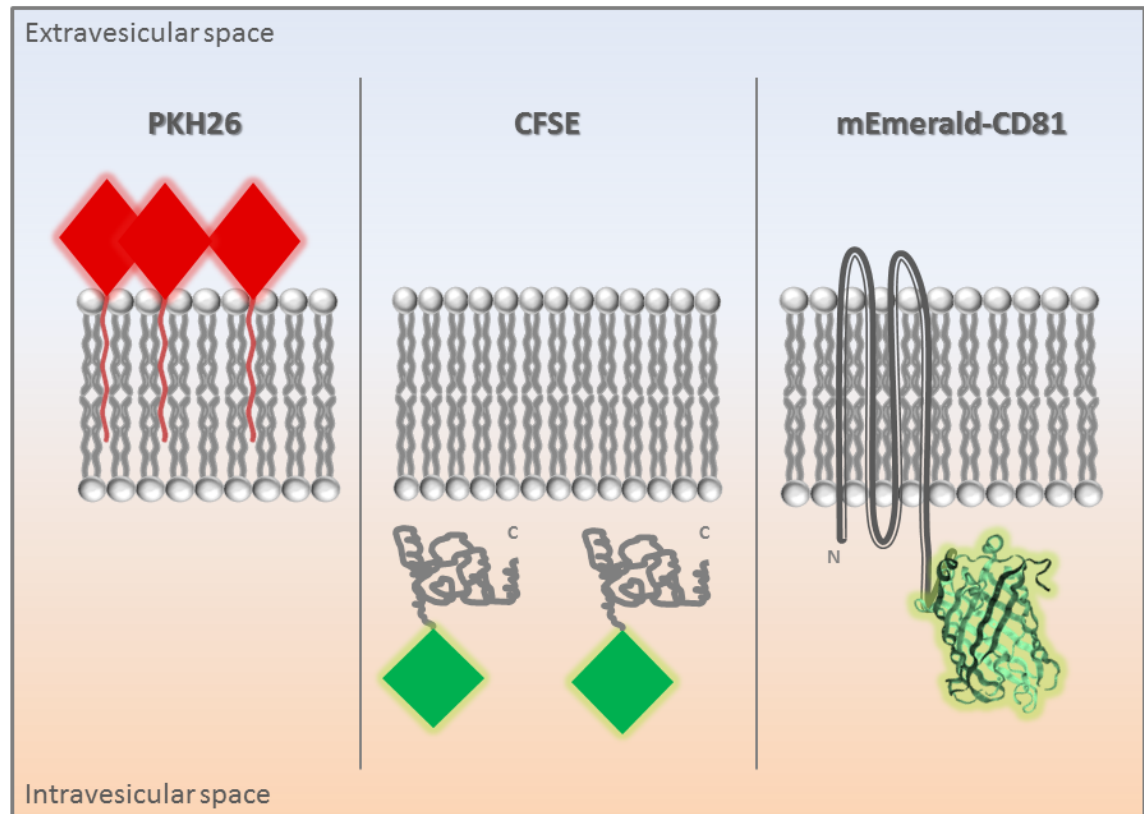


Figure 3.1 Schematic representation of the EV labelling methods used in this project.

PKH26 is a red fluorescent lipophilic dye carrying an aliphatic chain which binds to the lipid bilayer of EVs. CFSE fluoresces green only when it enters the EVs; it binds on the N-terminus of proteins. The green fluorescent protein mEmerald is fused at the C-terminus of the transmembrane protein CD81. Objects are not in scale.

3.2 Aim and objectives of chapter 3

Various methods of EV labelling have been proposed in the literature, however, it was hypothesised that not all labelling methods are equally effective. The aim of the experiments described in this chapter was to optimise an EV labelling technique that would allow the monitoring and accurate quantification of EV uptake. The objectives of this chapter were:

- To test labelling of EVs using PKH26
- To test labelling of EVs using CFSE
- To test dual labelling of EVs using a combination of PKH26 and CFSE
- To test labelling of EVs by transfecting cells with mEmerald-CD81

3.3 Methods

3.3.1 EV labelling using PKH26

EVs were isolated from T47D and MCF7 cells as described in section 2.2.1. A PKH26 kit (Sigma-Aldrich®) was used for EV labelling following the general protocol for cell labelling with minimal adaptations. Briefly, the EV pellet was resuspended in PBS and mixed with the same volume of diluent C. An equal volume of dye suspension in diluent C was added to the mixture (final dye concentration 2 μ M). 'No EV' controls were prepared by mixing equal volumes of PBS and dye suspension. To stop the staining process and quench any residual dye, an equal volume of 1% filtered BSA in PBS was added to the mixture. The labelled EVs were then passed through PBS equilibrated 2 ml Zeba™ Spin desalting columns, 7K MWCO (Thermo Fisher Scientific) (Holder *et al.*, 2016) and incubated with cells cultured on coverslips for 3 h. Cells were washed with PBS and fixed with 4 % PFA for 20 min at room temperature and then mounted with ProLong™ Gold antifade mountant with DAPI (Thermo Fisher Scientific) on slides for microscopic analysis.

To test whether quenching with BSA contributes to non-specific staining of EVs, during staining with PKH26, half of the samples were mixed with BSA, while the other half were brought to the same volume with diluent C (Figure 3.2). Control experiments were performed by mixing PKH26 dye ('no EV' control) with BSA or diluent C.

Uptake was then visualised using an LSM 880 confocal microscope and the Plan-Apochromat 63x/1.4 and 100x/1.4 oil objectives. Excitation and detection wavelengths were 405 and 420-480 nm for DAPI (laser power 1 – 5 %), and 561 and 570-695 for PKH26 (laser power 1 – 5 %, kept constant within the same experiment). Z-stacks of random positions on the slide were acquired for each sample.

In order to quantify the differences in fluorescence amongst treatment groups, projected images were created based on the sum of the intensity of each Z-stack using the open source software FIJI (Schindelin *et al.*, 2012). On the projected image, a region of interest was drawn around each cell using the free hand tool and parameters such as area, integrated density and mean grey value were measured. The corrected total cell fluorescence (CTCF) = integrated density – (area of selected cell $\times$ mean fluorescence of background readings), was calculated in Excel. The Shapiro Wilk test was used to assess distribution/normality of the data. The Mann Whitney test for non-parametric data was used to verify the significance between groups. This experiment was performed on MCF7 and T47D cells, three technical replicates per group.

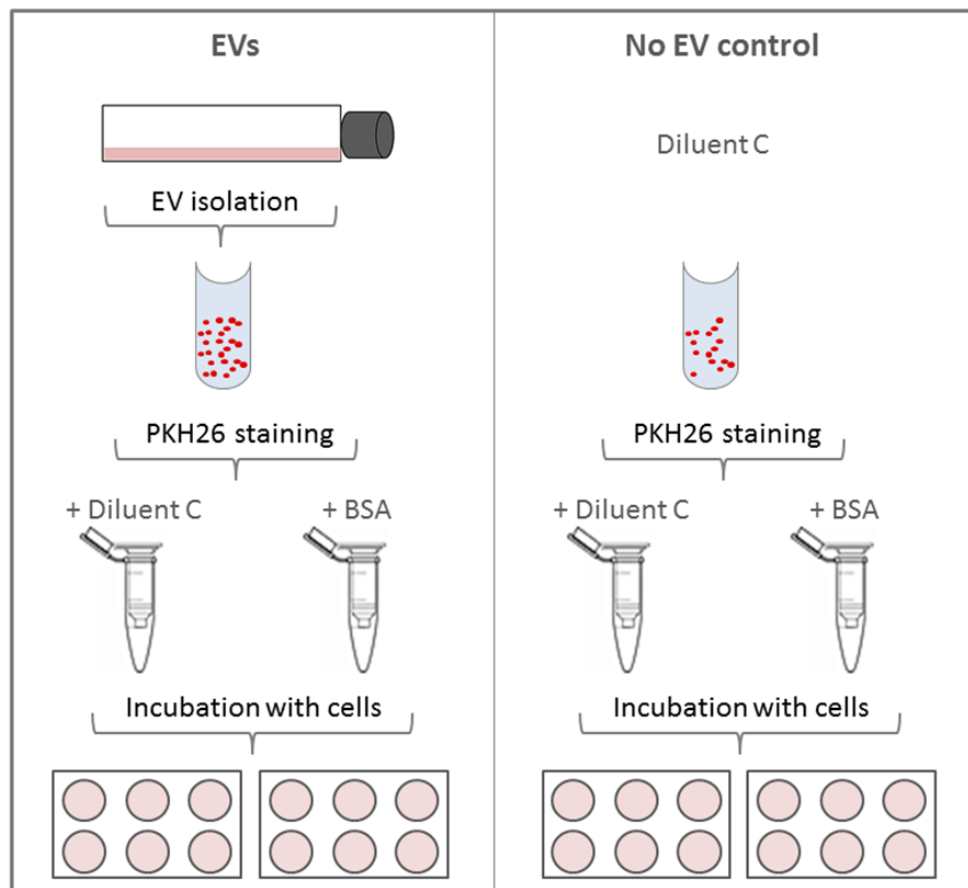


Figure 3.2 Schematic representation of the experimental procedure followed to assess the effect of BSA during PKH26 labelling of EVs.

EVs were isolated and stained with PKH26. Half of the samples were then mixed with BSA, while the other half were brought to the same volume with diluent C. The ‘no EV’ controls were performed at the same time. Samples were passed through Zeba™ columns and incubated with cells for 3 h.

3.3.2 EV labelling using CFSE

For the CFSE staining, an Exo-Green kit (System Biosciences, 10x stock) was used with some alterations from the manufacturer’s instructions. EVs were isolated from T47D cells as described in section 2.2.1. The EV pellets were resuspended in 400 µl PBS and stained with 40 µl CFSE for 10 min at 37°C. The ‘no EV’ control was prepared by mixing 400 µl PBS with 40 µl CFSE for 10 min at 37°C. EVs and ‘no EV’ controls were then passed through PBS equilibrated Zeba™ columns and incubated with cells cultured on 3mm glass bottom dishes (ibidi). Uptake of CFSE labelled EVs was monitored in real time using an LSM 880 confocal microscope and the Plan–Apochromat 40x/1.3 oil objective. Excitation and detection wavelengths were 488 and 490-535 nm (laser power 1 – 5 %, kept constant within the same experiment).

3.3.3 EV labelling using a combination of PKH26 and CFSE

Two protocols were used for labelling of EVs with PKH26 and CFSE: direct dual staining of EVs and isolation of EVs from dual stained cells.

Direct staining of EVs with PKH26 and CFSE

EVs were isolated from T47D cells as described in section 2.2.1. The EV pellet was firstly labelled with CFSE as in section 3.3.2 and immediately stained with PKH26 as described in section 3.3.1. Dual labelled EVs were incubated with cells cultured on coverslips for 3 h. Cells were washed with PBS and fixed with 4 % PFA for 20 min at room temperature and then mounted with ProLong™ Gold antifade mountant with DAPI on slides for microscopic analysis.

Staining of cells with PKH26 and CFSE

T47D cells were cultured in T175 flasks until they reached 50 % confluency. The cells were then stained with 0.5x CFSE (100 µl of the 10x stock in 20 ml medium) for 10 min. The CFSE staining solution was removed and the cells were washed with PBS and stained with 0.5 µM PKH26 for 15 min. The PKH26 staining solution was removed, the cells were washed with PBS, and EV depleted medium was added for 48 h. At the same time, a group of cells was stained only with CFSE and another only with PKH26. EV isolation was performed as described in section 2.2.1.

To image the labelled EVs, 2% low melting agarose (Appleton Woods) was dissolved in PBS by boiling and, when cool enough, was mixed with an equal volume of EVs. EVs in low melting agarose were applied onto a microscope slide, covered with a coverslip and imaged using an LSM 880 confocal microscope and the Plan–Apochromat 100x/1.4 oil objective.

3.3.4 Labelling of EVs using a fluorescently tagged EV marker

The EV marker CD81 fused with the fluorescent protein mEmerald (variation of GFP) was overexpressed in T47D and hTERT-HME1 cells to produce fluorescently labelled EVs. The cells were stably transfected with the mEmerald-CD81 plasmid (described in detail in section 2.1.2) (Figure 2.1). The plasmid was verified by sequencing of a small area containing part of mEmerald, the 30 nucleotides linker between mEmerald and CD81, and part of the CD81 gene using the common sequencing primer EGFP_C. Sequencing services and primer were provided by Source Biosciences. Isolation of EVs from the stably transfected cells was as described in 2.2.1.

3.4 Results

3.4.1 PKH26 labelled EVs are internalised by recipient cells

In order to test whether labelling EVs with PKH26 was an optimal method to study EV uptake, EVs from T47D and MCF7 cells were isolated and stained with PKH26 as described in section 3.3.1, and applied on unlabelled recipient cells for 3h at a ratio of approximately 700 vesicles/cell. An equal volume of 'no EV' controls was administered to cells under the same conditions to serve as a control for non-specific staining. Using confocal microscopy, it was demonstrated that both cell lines successfully took up labelled EVs (Figure 3.3 A), while cells that had received the 'no EV' control appeared to have a substantially lower, but still prominent, amount of red fluorescence (Figure 3.3 B). Cells treated with PBS as a control do not reveal any indications of autofluorescence (Figure 3.3 C). These data show that labelled EVs were internalised by recipient cells but do not eliminate the possibility of non-specific staining, perhaps due to dye carry-over during the clean-up process.

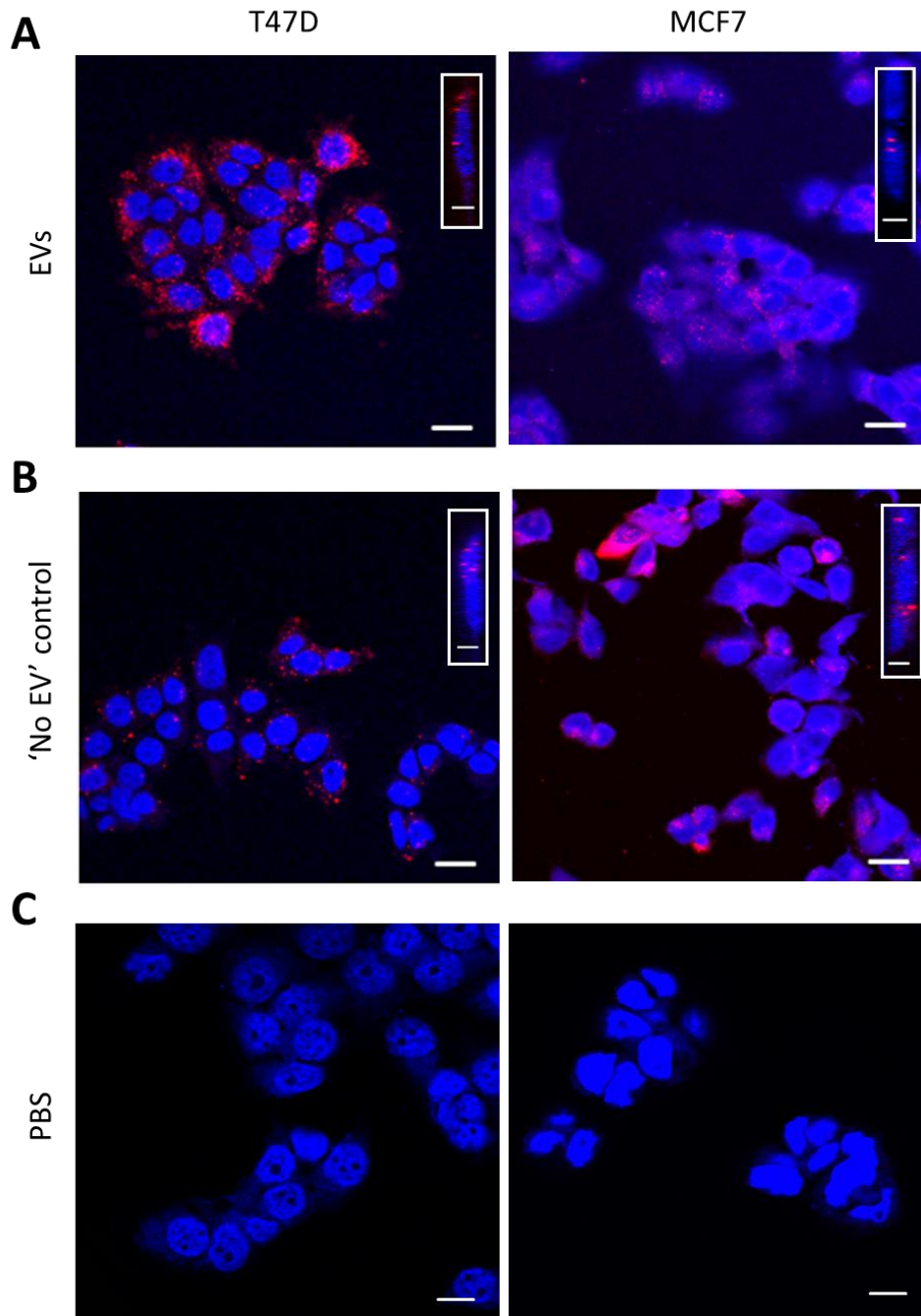


Figure 3.3 Uptake of PKH26 labelled EVs.

T47D (left) and MCF7 (right) cells after 3h incubation with A) PKH26 labelled autologous EVs, B) 'no EV' control, and C) PBS. After incubation, the cells were washed and fixed. Single Z-plane confocal images, DAPI blue, PKH26 red. Scale bar, 10 μ m. The insets show YZ view; scale bar, 5 μ m.

3.4.2 Use of BSA during PKH26 EV staining affects background fluorescence

To test whether BSA, used in the general labelling protocol, was responsible for the non-specific staining observed in Figure 3.3, the BSA quenching step was eliminated during EV labelling. Briefly, half of the samples were mixed with BSA, while the other half were brought to the same

volume with diluent C as shown in Figure 3.2. No difference in fluorescence between EV containing and 'no EV' control samples was observed when BSA was used to quench residual PKH26 dye (Figure 3.4). However, comparing the two EV samples, using BSA appeared to reduce fluorescence, probably eliminating staining by the free dye. These results were consistent for both cell lines examined (MCF7 and T47D). These observations suggest that using BSA during exosome staining with PKH26 may result in false fluorescent signals but eliminating this step does not improve the non-specific staining.

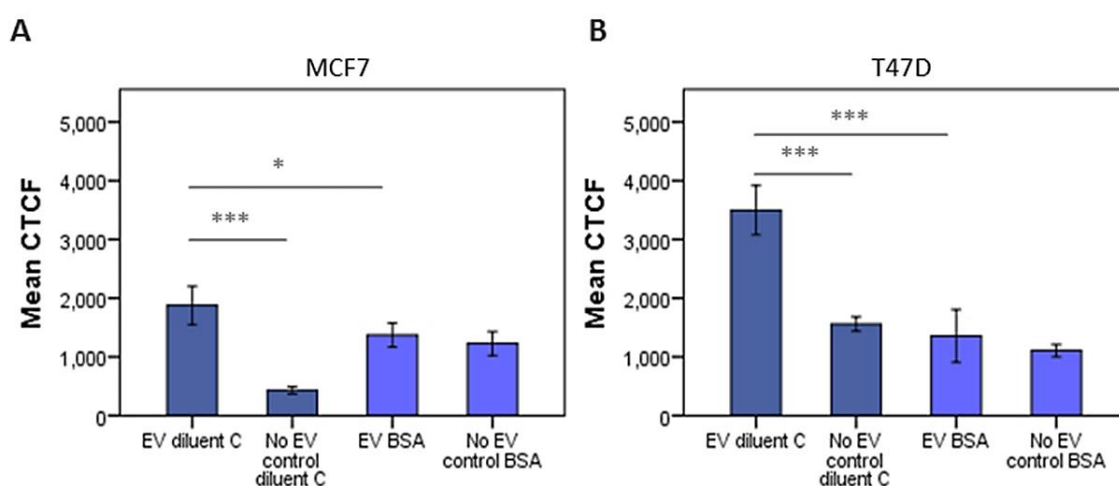


Figure 3.4 The effect of BSA on PKH26 labelling of EVs.

EVs and 'no EV' controls were either mixed with BSA to quench residual dye or diluted further with diluent C during PKH26 staining. A) MCF7 cells were incubated with PKH26 labelled MCF7 cell-derived EVs or 'no EV' controls for 3 h. B) T47D cells were incubated with PKH26 labelled T47D cell-derived EVs or 'no EV' controls for 3 h. Cells were then washed and fixed. Projected images were created according to the max intensity of each z-stack and the corrected total cell fluorescence (CTCF) was calculated. $N \geq 112$ cells per group, two biological replicates combined. Error bars, 95% CI. The Mann-Whitney test was used to determine the significance of differences. *** $P < 0.001$; * $P < 0.05$.

3.4.3 Uptake of CFSE labelled EVs might be masked by dye carry-over

To test whether labelling EVs with CFSE is an optimal method to study EV uptake dynamics, EVs from T47D cells were isolated and stained as described in section 3.3.2, applied on unlabelled recipient cells, and uptake was monitored in real time using confocal microscopy. The cells that had received stained EVs acquired green fluorescence immediately after the addition of the EVs (Figure 3.5 A). The non-EV control was clear of any fluorescence, demonstrating that there was no free CFSE in the preparations (Figure 3.5 B). In order to see how free CFSE would behave when added on the cells, a small amount of CFSE was added on T47D cells. The cells immediately incorporated the CFSE and became fluorescent (Figure 3.5 C). The time needed for EV uptake as

indicated by this experiment was rather too short and more comparable to the time needed for the free dye to enter the cells. According to previous studies, EVs start to enter the cells after approximately 10 min of administration reaching maximal uptake levels within a few hours (Tian et al., 2014, Heusermann et al., 2016). Even though the non-EV control indicates that there is no CFSE free dye in the preparations, the possibility that the EV preparation carries some free CFSE that could have caused the immediate staining of the cells cannot be excluded. Again, these experiments highlight the need for optimised EV labelling techniques that would allow the study of EV uptake.

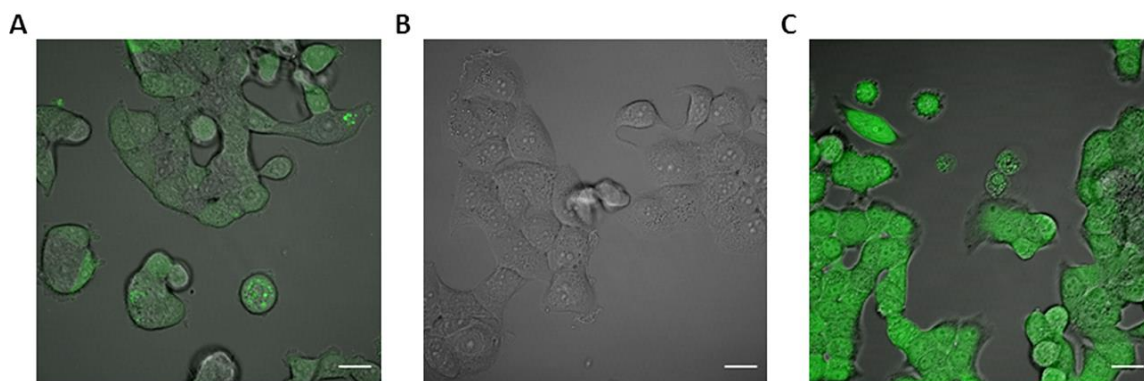


Figure 3.5 Confocal live cell imaging of CFSE labelled EV uptake.

Snapshots of T47D cells after 10 min incubation with A) CFSE labelled EVs, B) 'no EV' control, and C) CFSE dye. CFSE green. Scale bar, 20 µm.

3.4.4 CFSE and PKH26 dual labelling of EVs does not increase specificity of uptake

To test whether the combination of CFSE and PKH26 staining could increase the specificity of EV labelling and therefore uptake, EVs were dually labelled and administered to intact/ non-labelled cells. Live cell imaging showed that already at 10 min after administration of EVs, the cells became green but not red, indicating that the acquired fluorescence was not due to EV uptake. Red particles were accumulated in the extracellular region, similar to the 'no EV' control (Figure 3.6 A). The same experiment was repeated this time fixing the cells 30 min, 1h, 2h and 4h after EV administration. Using confocal microscopy, it became apparent that EVs were taken up by recipient cells within the first hour of incubation (Figure 3.6 B). Afterwards, the cells were observed to lose the brightness of green fluorescence, while accumulating red. These results suggest that the 'no EV' controls were clear of free CFSE dye but not of PKH26, which stained the cells in a punctate like manner over time.

Dual labelling of EVs did not result in a homogenous population of simultaneously red and green vesicles (Figure 3.6 C). In the 'no EV' control, red vesicle-like structures were observed, indicating that PKH26 might form micelles that could ultimately contribute to the non-specific labelling.

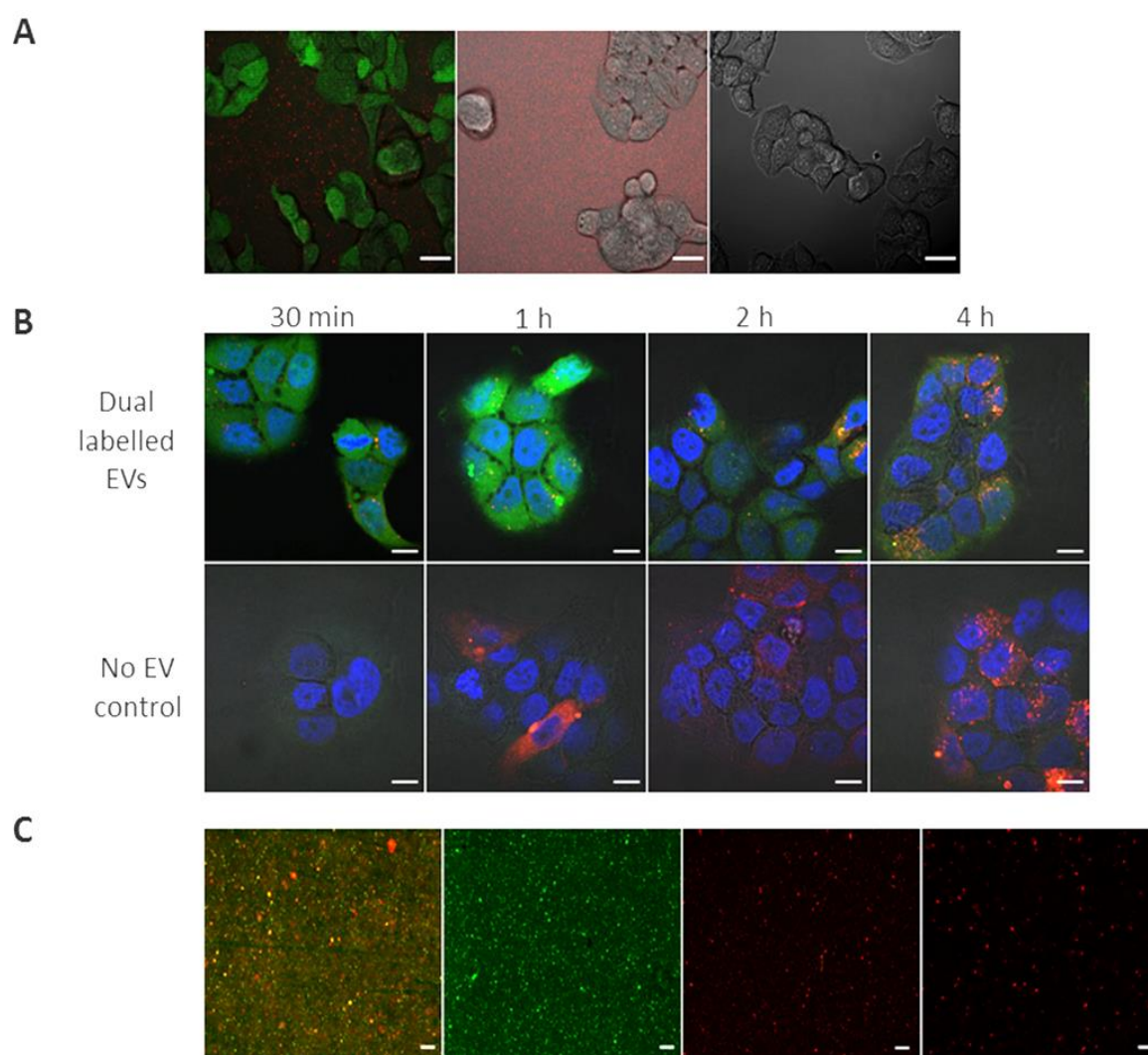


Figure 3.6 Dual labelling of EVs using PKH26 and CFSE.

A) From left to right: Live cell imaging of T47D cells 10 min after administration of PKH26 and CFSE dual labelled T47D-derived EVs, 'no EV' control and no treatment. Scale bar, 20 μ m. B) Uptake of dual labelled EVs by T47D cells over time. Labelled EVs were administered to T47D cells for 30 min, 1 h, 2 h and 4 h. Cells were then washed and fixed. Scale bar, 10 μ m. C) From left to right: PKH26 and CFSE dual labelled EVs, CFSE labelled EVs, PKH26 labelled EVs, PKH26 and CFSE dual labelling 'no EV' control. Scale bar, 5 μ m. DAPI blue, CFSE green, PKH26 red.

3.4.5 Labelling diversity in EVs produced by cells stained with CFSE and PKH26

Staining of EVs with CFSE, PKH26, or combination of the two produced non-specific uptake results. To test whether staining of the parental cells would increase the specificity of EV labelling, T47D cells were stained with PKH26, CFSE or both, as described in section 3.3.3, and

conditioned medium was collected 48h later for EV isolation. EVs from stained cells (Figure 3.7) were observed to be smaller in size compared with directly labelled EVs, clumps were not as common, and the proportion of green and red fluorescence was more balanced. However, similar to results obtained from direct EV staining, a population of simultaneously red and green particles was not achieved. These results show that stained cells release a heterogeneous population of EVs with variable labelling patterns (only green, only red, green and red).

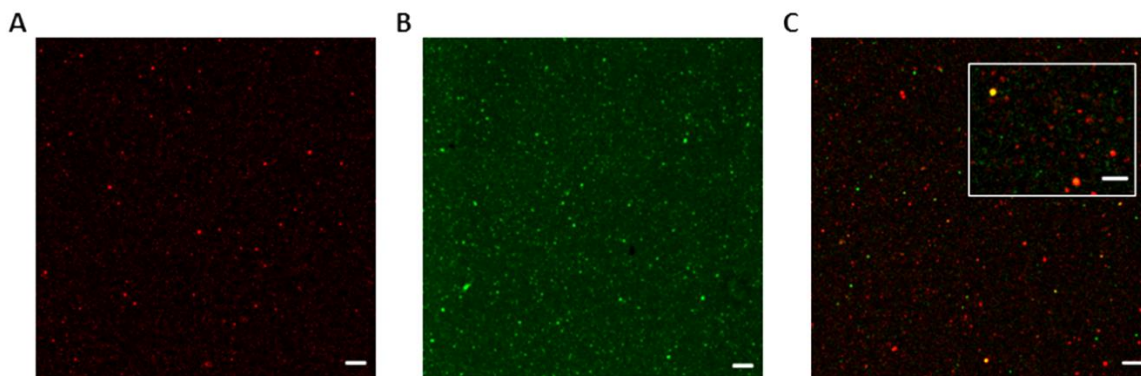


Figure 3.7 Indirect dual labelling of EVs.

Confocal imaging of EVs from A) PKH26, B) CFSE, and C) PKH26 and CFSE labelled cells. Scale bar, 5 μ m. The inset shows magnified area. Scale bar in the inset, 2 μ m.

3.4.6 mEmerald-CD81 EVs are favourable for EV uptake studies

To avoid the non-specific binding of the generic dyes, the next step was to fluorescently tag an EV protein marker. For this reason, the fusion protein of CD81 and mEmerald and stably overexpressed in hTERT-HME1 and T47D cells. Distribution of mEmerald-CD81 was as expected; mainly on the cell membrane and protrusions (Chang and Finnemann, 2007; Quast *et al.*, 2011), but was also found in subcellular compartments as shown in Figure 3.8 A. The mEmerald-CD81 EVs produced by the stable lines appeared to be smaller in size than when labelled with the dyes and the presence of clumps was minimal (Figure 3.8 B). The mEmerald-CD81 EVs were also labelled with immunogold against CD81 and imaged using TEM (Figure 3.8 C). To further characterise these EVs, western blot analysis, performed by Vicente Pagalday Vergara, Oxford Brookes University, showed the existence of both the parental CD81 (~ 25 kDa) and the overexpressed fusion protein mEmerald-CD81 (~ 50 kDa) in the transfected cells and EVs, compared with the non-transfected cells and EVs that only expressed the parental form of CD81 (Figure 3.8 D). When these EVs were added to non-transfected cells uptake could be observed from as early as 10 minutes and could be distinguished as individual events (Figure 3.8 E). Uptake pattern of mEmerald-CD81 EVs was considerably different to that of PKH26 or CFSE

labelled EVs, highlighting the possibility of false-positive signals by these dyes and compromise of uptake interpretation. Therefore, mEmerald-CD81 EVs were used in subsequent experiments to study uptake dynamics.

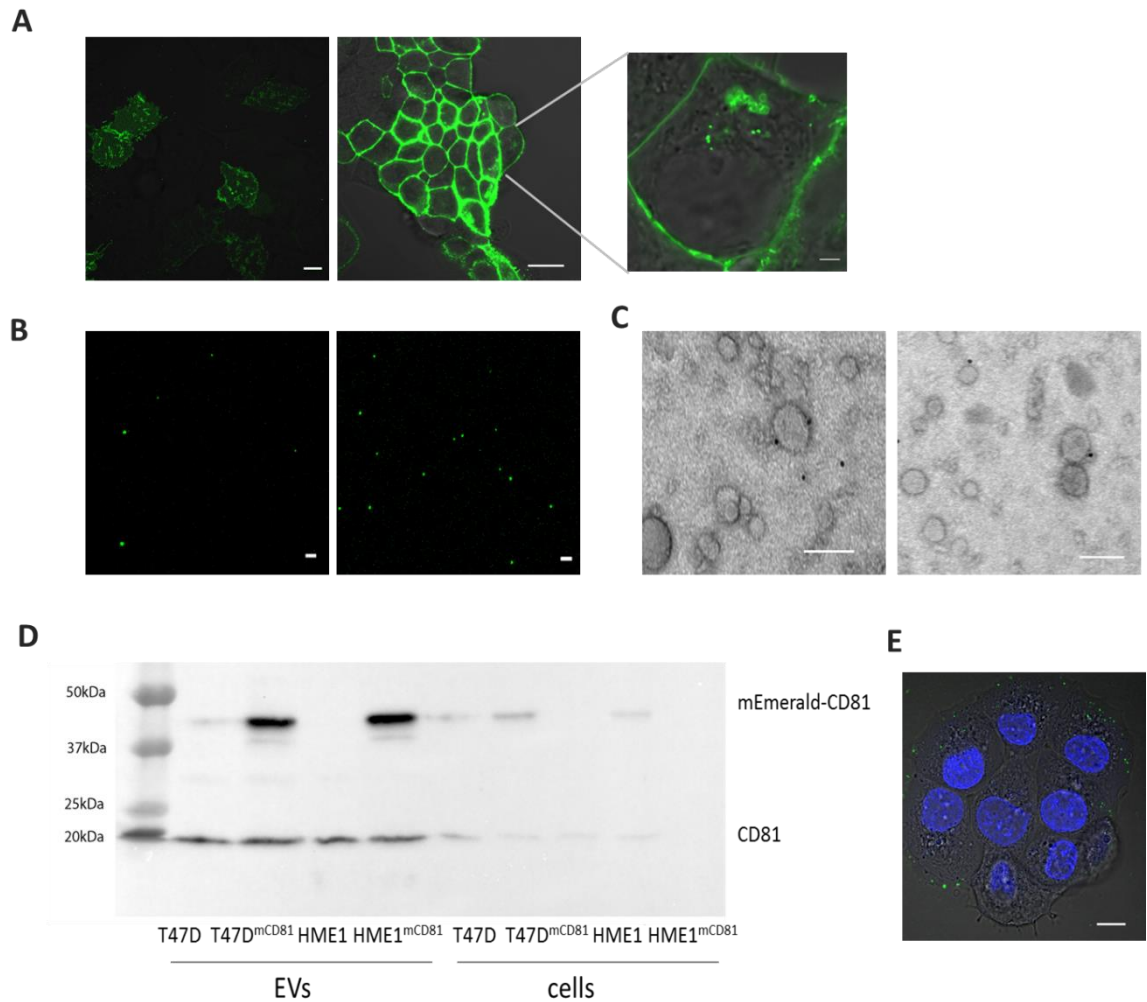


Figure 3.8 mEmerald-CD81 labelled EVs.

A) Confocal images of stably transfected hTERT-HME1 (left) and T47D (right) cells. Scale bar, 20 μm . Zoomed image acquired by airy scan. Scale bar, 2 μm . B) Confocal images of mEmerald-CD81 EVs from hTERT-HME1 (left) and T47D (right) cells in low melting agarose. Scale bar, 2 μm . C) Electron micrographs of mEmerald-CD81 EVs from hTERT-HME1 (left) and T47D (right) cells. Immunogold labelling against CD81. Scale bar, 100 nm. D) Western blot of parental cells (hTERT-HME1 and T47D), transfected cells (mEmerald-CD81 hTERT-HME1 and T47D), and EVs from parental and transfected cells. E) Uptake of mEmerald-CD81 T47D EVs by recipient non-transfected T47D cells after approximately 2 h of administration. Scale bar, 10 μm . Hoechst blue, mEmerald green.

3.5 Discussion

EV labelling using PKH26

PKH26 is one of the most common dyes used for EV labelling. This chapter highlights that PKH26 labelling is not EV-specific, and free dye carry-over in EV and 'no EV' preparations can bind and thus stain membrane-rich compartments of target cells. Even though the pattern of labelling shown in Figure 3.3 is characteristic of EV uptake as published in numerous studies (Singh *et al.*, 2016; Sento *et al.*, 2016; Bilen *et al.*, 2017), the 'no EV' control reveals non-specific labelling of the same pattern, which was unexpected at the time of these experiments. In an attempt to troubleshoot this, quenching with BSA during PKH26 labelling was excluded from the routine protocol. However, it became apparent that BSA was useful to quench residual dye, as the EV preparations that were not treated with BSA yielded subsequently increased fluorescence in the recipient cells compared with the BSA treated EVs (even though same EV numbers were used) (Figure 3.4). Due to its long aliphatic chain, PKH26 is able to form micelles of size equal to that of EVs, therefore making them difficult to distinguish from EVs using size-based methods like ultracentrifugation or Zeba™ columns. It is slowly becoming clear in the field that the use of PKH26 and other lipophilic dyes for EV labelling might contribute to non-specific labelling of cells, masking the actual EV effects (Takov *et al.*, 2017). The first systematic study on PKH26 formed nanoparticles during EV labelling showed that micelles are internalised by cells possibly by endocytosis, similar to EVs (Puzar Dominkus *et al.*, 2018). The same study also suggested that PKH26-labelled EVs should be isolated using methods that depend on the density of the vesicles (e.g. sucrose gradient centrifugation) in order to acquire a more homogeneous population of labelled EVs, clear of 'hollow' dye particles. Another important characteristic of PKH dyes, which serves as a disadvantage in this case, is their long half-life which outlasts that of EVs (Lai *et al.*, 2014). Therefore, for the purposes of this project – the study of EV uptake dynamics –, labelling of EVs with PKH26 was concluded to be not ideal.

CFSE labelling of EVs

Another method to label EVs is by staining their cargo. CFSE has been used to fluorescently label the protein load of vesicles (Pospichalova *et al.*, 2015; Aliotta *et al.*, 2015). In the experiments reported here, administration of CFSE labelled EVs rapidly and homogeneously stained the recipient cells, a phenomenon probably caused by CFSE dye carry-over; even though the 'no EV' controls were clear of fluorescence. Cells treated with CFSE labelled EVs seemed to become fluorescent in the same way as when treated with CFSE dye alone. These observations suggest that EV labelling using CFSE were not suitable for uptake experiments.

Dual labelling of EVs

To increase the sensitivity of EV labelling, a combination of CFSE and PKH26 was used. Dual staining of EVs using CFSE and PKH26 has not been reported before. This method provides simultaneous labelling of the EV membrane and cargo, potentially allowing the study of EV uptake dynamics. Directly stained EVs, however, did not yield encouraging results, as CFSE homogeneously stained the recipient cells almost immediately after administration, while PKH26 was internalised at later points. Therefore, this experiment indicated that the changes in the accumulation of fluorescence in the recipient cells were not specific to EV uptake, as this would suggest simultaneous internalisation of CFSE and PKH26. Imaging of the EVs showed that a large population of them were only red or/ and only green and that PKH26 micelles were present in the 'no EV' control samples. The next step was to isolate EVs from PKH26 and CFSE dual stained cells. Staining the cells and then collecting the EVs they release is an uncommon method, but has been reported before (Pospichalova *et al.*, 2015; Monopoli *et al.*, 2018). The populations of the collected EVs were equally diverse, consisting of not only yellow (dual labelled) but also red and green EVs; similar to the EV diversity observed by Heusermann *et al.* (2016) when they used CD63-GFP/CD63mCherry co-transfected cells. Using these EVs in uptake experiments would not allow the discrimination between the single labelled EVs and the ones that have dissociated, for example, due to cargo delivery.

mEmerald-CD81 labelled EVs

EV-enriched proteins conjugated with fluorescent proteins have been utilised as a method to visualise EVs, and more specifically to investigate EV uptake and delivery mechanisms (Heusermann *et al.*, 2016; Purushothaman *et al.*, 2016). For the purposes of this project, the CD81 protein, which is enriched in small EVs (Kowal *et al.*, 2016), was fused with the fluorescent protein mEmerald and stably overexpressed in hTERT-HME1 and T47D cells. The mEmerald-CD81 cells released bright green fluorescent EVs that carried the fusion protein. The hTERT-HME1 and T47D mEmerald-CD81 EVs were characterised by TEM and western blot. Uptake of these vesicles was clear of the artefacts produced by the common dyes used previously and appeared smaller in size than the PKH26 and/or CFSE labelled EVs. Another advantage of the mEmerald-CD81 EVs is that this type of labelling is predicted not to interfere with EV surface molecules that might be useful for EV – cell interactions, uptake, etc.

One of the challenges faced when imaging EVs is their small size that is below the diffraction limit of light. However, provided that the EVs are bright enough, even though they are below the resolution limit of the light microscope (200 nm), they will still be visible. This is due to point

spread function that makes fluorescent objects appear larger than they really are. Here, mEmerald, a molecule brighter than GFP was used. On the other hand, brightness also depends on the number of CD81 molecules present on the membrane of EVs, which has not been established yet. One way of making EVs brighter and easier to follow would be the use of molecules such as the nano-boosters (Chromotek), which are fluorescence enhancers highly specific for GFP and other fluorescent proteins. Another way to overcome the limitations imposed by the diffraction limit of light is to use super-resolution imaging techniques, such as Airyscan (Miranda *et al.*, 2018), PALM/STORM (Chen *et al.*, 2016), TIRF (Heusermann *et al.*, 2016), etc.

Other EV labelling and uptake monitoring methods

Other EV labelling methods have also been described and are listed in Table 3.1. An interesting generic method to label EVs is by targeting the thiol (-S-H groups) with a maleimide group conjugated with a fluorophore (Roberts-Dalton *et al.*, 2017). Even though this method could be used to label different sized EV populations, it could affect EV behaviour by altering membrane fluidity through protein crosslinking. Lai *et al.* (2015) developed fluorescent EV reporters by linking a palmitoylation signal to a fluorescent protein (PalmGFP and PalmtmTomato). This targeted the fluorescent protein to the plasma membrane, thus labelling the cells and allowing the release of labelled EVs. This method allowed the labelling and study of different subtypes of EVs and the tracking of EV exchange between differentially labelled cells. Another interesting method of monitoring EV uptake was developed by (Zomer *et al.*, 2015; Zomer *et al.*, 2016) and is based on the Cre-loxP system. Briefly, Cre-reporter cells were fluorescently labelled green when they took up EVs derived from Cre-recombinase expressing cells, while cells that did not internalise EVs were labelled red. Even though the knowledge around EV biology has rapidly evolved in the last years, further technological advances are required to fully characterise EV uptake and EV-mediated communication.

3.6 Key findings

- PKH26 labelling produces non-EV associated particles that result in false-positive signals.
- The use of BSA during PKH26 EV staining decreases but does not eliminate the non-specific fluorescence/ binding of the dye.
- Using CFSE labelled EVs in uptake experiments results in non-EV specific staining of recipient cells.
- Dual labelling of EVs with CFSE and PKH26 does not improve specificity of the labelling, and further confirms the unsuitability of these dyes for uptake tracking.

- Cells stained with CFSE and PKH26 release a diverse population of EVs with heterogeneous labelling.
- Cells stably expressing mEmerald-CD81 release fluorescent EVs with the typical morphology and are suited for EV uptake studies using light microscopy.

Investigations into the dynamic uptake of EVs

Chapter 4

4 Investigations into the dynamic uptake of EVs

4.1 Background

Once released into the extracellular milieu, EVs may reach their target cells and trigger a signalling cascade that would bring about phenotypic changes. The first step towards this type of communication is the docking of EVs onto the cell membrane. Target specificity is mediated through EV surface molecules and corresponding receptors on the cell. Such molecules include glycans and lectins (Barres *et al.*, 2010; Christianson *et al.*, 2013), tetraspanins (Rana *et al.*, 2011), integrins (Morelli *et al.*, 2004; Hoshino *et al.*, 2015), lipids (Frey and Gaip, 2011) and the major histocompatibility complex (Lynch *et al.*, 2009). After docking to the plasma membrane, EVs may stay bound (Denzler *et al.*, 2000) or be internalised via several different routes (Mulcahy *et al.*, 2014). Uptake of EVs has been shown to occur via: a) direct fusion between the EV and the cell membrane (Parolini *et al.*, 2009), b) lipid rafts (Svensson *et al.*, 2013), c) clathrin – dependent endocytosis (Escrevente *et al.*, 2011), d) caveolin-dependent endocytosis (Nanbo *et al.*, 2013), e) macropinocytosis (Fitzner *et al.*, 2011), and f) phagocytosis (Feng *et al.*, 2010), as described in section 1.1.3. After uptake, EVs follow the endocytic pathway, entering early endosomes and eventually finding their way to the multivesicular bodies. They are then targeted towards degradation in the lysosomes or release their contents in the cytoplasm by fusion with the limiting membrane of the multivesicular endosomes (Tian *et al.*, 2010; Bissig and Gruenberg, 2014).

Current understanding of EV uptake routes is highly dependent on the type of cells, type of EV labelling, method of observation and uptake inhibitors used for each study. It still remains unclear whether different mechanisms are employed by different cells and whether these are affected by diverse conditions. Interestingly, Riches *et al.* (2014) demonstrated that there is a positive feedback loop regulating EV release triggered by autologous EVs, which did not stand true for non-self EVs. More specifically, using a normal immortalised breast cell line and its cancerous counterpart, they showed that even though autologous EVs further enhance EV release, incubation with cancer EVs inhibited EV secretion by normal cells. Nakase *et al.* (2015) showed that EGF in EVs increases their uptake efficiency via macropinocytosis. Taken together, these studies suggest that EVs from certain cells are preferentially internalised by specific targets.

The mechanisms through which EVs interact with the plasma membrane of the target cells also vary among different cell types. For example, leukaemia cell-derived EVs were preferentially

incorporated by macrophages (phagocytic cells) but remained docked to the membrane of non-phagocytic cells (Feng *et al.*, 2010). Likewise, microglia-derived EVs moved along the membrane of microglia cells towards the nucleus before their internalisation, however, they stay attached to the surface of astrocytes (Prada *et al.*, 2016). These observations suggest preferential interactions of EVs with specific cell types both during docking and uptake. The molecular level of such interactions has been studied before and part of this specificity is attributed to molecules on the EV and cell surfaces. Such molecules include tetraspanins, extracellular matrix proteins, integrins, proteoglycans and lectins (French *et al.*, 2017). Interestingly, the role of lipids in EV-membrane interactions has not been studied exhaustively, even though lipids, for instance in lipid rafts, have been reported to participate in EV uptake (Svensson *et al.*, 2013). Several studies have modified the properties of the plasma membrane to investigate whether they affect uptake (Holder *et al.*, 2016; Escrevente *et al.*, 2011; Svensson *et al.*, 2013). Such modifications lead to abrogation of active internalisation processes such as endocytosis and macropinocytosis, but also passive by changing the membrane permeability through disruption of lipid rafts (cholesterol rich areas providing membrane rigidity). Classically, investigations disentangling the molecular interactions between EV and plasma membrane during docking and uptake involve the use of pharmacological drugs such as amiloride, simvastatin, dynasore and others. In this study, to examine the possible routes of EV binding and uptake in all combinations of cells and EVs, three drugs, some also employed by other studies to inhibit uptake, were used; amiloride, dynasore, and cholesterol oxidase.

Amiloride

Amiloride is a pyrazine containing compound, extensively used in the clinic to treat conditions like high blood pressure and liver cirrhosis (WHO). Its structure allows for the efficient inhibition of Na⁺/H⁺ channels that further leads to macropinocytosis suppression. Because amiloride shows diverse affinities for the different Na⁺/H⁺ channels, various analogues have been synthesised. The type of amiloride used in most studies (Nakase *et al.*, 2015; Koivusalo *et al.*, 2010; Escrevente *et al.*, 2011) and also used here was the (5-(N-ethyl-N-isopropyl)) amiloride, which is specific for the sodium-hydrogen antiporter 1. The exact mechanism through which amiloride blocks macropinocytosis is not completely understood. Koivusalo *et al.* (2010) showed that amiloride decreases sub-membranous pH, inhibiting Rac1 and Cdc42 signalling, that further blocks macropinocytosis. Moreover, they demonstrated that macropinocytosis is not directly sensitive to amiloride but rather to the acidification caused by this drug. Acidification also affects other types of endocytosis such as the clathrin-mediated endocytosis, though lower pH values are required to confer changes compared to macropinocytosis (Koivusalo *et al.*, 2010).

Dynasore

Dynasore is a carbohydrazide, originally introduced as a dynamin inhibitor (Macia *et al.*, 2006). Dynamin belongs to the GTPase family of proteins and is essential for the membrane fission and subsequent production of free vesicles in the cell during endocytosis. Dynamin is an essential player of clathrin-mediated endocytosis; it exists in tetramers that form rings and helices to allow vesicle budding (Hinshaw, 2000). Several studies have used dynasore as an inhibitor of endocytosis. However, accumulating evidence suggests that dynasore can confer dynamin-independent responses, in addition to the dynamin-dependent effects (Preta *et al.*, 2015a). These include the remodelling of actin filaments, reduction of plasma membrane cholesterol due to impairment of membrane recycling, and finally disruption of lipid rafts (Park *et al.*, 2013; Preta *et al.*, 2015b).

Cholesterol oxidase

Cholesterol oxidase is a flavoenzyme that catalyses the oxidation and isomerization of cholesterol into cholest-4-en-3-one, thus changing its membrane-ordering properties. Cholesterol, provides a higher degree of order and rigidity to fluid membranes, while saturated lipid acyl chains still bear lateral mobility (Kreit and Sampson, 2009). Cholesterol oxidase does not remove cholesterol from the membrane but instead changes its form, therefore it is considered less harmful for the cells. Cholesterol oxidase has not been previously used in EV uptake experiments, however, filipin III, an inhibitor of lipid raft- and caveolin- dependent endocytosis, has been shown to significantly lower EV uptake in endothelial cells (Svensson *et al.*, 2013). Therefore, it is anticipated that cholesterol oxidase would influence uptake, indicating that a passive mechanism might also be employed during EV internalisation.

4.2 Aim and objectives of chapter 4

Several mechanisms have been proposed to be responsible for EV docking and uptake, involving either active or passive cellular responses. It cannot be discounted that cells may employ more than just one internalisation mechanism, most of which critically rely on the plasma membrane properties. Here, it was hypothesised that EVs of different origin may employ different EV uptake routes and the focus was placed on their interactions with the plasma membrane of target cells. The aim of this chapter was to disentangle EV specificity in cellular docking and uptake and the role of plasma membrane properties. This overarching aim was tackled with the following specific objectives:

- To explore the specificity of EV docking and uptake across different cell lines.

- To investigate macropinocytosis, endocytosis and membrane rigidity as possible routes of EV uptake.
- To characterise EV-plasma membrane interactions and the role of cholesterol.

4.3 Methods

4.3.1 Experimental setting

EVs were isolated from mEmerald-CD81 T47D and mEmerald-CD81 hTERT-HME1 stably transfected cells as described in section 2.2.1. T47D, hTERT-HME1 and HeLa cells were seeded in glass bottom μ -slide 8 well plates (ibidi) in complete medium until approximately 60-70% confluency. Drug treatments were performed as described in section 2.3.1. Prior to each experiment, cells were washed with PBS and stained for 3 min with Cell Mask Deep Red (1:1000 dilution in medium, ThermoFisher). EVs were diluted in EV depleted medium and administered to the cells (total volume 200 μ l). Based on some preliminary experiments (not shown) the amount of EVs applied was kept the same for all experiments (30,000 EVs, as counted by NTA, per seeded cell). Live cell imaging was performed using the LSM 880 confocal microscope and the Plan-Apochromat 100x/1.4 Oil objective maintaining conditions of 37°C and 5% CO₂. Excitation and detection wavelengths were 488 and 493-585 for mEmerald (laser power 1.5 %), and 633 and 640-748 for Cell Mask Deep Red (laser power 1 – 5 %). Videos consisted of 100 frames taken at a single plane spanning the cell monolayer on the Z-axis (1.27 sec per frame) at time points 1, 2, 3 and 4 representing 10 min, 40 min, 70 min, and 100 min after EV administration, respectively. Overall, three cell lines (T47D, hTERT-HME1, HeLa), two types of EVs (mEmerald-CD81 T47D and mEmerald-CD81 hTERT-HME1), 4 treatments (amiloride, dynasore, cholesterol oxidase, and control) were tested in all possible combinations. Representative images of the videos can be found in Appendix 2 (Figure 7.3).

4.3.2 Image analysis

EV counting

EVs were counted as individual events using FIJI. Briefly, the first ten of 100 frames of the confocal image plane time series (for a given experimental time-point (i.e. points 1,2,3 or 4)) were sum projected (the sum of the intensity of each of the 512x512 pixels is combined and projected onto one image) and the image was converted to 8-bit (this reduces file size without compromising quality) (Figure 4.1 A). Regions of interest (here membrane and/or cytoplasm) were drawn using the free hand tool from the FIJI toolbar. Thresholding was applied empirically

to produce binary images, so that all pixels that were part of the same object (EV) had the same, unique value (Figure 4.1 B). The 'analyse particles' plugin detects and measures all foreground areas as individual events, and therefore was used to find and count EVs of any size and circularity of 0.5 – 1. All data were further analysed using excel and SPSS for statistical analysis.

EV tracking

The full 100 frame time series were analysed using FIJI and the 'TRACKMATE' (Tinevez *et al.*, 2017). The videos were opened in FIJI, regions of interest were drawn to include the membrane or cytoplasm of the cells, and the TRACKMATE plugin was initiated. The Laplacian of Gaussian (LoG) detector was applied to filter the image with a sigma (pixel distribution based on local maxima) suited to the 'blob estimated size' (here 0.1 μm). Threshold was set empirically, to ensure all spots (EVs) were detected and the threshold on 'spot quality' was set automatically. The HyperStack Displayer was used to overlay the spots on the current hyperstack window. The Simple Linear Assignment Problem (LAP) tracker was used to link spots from frame to frame to build track segments; 'linking max distance' was set to 0.2 μm (equal to the sum of the diameters of two EVs), 'gap closing distance' was set to 0.2 μm and 'gap closing max frame' was set to 2 frames (Figure 4.1 C and D). To increase stringency and confidence that tracks represent genuine EV movement only tracks built up of more than 4 spots were selected for further analysis using excel and SPSS.

Statistical analysis of results

In most cases both ANOVA with Tukey post hoc test and Kruskal-Wallis multiple comparison tests were performed, but p values for differences between groups were calculated using the non-parametric Mann-Whitney test, as the data did not follow the normal distribution (confirmed using the Shapiro-Wilk test).

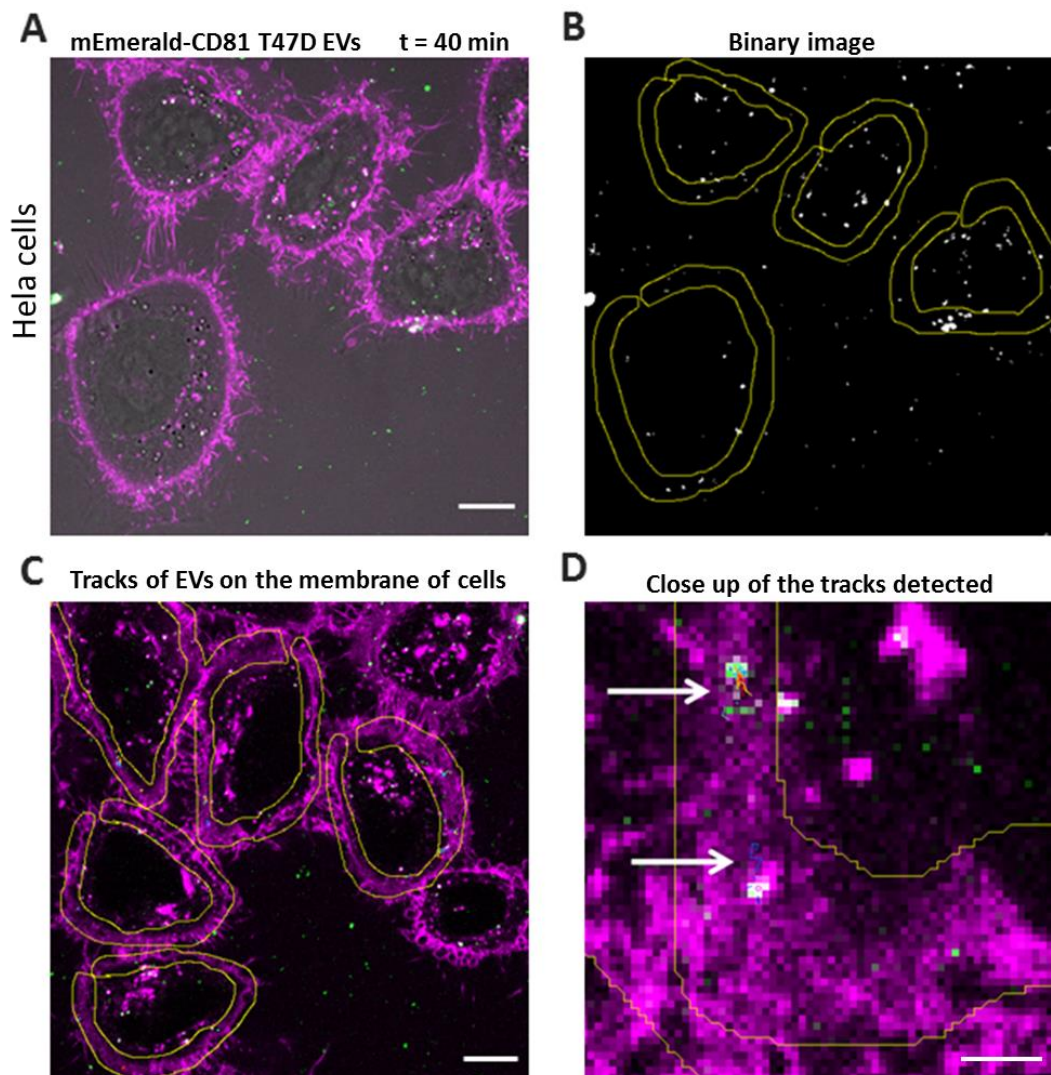


Figure 4.1 EV counting and tracking.

A) HeLa cells 40 min after mEmerald-CD81 T47D EV administration, the sample was randomly selected to explain the methodology followed. Scale bar, 10 μm . B) Regions of interest were selected (here membranes - yellow outline) and threshold was applied to create binary images. The white objects were counted using FIJI. C) Overlay of the different channels (green and magenta) and the tracks within the regions of interest as created by TRACKMATE. Scale bar, 10 μm . D) Zoomed area. Arrows show EV tracks (each track is labelled with a different colour). Scale bar, 2 μm . Cell mask deep red (membrane) magenta, mEmerald (EVs) green.

4.4 Results

4.4.1 Uptake depends on the recipient cell type

To elucidate EV binding and uptake specificity, three types of cell lines were employed; epithelial breast cancer cells (T47D), epithelial-like breast immortalised cells (hTERT-HME1), and epithelial cervical cancer cells (Hela). Fluorescent EVs from mEmerald-CD81 T47D and mEmerald-CD81 hTERT-HME1 cells (referred to as T47D^{mCD81} and HME1^{mCD81} EVs in the main text of this chapter)

stably transfected cells were isolated and incubated with the aforementioned cells (Figure 4.2 A). EVs were counted on the membrane and in the cytoplasm of the recipient cells. Figure 4.2 (B and C) shows the number of EVs docked at the plasma membrane in contrast to the ones incorporated by the three types of cells used here. Similar total numbers of T47D^{mCD81} EVs were found in all three cell lines. Interestingly, the number of T47D^{mCD81} EVs on the membrane did not vary much across cell lines, but some differences were found in the incorporated EVs. Notably, more T47D^{mCD81} EVs were internalised by hTERT-HME1 and HeLa cells in comparison to T47D cells ($p=0.062$ and $p=0.04$, respectively) (Figure 4.2 B). Interestingly, when uptake was investigated using HME1^{mCD81} EVs, each recipient cell line showed different total docking and internalisation levels (Figure 4.2 C), with HeLa cells showing the highest overall value. More specifically, lower numbers of EVs were found in the cytoplasm of T47D cells compared to the other two cell lines ($p=0.001$ in both cases), while hTERT-HME1 and HeLa cells incorporated similar high numbers of EVs. Observing the EVs on the membrane, T47D cells and hTERT-HME1 had a similar value, however, HeLa cells had significantly more EVs. These results suggest that uptake depends on the recipient cell type, and perhaps also on the EV type.

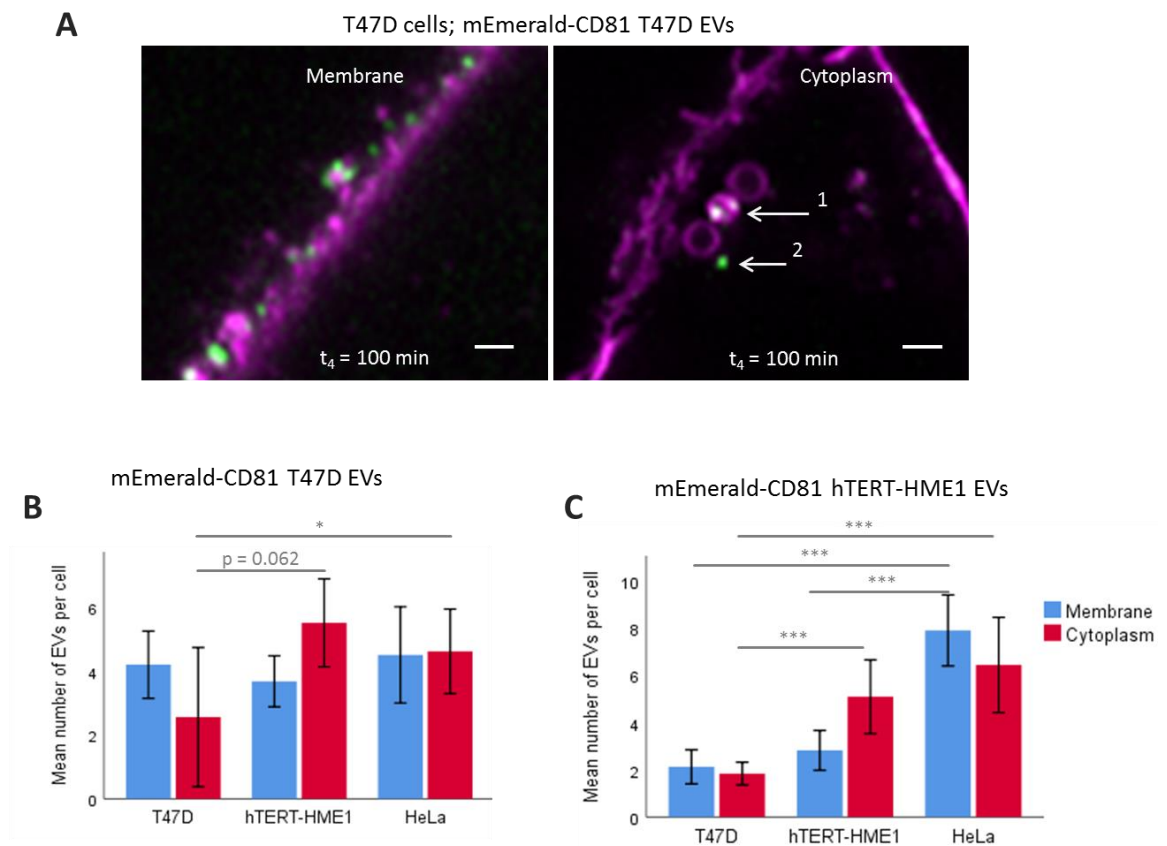


Figure 4.2 Uptake of EVs by different cell types.

A) Airyscan live cell imaging of mEmerald-CD81 T47D EVs by T47D cells. Scale bar, 1 μm . Left: EVs interacting with the plasma membrane. Right: EVs in the cytoplasm, in association with membranous structures (arrow 1) and alone (arrow 2). B) mEmerald-CD81 T47D EV numbers counted on the membrane and in the cytoplasm of T47D, hTERT-HME1, and HeLa cells. C)

*mEmerald-CD81 hTERT-HME1 EVs numbers counted on the membrane and in the cytoplasm of T47D, hTERT-HME1, and HeLa cells. $N_{\text{cells}} \geq 32$. Error bars, 95% CI. The Kruskal-Wallis test was used for multiple comparisons and the Mann-Whitney test to determine the significance of differences between groups. ***, $P < 0.001$; *, $P < 0.05$.*

4.4.2 EV origin affects docking on the membrane of recipient cells

To examine whether EV type affects uptake, further statistical analysis of the data was performed. It was apparent that T47D cells attracted more T47D^{mCD81} than HME1^{mCD81} EVs on their membrane, while HeLa cells showed the opposite (Figure 4.3 A). No differences were observed regarding the type of EVs in the cytoplasm of these cells (Figure 4.3 B). Similar numbers of both types of EVs were found on hTERT-HME1 cells, both on the membrane and in the cytoplasm. These results suggest that different types of EVs dock preferentially to recipient cell membrane, regardless of internalisation.

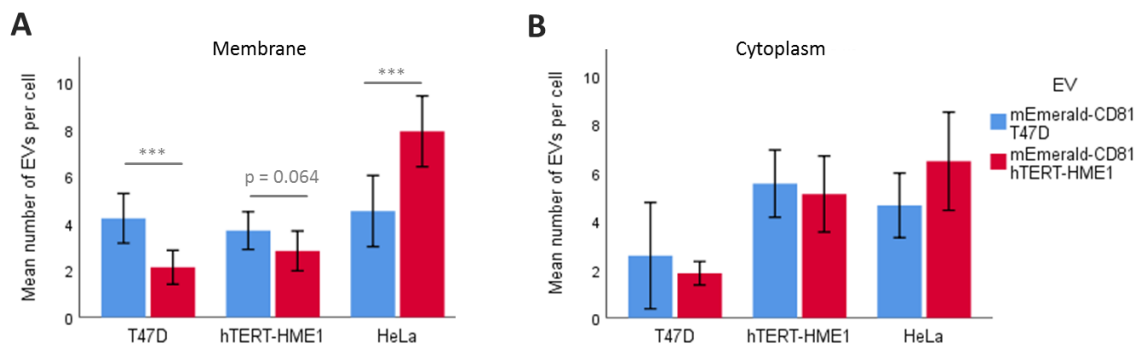


Figure 4.3 Uptake of different EV types.

*A) EV counts on the membrane of T47D, hTERT-HME1 and HeLa cells. B) EV counts in the cytoplasm of T47D, hTERT-HME1 and HeLa cells. $N_{\text{cells}} \geq 32$. Error bars, 95% CI. The Mann-Whitney test was used to determine the significance of differences between groups. ***, $P < 0.001$.*

4.4.3. Dynasore and cholesterol oxidase treatment affects EV uptake in a variable manner depending on the cell and EV type

To further explore the interactions of EVs with the plasma membrane, T47D, hTERT-HME1 and HeLa cells were pre-treated with amiloride, dynasore and cholesterol oxidase before EV addition. EV numbers were counted on the membrane of each cell for each treatment group. It was shown that the drugs differentially affected the amount of EVs found on the membrane of each cell type (Figure 4.4). Cholesterol oxidase increased the amount of both T47D^{mCD81} and HME1^{mCD81} EVs on the membrane of T47D cells ($p=0.014$, in both cases), while it had the opposite

results for these two types of EVs on HeLa cells ($p > 0.001$ for HME1^{mCD81} EVs and $p = 0.044$ for T47D^{mCD81} EVs). In hTERT-HME1 cells treated with dynasore, the amount of both types of EVs was significantly elevated ($p = 0.001$, in both cases). Amiloride appeared to be less effective, even though it increased the amount of both types of EVs on the membrane of hTERT-HME1 cells ($p = 0.013$ for HME1^{mCD81} EVs and $p = 0.012$ for T47D^{mCD81} EVs). In order to reduce the variables and focus on the role of lipids in EV uptake and interaction with plasma membrane, amiloride, which blocks an active mechanism of endocytosis, was omitted from subsequent analyses. Nevertheless, taken together these results show that drugs that modify the plasma membrane affect EV docking in various ways, depending on the cell and EV type.

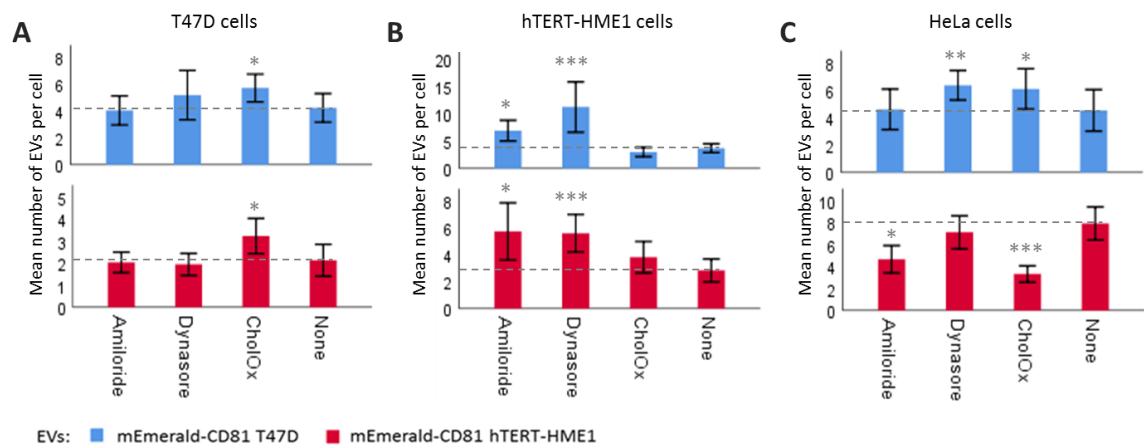


Figure 4.4 The effect of amiloride, dynasore and cholesterol oxidase on membrane EV numbers.

The drugs were added to A) T47D, B) hTERT-HME1 and C) HeLa recipient cells before EV administration and EV numbers were counted on the membrane of recipient cells. X-axis: treatment. Dashed line allows comparisons with the 'no-treatment' control. The Mann-Whitney test was used to calculate significance of difference between treatment and 'no-treatment' control. Error bars, 95% CI. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

4.4.4 Lipids may be involved in EV-membrane interactions

The next step was to examine whether the proportion of EVs on the membrane and in the cytoplasm changes over time. Here, EV numbers were expressed as percentages of EVs on the membrane and in the cytoplasm (Figure 4.5 C). To tackle variability effects among cells, EVs of all 8 cells were summed. For each condition, the proportion of EVs found on the membrane was expressed as a percentage of the total EVs (EVs on the membrane and in the cytoplasm of all 8 cells together made up 100% of the EVs). This analysis is not per cell, however, the same exact cells were monitored for different time-points. Graphs including all time-points tested can be

found in Appendix 2 (Figure 7.4 and Figure 7.5). Cells appeared to have already internalised EVs at the first time-point tested (10 min after EV administration). To compare changes between the first minutes and the overall duration of the experiment (100 minutes after EV administration), the difference between time-point 2 (40 min) or 4 (100 min) and time-point 1 (10 min after EV administration) (Δt_2-t_1 and Δt_4-t_1) was calculated and plotted for each cell line, EV type, and treatment (Figure 4.5 A).

It was shown that HeLa cells were responsive to cholesterol oxidase treatment, revealed by changes in the proportion of EVs on the membrane, regardless of EV type and time range (Figure 4.5 A i and ii). More specifically, both cholesterol oxidase and dynasore increased uptake of T47D^{mCD81} EVs by HeLa cells, while for HME1^{mCD81} EVs, only cholesterol oxidase appeared to increase it (Figure 4.5 B). Both cholesterol oxidase and dynasore act by modulating cholesterol content in the plasma membrane (known to disrupt lipid rafts), and thus altering membrane fluidity. These data reveal the importance of lipid packaging and membrane rigidity in uptake of EVs by HeLa cells.

In T47D cells, which were the least efficient in internalising EVs as shown previously, the percentage of membrane T47D^{mCD81} EVs was reduced in both dynasore- and non-treated cells, but only at the latest time-point (Figure 4.5 A iii). The same cells showed different behaviour with HME1^{mCD81} EVs; the percentage of membrane EVs rapidly reduced in the first 40 minutes after administration to untreated cells, but increased for dynasore- and cholesterol oxidase-treated cells (Figure 4.5 A iv). This pattern was kept the same when looking at the last time-point alone, but the increment of change was not as profound. As opposed to HeLa cells, cholesterol oxidase and dynasore block EV entry in T47D cells, presumably because changes in lipid packaging can affect EV uptake in various ways depending on the cell type.

In hTERT-HME1 cells, both dynasore and cholesterol oxidase favoured EV internalisation in the first 40 min of administration. However, overall at the final time-point of 100 min, cholesterol oxidase showed the opposite effect (Figure 4.5 A v and vi). Therefore, this type of cell shows time-dependent changes in response to cholesterol oxidase that may be attributed to the fast membrane turnover as implied by the accumulating intraluminal membrane fragments as observed in the videos (Figure 7.3). Overall, these data show that dynasore and cholesterol oxidase can affect the proportion of EVs on the membrane (compared to cytoplasm), but these effects vary for the different cell lines and EV types, and in some cases may be time dependent. Moreover, these results highlight the importance of lipids and membrane organisation in EV docking and internalisation.

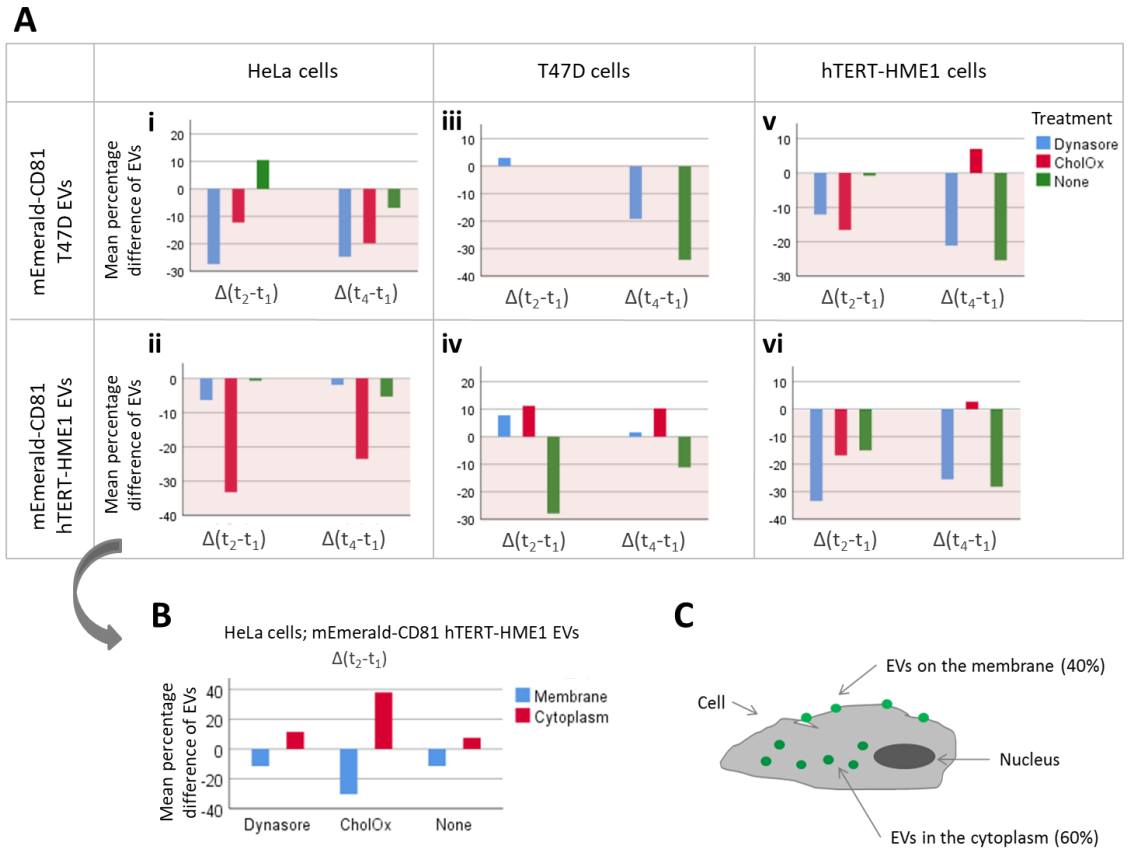


Figure 4.5 Increment of membrane EV change between early and late time-points.

A) The mean percentage change of membrane EVs during the first 40 min (time-point 2) and 100 min (time-point 4) compared to time-point 1 (10 min after EV administration) was calculated and plotted for each cell line, EV type, and treatment. Numbers were combined for all cells counted. B) Example of the mean percentage change of mEmerald-CD81 hTERT-HME1 EVs in the cytoplasm in comparison to the membrane of HeLa cells at Δt_2-t_1 . C) The amount of EVs on the membrane and in the cytoplasm was expressed as a percentage of the total amount of EVs counted.

4.4.5 Cholesterol on the membrane of target cells is involved in the early interactions with EVs

To further characterise EV-plasma membrane interactions, which represent an early but critical step towards EV uptake, the kinetics of the EVs on the membrane were explored. Single particle tracking was performed and variables like speed, total distance travelled, and confinement were calculated for each track. To assess diffusion rates, the speed of each track was plotted against the total distance travelled for each type of cell, EV, and treatment (Figure 4.6). This representation allowed the discrimination of two distinct distributions in almost all cases; a) particles with high speed that travel short distance (short range diffusion) and b) particles that linger on the membrane and have reduced speed (long range diffusion). To further confirm the presence of these subpopulations, the data was split in tracks with distance longer than and less

than 1 μm and correlation curves and coefficients (Spearman's correlation coefficient) were calculated (Figure 4.6 B). The slope of the curve represents the rate of change and therefore was used to assess the sensitivity of the treatment. For example, a steeper slope would mean that EVs performed faster interactions with the plasma membrane in response to the drug. The slopes were plotted as bar charts for each type of cell, EV, and treatment for the short-range diffusion only, as they are representative of the fast and short interactions with the plasma membrane (Figure 4.6 A). Data in the long range diffusion were limited and therefore were not analysed further. Besides, due to limitations of the acquisition technique it would be difficult to accurately calculate changes and differentiate between free and hindered diffusion in long distances as shown in Figure 4.6 A right panel. Nevertheless, analysing the short-range diffusion of EVs could help understand the very early events in EV-membrane interactions and subsequently uptake. Only the distributions that were correlated above 20% were used to draw conclusions (if not they were marked with an asterisk). In most cases, short range diffusion occurred at a similar speed for the same EV type incubated with different types of cells, regardless of drug treatment. The one exception to this observation was treatment with cholesterol oxidase, which appeared to alter short range diffusion (and therefore interaction sensitivity) of EVs across the different cell types. This is in agreement with the previous result showing that cholesterol oxidase has different effects on the different cell types. Within the same cell type, starting with T47D cells, the response to cholesterol oxidase was 10% faster than to dynasore in both EV types. On the contrary, in HeLa cells the response to cholesterol oxidase was 10% slower than that to dynasore in both EV types. Interestingly, in hTERT-HME1 cells, cholesterol oxidase affected the nature of EV-membrane interactions by 40% between the two EV types. This may underline the importance of EV content or surface molecules in their interaction with the plasma membrane and may set the ground for selective uptake. Between the two EV types, on average T47D^{mCD81} EVs were more responsive to drug treatment, meaning faster interactions with the plasma membrane. Unfortunately, the no treatment control for HME1^{mCD81} EVs was discarded during data mining and therefore no conclusion could be drawn for the direction of change between drug treatment and no treatment in T47D cells. However, judging from the other two cell types, it is clear that HME1^{mCD81} EVs perform faster interactions with the non-treated cells compared to dynasore and cholesterol oxidase treated cells. No statistically significant changes in the confinement ratio of EVs were observed among treatments. Regardless of cell or EV type, when cells were treated with cholesterol oxidase, there was a positive correlation between the accumulation of EVs on the membrane ($\Delta t_2 - t_1$) and the speed (short range diffusion slope) of interactions (Spearman's $\rho = 0.77$, $p = 0.076$). This further supports the involvement of cholesterol in the initial steps of EV uptake by controlling

EV-membrane dynamics. Further experiments are needed to establish the role of lipids in these first recognitory events in order to decipher uptake specificity.

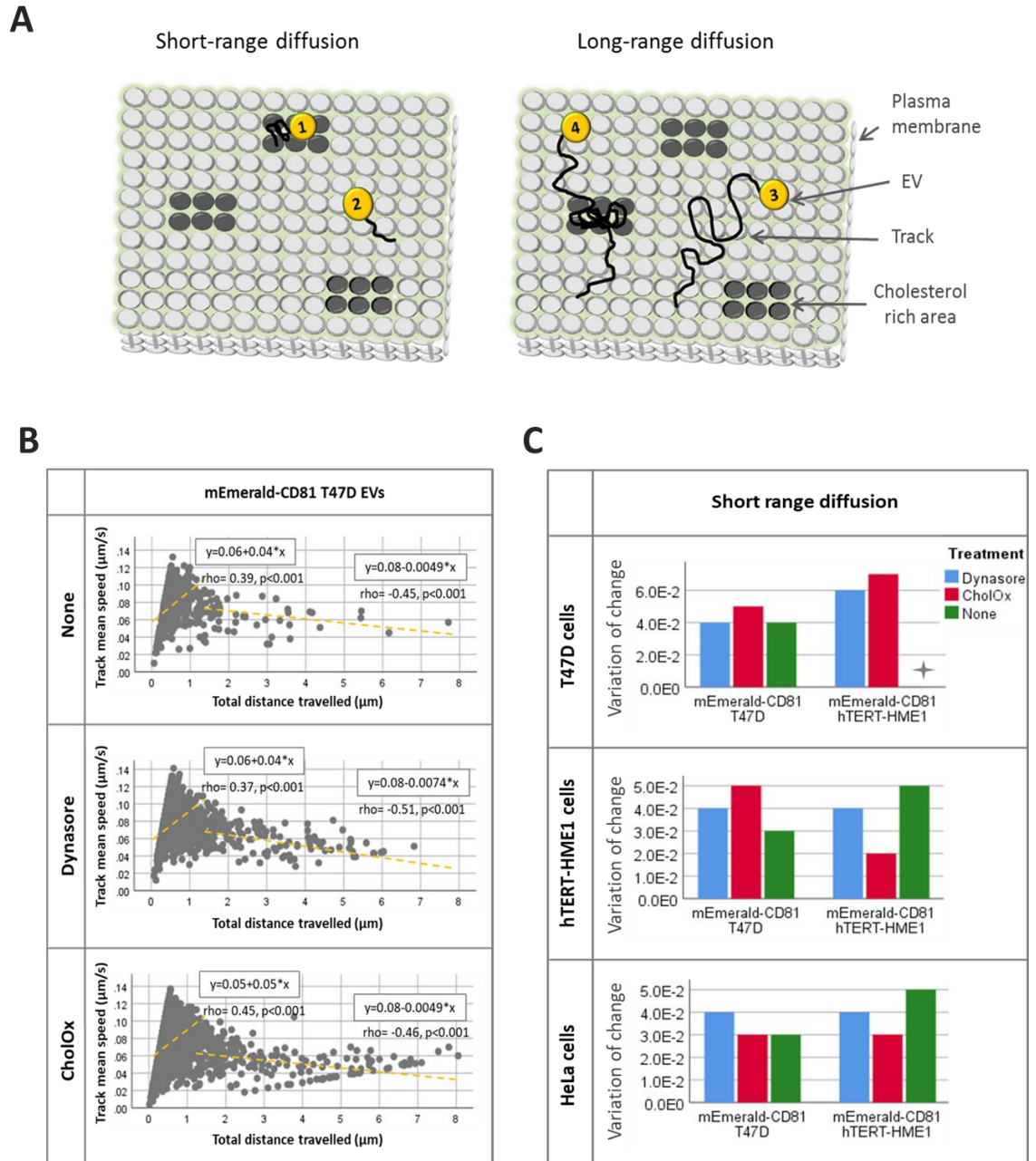


Figure 4.6 Tracking of EVs on the plasma membrane of target cells.

A) Schematic representation of short- and long-range diffusion modes of EVs on the plasma membrane of recipient cells. Diffusion can be free (2 and 3) or hindered in the highly organised domains of the membrane (1 and 4). B) Representative scatter plots of tracks (here mEmerald-CD81 T47D EVs on T47D cells). Two distinct distributions were observed and confirmed by Spearman's correlation. Correlation curves and coefficients, as well as p values are given on the graphs. C) The slopes of the short range diffusion curves were plotted as indicative of variation of change (regarding short EV-membrane interactions), for each cell line, EV type, and treatment.

4.5 Discussion

The aim of this chapter was to describe EV uptake dynamics in different types of cells using autologous and heterologous EVs. During this screen, it became apparent that different cells exhibit different EV uptake behaviours, such as the number of EVs internalised, the number of EVs that interact with the plasma membrane, the time-frame for EV interactions and uptake, sensitivity to uptake inhibitors and the involvement of lipids in such interactions.

An open question in the field of extracellular vesicles is whether EVs incorporation by recipient cells is a selective or a non-selective process. Peinado *et al.* (2012) showed that EVs from melanoma cells were transferred solely to the lung. Hoshino *et al.* (2015) further supported this concept by elucidating the role of specific integrin subtypes in organotropic metastasis and niche priming by EVs. On the other hand, Svensson *et al.* (2013) showed that EVs from a glioblastoma cell line were able to be incorporated by many different cell types including the autologous glioblastoma cells, endometrial cancer cells (HeLa), endothelial cells, murine ovarian cells and others, endorsing the concept of non-selective uptake. These controversies in the literature might reflect the heterogeneity in both the EV donor and acceptor cells.

In this project, a breast cancer cell line (T47D) and a normal immortalised breast cell line (hTERT-HME1) were used to investigate the EV uptake capacity of different cells. Fluorescent EVs from the stably transfected mEmerald-CD81 T47D and hTERT-HME1 cell lines were produced and used for the experiments of this chapter. A third non-breast cell line was added to the panel of recipient cells, the cervical cancer cell line (HeLa), which has been well characterized for EV uptake experiments (Roberts-Dalton *et al.*, 2017). All three of the cell lines used have epithelial or epithelial-like morphology. EVs were counted on the membrane and in the cytoplasm of recipient cells at four time-points (from 10 to 100 min after EV administration). It was observed that the three different cell lines incorporated EVs at different rates, with T47D cells being the least efficient in internalising any of the two types of EVs utilised in this study. The type of EVs did not affect their uptake, but rather their docking on the plasma membrane of target cells. For instance, T47D cells internalised the same amount of HME1^{mCD81} and T47D^{mCD81} EVs, but a higher amount of T47D^{mCD81} EVs was found on their membrane compared to HME1^{mCD81} EVs. hTERT-HME1 cells did not show a preference on the type of EVs, while HeLa cells accumulated more HME1^{mCD81} EVs on their membrane.

Using super resolution microscopy (Airyscan), EVs were observed to interact with the plasma membrane as single entities or clumps, while in the cytoplasm EVs were found either free or enclosed in membranous fragments (Figure 4.2 A). These observations might imply that different

mechanisms are activated during EV uptake. Recently, Horibe *et al.* (2018) demonstrated that EVs are non-selectively internalised by recipient cells and the uptake rate and mechanism depend on the recipient cell. In this project, two widely used EV uptake inhibitors, amiloride and dynasore, were employed to decipher the uptake mechanisms in the current experimental settings. Cholesterol oxidase, which has not previously been used in EV uptake experiments, was also employed here to help understand the role of cholesterol in EV-plasma membrane interactions and uptake. The drugs were selected to block a range of endocytic mechanisms: a) amiloride blocks macropinocytosis, an active actin-dependent pathway (Koivusalo *et al.*, 2010), b) dynasore blocks clathrin-dependent endocytosis (active) but also has other secondary effects including impairment of membrane turnover and consequent lipid disequilibrium (Preta *et al.*, 2015a), and c) cholesterol oxidase alters the conformation of cholesterol affecting membrane fluidity (passive)(Kreit and Sampson, 2009). It was observed that even though macropinocytosis might be one of the mechanisms of EV uptake, the response to amiloride was not as strong as the response to dynasore and cholesterol oxidase in the current experimental setting. Even though several studies support inhibition of EV uptake by use of amiloride (Costa Verdera *et al.*, 2017; Escrevente *et al.*, 2011; Tian *et al.*, 2014), it has also been reported that amiloride had no effect on EV uptake by endothelial HUVEC cells regardless of concentration (Svensson *et al.*, 2013). Nanbo *et al.* (2013) showed that amiloride was not able to inhibit uptake of Epstein virus positive Hodgkin lymphoma EVs by nasopharyngeal carcinoma cells, while Nishida-Aoki *et al.* (2017) showed that amiloride can even increase EV uptake by macrophages. Dynasore and cholesterol oxidase considerably affected the proportion of EVs on the plasma membrane compared to cytoplasm, but the effects varied for the different cell lines and EV types. However, it should not be discarded that changes in the proportion of EVs on the membrane compared to cytoplasm could be due to the fact that EVs might detach from the plasma membrane or incorporated EVs might be metabolised. In EV studies, dynasore has been used to inhibit EV uptake via endocytosis. Indeed, in most cases, dynasore reduced the amount of internalised EVs (Feng *et al.*, 2010; Newton *et al.*, 2006), however, care should be given when assumptions regarding the mechanism of EV uptake are made, especially due to the range of pathways affected by dynasore. Given the fact that both dynasore and cholesterol oxidase affect lipid composition, it was hypothesised that lipid rafts and more specifically cholesterol might play a role in EV-plasma membrane interactions and further affect uptake. Particularly, cholesterol oxidase favoured EV uptake by HeLa cells, while blocked it in T47D cells. These two types of cells previously showed the opposite preference for the two EV types tested. Taken together, these results highlight the importance of lipids in the specificity of EV uptake. In hTERT-HME1 cells, the effect of cholesterol oxidase was different at the early time-points compared to later time-

points, probably due to the fast membrane turnover observed in this type of cells and cholesterol replenishment.

EV-membrane interactions are crucial for EV uptake and in some cases may even be enough to trigger intracellular signalling pathways (Mulcahy *et al.*, 2014; Fabbri *et al.*, 2012). To further explore the EV-membrane interactions, the focus was given on the kinetics of EVs found on the membrane of recipient cells. Single particle tracking revealed two distinct distributions reflecting a) EVs that interact fast and travel short distance on the plasma membrane and b) EVs that interact longer and travel further. Focus was given on short interactions, where free and hindered diffusion can clearly be distinguished, while longer interactions may involve a combination of free and hindered diffusion modes, which are difficult to resolve with the technology available in this project. The same EV type appeared to maintain constant interaction dynamics among the various cell types, unless cells were treated with cholesterol oxidase. In hTERT-HME1 cells treated with cholesterol oxidase the two EV types exhibited considerably different properties; HME1^{mCD81} EVs were 40% slower than T47D^{mCD81} EVs. Apart from the role of membrane organisation in target cells, these results indicate that molecules on the EV surface, being lipids or others, may also be crucial for the initial interactions with the cell. It still remains to uncover whether these short and fast interactions reflect fast uptake or bouncing of EVs on the membrane.

This study has highlighted the importance of cholesterol in early interactions of EVs with the plasma membrane of target cells. The next step would be to analyse the diffusion of lipids on the EV entry points on the plasma membrane in order to further validate the interaction and identify its nature. Due to time limitations, such analysis was not feasible during the course of this project; however, future work will analyse the videos acquired in this study by raster imaging correlation spectroscopy (RICS) (Brown *et al.*, 2008), which is a novel method to detect and quantify diffusion coefficients of molecules whilst also indirectly detect binding. Other cholesterol depleting drugs, such as simvastatin or methyl- β -cyclodextrin should further support these findings. In our hands, methyl- β -cyclodextrin prevented staining of the membrane with Cell Mask Deep Red, the membrane dye used for these experiments, and therefore was not selected for this project. Moreover, methyl- β -cyclodextrin completely depletes cholesterol leading to a more extreme phenotype, while cholesterol oxidase reduces cholesterol altering its conformation and packaging on the membrane.

Overall, this project has highlighted the diversity in EV uptake among the different types of cells and EVs, has addressed the role of cholesterol and membrane organisation in the early EV-

plasma membrane interactions, and has provided grounds for further investigations that could resolve the question of selective uptake.

Experimental limitations

Overall, the experimental setting allowed monitoring of EVs on the plasma membrane and the cytoplasm of recipient cells. The selection of cells, EVs and treatments was adequate to cover a range of responses. For this project, confocal scanning microscopy was used and videos were acquired in a single focal plane (approximately 300 nm resolution in the Z-axis), therefore information on whole cell might have been lost. However, acquisition in three dimensions would have significantly decreased the scanning speed and EV movement might have not been easy to track. A technology that could be alternatively used is biplane microscopy (Prabhat *et al.*, 2004), which is based on a camera (faster than scanning mode). This system allows illumination of two to three focal planes at a time, providing high spatiotemporal resolution. Due to unavailability of this system, confocal microscopy was adapted for the purposes of this project. Moreover, due to the large amount of data collected during this screening process, especially regarding tracking of EVs, analysis of all variables and possible scenarios was not feasible during the time-frame of this thesis, and focus was given on exploring the passive mechanisms (such as membrane permeability) that might mediate EV uptake.

4.6 Key findings

- EV uptake depends on the recipient cell type. Different cell types incorporate EVs in different rates and amounts.
- EV type/origin affects docking on and possibly interactions with the plasma membrane of target cells.
- Dynasore and cholesterol oxidase, both disturbing the cholesterol equilibrium of the plasma membrane, affect EV uptake in a variable manner depending on the cell and EV type.
- Two patterns of EV behaviour were identified on the membrane of target cells: fast and short distance diffusion, versus slow and long distance diffusion.
- Cholesterol is involved in the early interactions of EVs with the plasma membrane.

The effect of chemotherapy-induced EVs on breast cancer metastasis

Chapter 5

5 The effect of chemotherapy-induced EVs on breast cancer metastasis

5.1 Background

Metastasis is a crucial process in cancer progression. In the past two decades, EVs have been shown to play important roles in cancer metastasis by acting as mediators of intercellular communication (Becker *et al.*, 2016; Li *et al.*, 2018). It has been reported that EVs from aggressive breast cancer cells can increase the motility and invasiveness of recipient less aggressive breast cancer cells (O'Brien *et al.*, 2013). Moreover, cells incubated with autologous EVs become more invasive. EVs secreted by invadopodia, structures required for cell invasion, stabilise and/ or promote *de novo* formation of invadopodia in an autocrine manner (Hoshino *et al.*, 2013). Here, it was hypothesised that EVs from chemotherapy-treated cells could induce pro-metastatic effects in autologous intact recipient cells. This hypothesis was also based on studies showing that EVs released by cells that have been treated with other forms of cancer treatment, such as radiation and hyperthermia, increased invasion *in vitro* (Bewicke-Copley *et al.*, 2017; Arscott *et al.*, 2013).

5.1.3 Methods to evaluate the different steps of the metastatic cascade *in vitro*

For the purposes of this project, the T47D non-invasive breast cancer cell line (Mierke *et al.*, 2008; Goicoechea *et al.*, 2009), was treated with docetaxel and mitomycin C and EVs from the surviving cells were collected and administered to intact recipient T47D cells. The metastatic competence of the recipient cells was tested by evaluating individual steps of the metastatic cascade *in vitro*.

EMT

EMT is the transformation of epithelial cells to mesenchymal (Hay, 1995). During EMT, epithelial cells lose their polarity by eliminating cell adhesion structures such as gap junctions, while cell adhesion molecules like CDH1 (E-cadherin) are downregulated (Nakajima *et al.*, 2004). The morphology of the cells gradually changes from cuboidal to spindle-shaped, which is a result of alterations in cytoskeleton dynamics. Particularly, vimentin, a subtype of intermediate filaments proteins is highly expressed in cells that have acquired the mesenchymal phenotype (Robert *et al.*, 2016). In this project, to test whether cells underwent EMT, the expression levels of CDH1 and vimentin were measured by qPCR.

Cell motility

To study cell motility *in vitro* several assays have been developed (Paul *et al.*, 2017). Here, the wound-healing or 'scratch' assay was employed to study motility/migration in 2-dimensions of cells that received EVs from chemotherapy-treated cells.

Briefly, the cells are cultured to confluence and an artificial 'wound' is created by 'scratching' the monolayer with a sharp object (pipette tip). Cell migration is quantified by measuring the reduction of the 'scratch' area over time (Liang *et al.*, 2007). To ensure that the gap closure is due to cell migration and not multiplication of cells, a proliferation assay is usually performed in parallel under the same conditions.

Invasion

Invasion refers to the ability of cells to adhere to and lyse the extracellular matrix in order to escape the borders of the tumour mass and migrate further (Friedl and wolf, 2009). The molecular mechanisms governing invasion have been extensively studied but are still incompletely understood. Cell-cell and cell-matrix adhesion molecules are important to transmit information from the surrounding environment that enables the cell to act and/or interact with its local environment. Equally, proteolytic enzymes that are known to degrade the extracellular matrix, have also a role in signalling regarding tumour cell growth or inhibition (Cairns Khoka Hill, 2003).

Assays to test the invasive capability of cells contain a matrix component for the cells to invade through. Here, the transwell invasion assay (Boyden, 1962) was employed to test invasiveness of cells treated with chemotherapy-derived EVs. The system is composed of two compartments separated by a cell permeable porous membrane (Brooks, 2001). The cells are seeded on a thick layer of extracellular matrix (Matrigel®) in transwells (in this case along with the appropriate amount of EVs) in serum free medium and allowed to invade for a period of time. The lower chamber contains medium supplemented with serum to serve as attractant. Invasive cells hydrolyse the Matrigel® and pass through the pores of the membrane. The diameter of the pores is smaller than the cell size to avoid non-specific cell passage. The invaded cells should be on the bottom side of the membrane, while the non-invaded cells should be on the upper surface of the membrane and are removed using cotton swabs. Invaded cells are then stained, here with a nuclear dye, and counted (Brooks, 2001).

Adhesion to the endothelium

After invading the surrounding tissue, metastatic cells reach the endothelial walls, where they adhere to in order to intravasate and later extravasate, to form distant tumour foci (Kramer and Nicolson, 1979). In this project, to test the ability of cells treated with chemotherapy EVs to adhere to the endothelium, the static adhesion assay was employed. Briefly, endothelial cells are cultured in a monolayer and activated with pro-inflammatory cytokines before the experiment. Fluorescently labelled cancer cells are then added to the endothelial monolayer. Non-bound cells are removed, and the remaining bound cells are fixed and counted (Glinsky 2000, wilhemsen 2013). This type of adhesion assay better represents cells that have arrested in capillary beds, contrary to flow adhesion assays that are performed under constant movement and are reflective of cell adhesion under physiological flow rates (Bapu 2014).

5.2 Aim and objectives of chapter 5

Chemotherapy can trigger metastatic phenotype in tumour cells. EVs, as mediators of cell-cell communication, have been implicated in all steps of the metastatic cascade. Here, it was hypothesised that non-invasive cells treated with chemotherapy secrete EVs able to confer a metastatic phenotype to autologous cells.

The aim of this chapter was to investigate the effect of chemotherapy-induced intercellular communication via EVs on cancer metastasis. The objectives were:

- To define sub lethal concentrations of docetaxel and mitomycin C.
- To isolate and count EVs from T47D breast cancer cells that have been treated with sub lethal concentrations of docetaxel and mitomycin C (chemotherapy-derived EVs).
- To examine the effect of chemotherapy-derived EVs on the invasiveness of intact recipient cells.
- To investigate the effect of chemotherapy-derived EVs on the motility and proliferation of intact recipient cells.
- To test whether chemotherapy-derived EVs can trigger EMT in intact recipient cells.
- To explore the effect of chemotherapy-derived EVs on the glycosylation profile of intact recipient cells as revealed by HPA-labelling.

5.3 Methods

5.3.1 Viability / proliferation assay

To assess the proliferation rate or viability of cells, the MTT assay, a colourimetric assay, was used. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow soluble tetrazole that, when metabolised by living cells, transforms to its non-soluble purple formazan product. When cells are lysed, formazan creates a coloured solution, the absorbance of which can be measured at 570 nm by a spectrophotometer. MTT assay is an indirect method to evaluate cell growth as the intensity of the coloured product depends on the metabolic activity of the cells.

Cell preparation

The MTT assay was used to a) establish the IC₅₀ values (the concentration of drug that kills half of the cells) against Docetaxel and Mitomycin C, and b) test the proliferation rate of cells after treatment with EVs or EV depleted medium. For IC₅₀ viability assay, cells were seeded in 48-well plates and, when 70% confluent, treated with different concentrations of each drug. After 24h, the cells were washed with PBS and the MTT assay was performed. For proliferation, cells were seeded in four 48-well plates at 12,000 cells per well in complete medium. Next day, the medium was changed in three of the four plates to EV depleted medium and the appropriate amount of EVs was added for 24 h, 48 h and 72 h. The MTT assay was performed on one plate every day. The cells in the fourth plate were washed with PBS and the MTT assay was performed to determine proliferation at time-point 0 (time of EV addition). For experiment overview see Figure 5.1.

MTT assay

MTT (Sigma-Aldrich®) was dissolved in PBS at a concentration of 5 mg/ml and passed through 0.22 µm filters. A total volume of 300 µl of 0.75 mg/ml MTT in complete or EV depleted medium was added to each well and incubated for 3 h at 37°C in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere. After this time, MTT was removed, 300 µl of solvent (4 mM HCl, 0.1% IGEPAL® in isopropanol) was added to each well, and absorbance was measured at 570 nm using a SpectraMax i3x plate reader (Molecular Devices). The four technical replicates were averaged and the ratio of the treatment group to the control was calculated for each one of the independent experiments. Data were combined for statistical analysis.

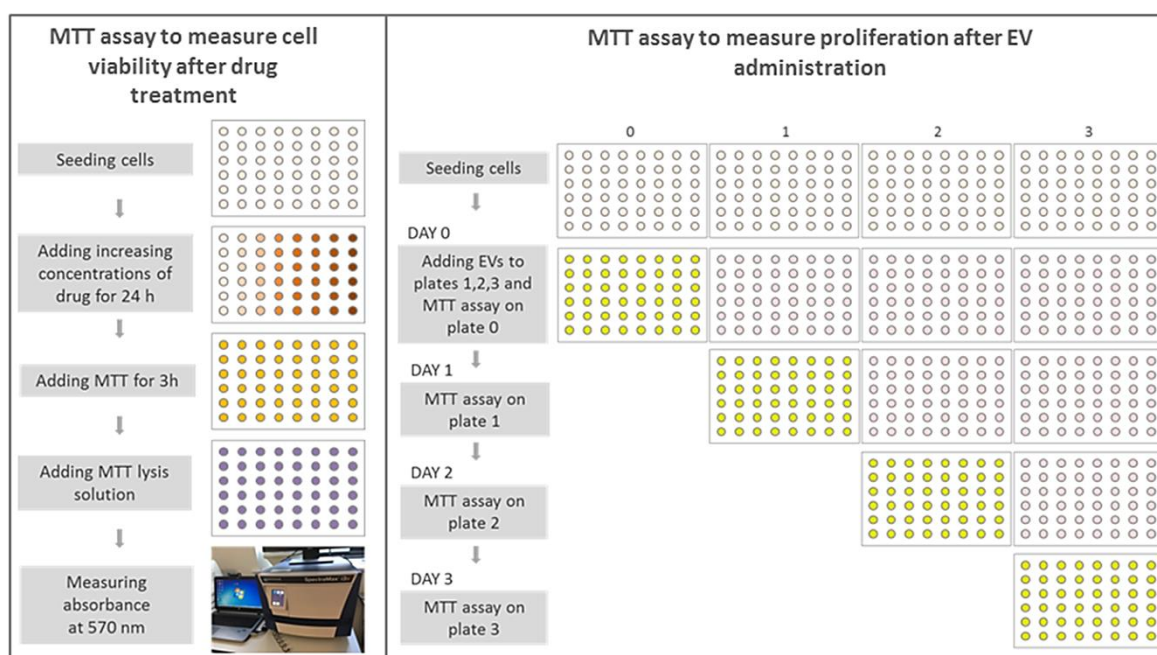


Figure 5.1 Schematic representation of the viability / proliferation experiments.

The MTT assay was used to assess viability of T47D cells at increasing doses of Docetaxel and Mitomycin C (left) and to measure proliferation of T47D cells that received EVs (right).

5.3.2 NTA

Nanoparticle tracking analysis was performed on the isolated EVs to assess their concentration and size, as described in section 2.2.2.

5.3.3 Real-time qPCR for epithelial and mesenchymal marker expression

To investigate the expression levels of the epithelial marker CDH1 and the mesenchymal marker vimentin, real-time qPCR was performed. The primers, probes, assays and amplification conditions are given in section 2.4.

To test the efficiency of the assays for multiplexed real-time qPCR, 5-fold serial dilutions of the same cDNA sample (derived from T47D cells) were prepared. Quantification cycle (C_q) values were obtained from the amplification plots of each sample (Figure 5.2 A and B) and plotted against the log₁₀ of cDNA dilution (Figure 5.2 C and D). Analysis of qPCR results assumes perfect doubling of initial template at each cycle. Therefore, the distance between consequent amplification curves (n) should be given by the equation $n = \log_2(\text{dilution factor})$. Here, 5-fold dilutions were prepared, therefore, $n = \log_2(5) = 2.32$. The slope (a) of the linear regression curves $y = a \cdot x + b$ should be -3.32 for 100% efficiency. The equation to obtain the efficiency of the assay is $e = (10^{(-1/a)} - 1) \cdot 100$. Here, in the multiplexed reactions, CDH1 efficiency was 108.5%

with ACTB in the same reaction 107.5%, vimentin efficiency was 105.6% with ACTB in the same reaction 106.1%.

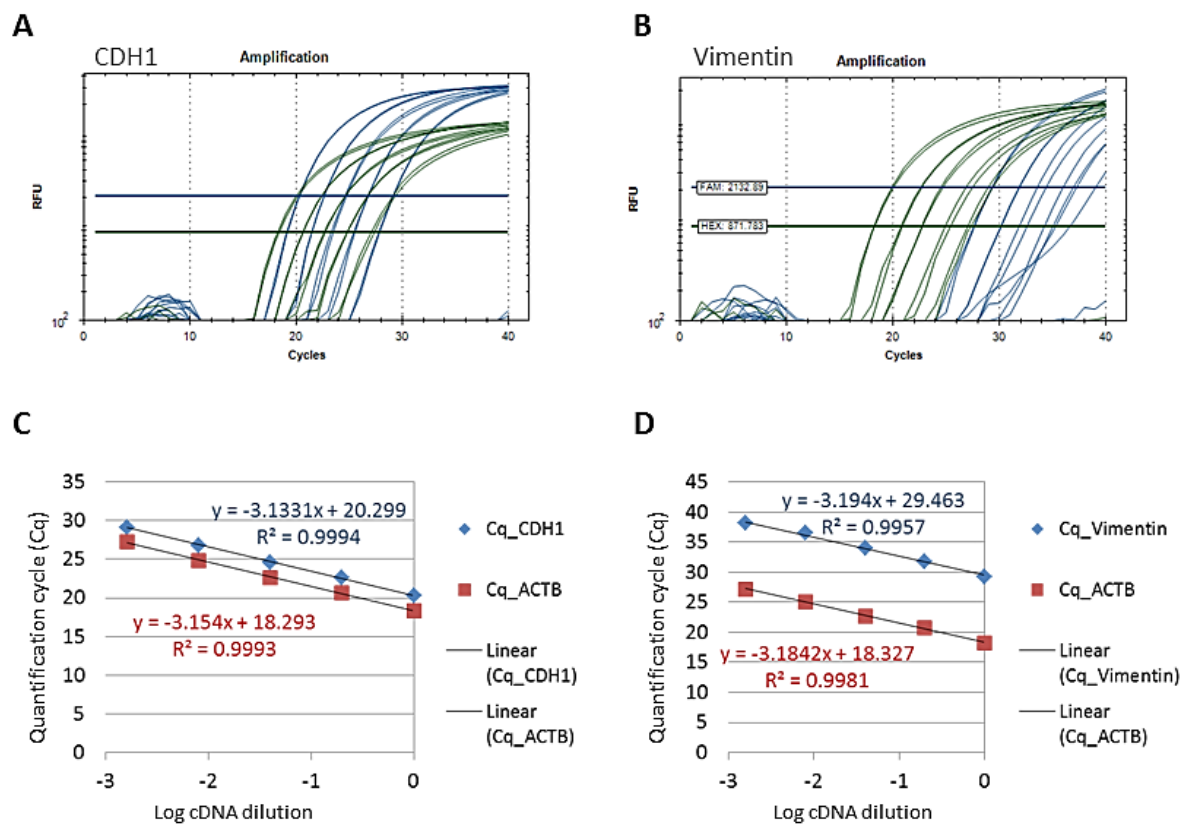


Figure 5.2 Optimization of multiplexed qPCR for the expression analysis of EMT markers.

Amplification plots of 5-fold serial dilutions of the same cDNA obtained using multiplexed A) CDH1 (blue) and ACTB (green) assays and B) vimentin (blue) and ACTB (green) assays. Y-axis: relative fluorescence units (RFU), x-axis: PCR cycles. Cq values were obtained from the amplification plots of each sample and plotted against the $\log_{10}$ of cDNA dilution. The equations of the linear regression lines, along with the coefficient of determination (R^2) are given in C) for CDH1 and ACTB and D) for vimentin and ACTB, and were used to calculate the efficiency of each assay in the multiplexed reactions. Single experiment, two technical replicates.

Usually, efficiencies over 100% signify inhibition of PCR in the higher concentrations of the standard curve. This could be due to the presence of ethanol, other solvents and residues from the RNA purification and cDNA synthesis, or too much template. As the inhibitor is diluted in the higher dilutions of cDNA, the effect fades, causing the efficiency to appear over 100%. Typically, acceptable efficiencies range between 90% and 110% (Rogers-Broadway and Karteris, 2015). The coefficient of determination R^2 for the regression plot should be greater than 0.98. And finally, the efficiency of endogenous and target genes should be identical when multiplexed (difference not greater than 5%, parallel curves). All these requirements were met, and the assays were used without further optimisation to determine the expression levels of CDH1 and vimentin in cells treated with EVs as described in section 2.4.3.

5.3.4 Matrigel® invasion assay

To assess the invasive capacity of cells after treatment with EVs, the Corning® BioCoat™ Matrigel® assay was used (Figure 5.3).

Preparation of the Matrigel® plates

The plates were allowed to equilibrate to room temperature for 30 min. After this time, the Matrigel® matrix was rehydrated by adding 500 µl pre-warmed serum-free medium in both the inserts and the wells, and the plates were placed in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere for 2 h. The medium was discarded and 750 µl complete medium was added in each well of the plate. T47D cells, which were cultured in serum-free medium for 24 h prior to the invasion assay, were mixed with the appropriate amount of EVs and added to the inserts (20,000 cells in a total of 1 ml serum-free medium per insert). The cells were allowed to invade for 48 h.

Fixation and staining of invaded cells

The inserts were washed with PBS and invaded cells were fixed with 4 % PFA, pH 6.9, for 20 min. Cells were treated with 0.1 % Triton X-100 (Sigma-Aldrich®) in PBS for 10 min, stained with Hoechst stain (Sigma-Aldrich®) for 2 min and washed with PBS. Non-invaded cells were removed from the inserts by wiping with a lightly moistened cotton swab. Membranes were cut from the inserts using a scalpel (Swann-Morton™) and mounted in ProLong™ Diamond antifade mountant (Thermo Fisher Scientific).

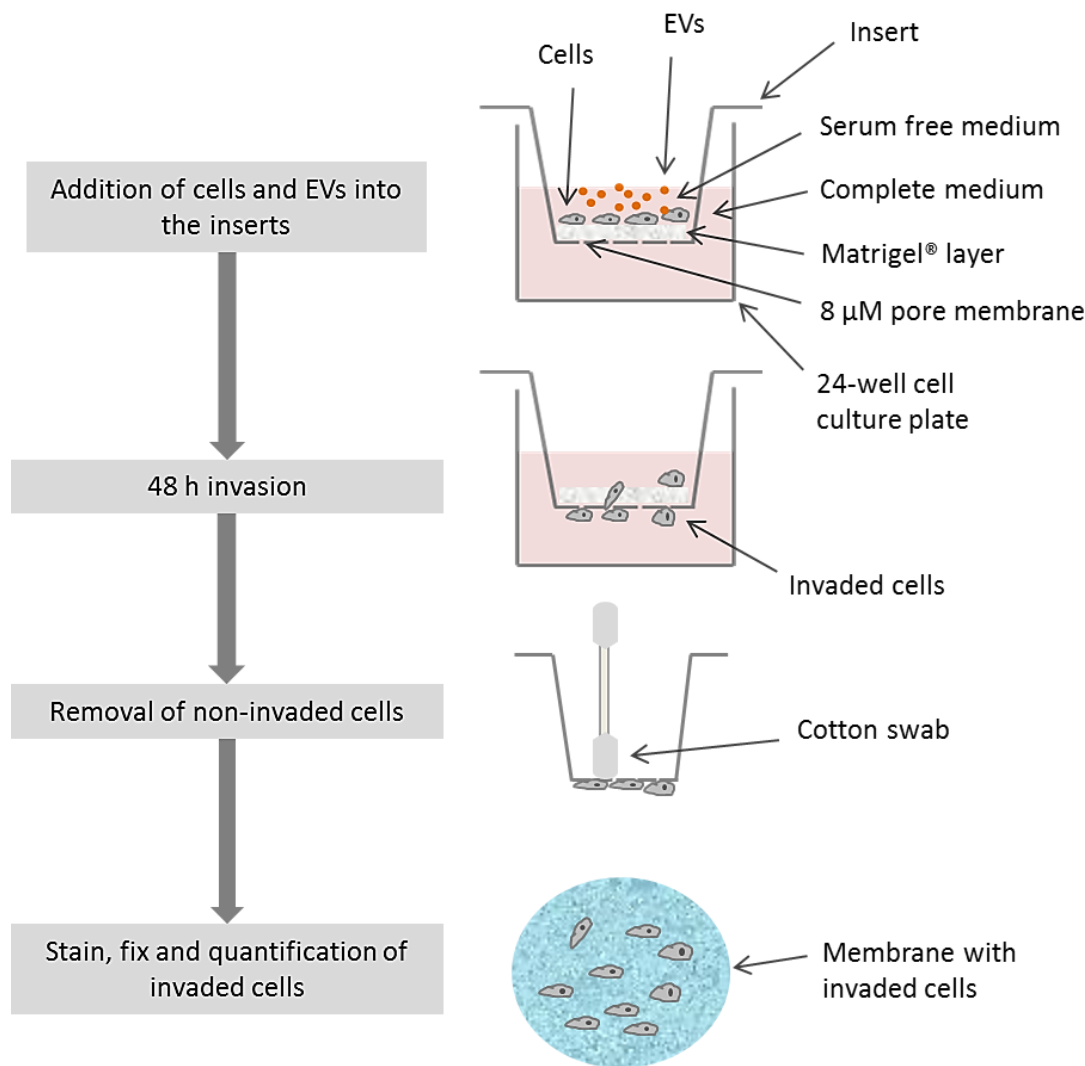


Figure 5.3 Schematic representation of the Matrigel® invasion assay.

The Matrigel® membrane was rehydrated and cells along with EVs were added in the inserts in serum free medium, while complete medium was added to the wells of the plate. The cells were allowed to invade for 48 h and then the medium, Matrigel® and non-invaded cells were removed and the membrane was fixed and stained. Invaded cells were imaged and counted using the cell counter plugin in FIJI. Objects not to scale.

Imaging and analysis of invasion

To image the invaded cells, a Zeiss Axio Imager Z2 upright microscope fitted with an ORCA-Flash 4.0 Digital CMOS camera (Hamamatsu) was used and tiled images were taken (7 x 7 tiles) to include the whole membrane. The Plan-Apochromat 10x/0.45 lens (Zeiss) and the 405 nm laser were used to visualise stained nuclei, which were counted using the cell counter plugin in FIJI. The three technical replicates were averaged and the ratio of the treatment group to the 'no EV' control was calculated for each one of the four independent experiments (three in the case of Mitomycin C EVs). Data were combined for statistical analysis.

5.3.5 Cell migration / ‘scratch’ assay

One of the most frequently used methods to study cell migration *in vitro* is the ‘scratch’ or wound–healing assay. This method includes the creation of an artificial gap (‘scratch’ or ‘wound’) on a confluent layer of cells and the observation of the cells on the two edges closing this gap (Liang *et al.*, 2007). Here, the ‘scratch’ assay was employed to assess cell motility after EV treatment.

Cell preparation, ‘scratch’, and imaging of gap closure

Cells were cultured to complete confluence in 24-well plates. Using a p200 pipette tip and a ruler, the cell monolayer was scraped to produce a straight ‘scratch’. The cells were washed with PBS to remove floating cells and EV depleted medium along with the appropriate amount of EVs (final volume 1 ml per well) was added. Plates were kept in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere. To obtain the same field during the image acquisition, wells were previously marked on their base with a straight line perpendicular to the ‘scratch’ line using a permanent marker. Images were taken at time-points 0, 6, 24 and 48 h using a Primovert microscope with an LD5xPh1 objective (Zeiss).

Image analysis

Data were analysed using the MRI Wound Healing Tool plugin on FIJI with empirically set but constant experimental parameters. This plugin measures the area unoccupied by cells in each image. The final gap size was subtracted from the gap size at time-point 0 h to calculate the migration area. The four technical replicates were averaged and the ratio of the treatment group to the ‘no EV’ control was calculated for each one of the three independent experiments. Data were combined for statistical analysis.

5.3.6 Static adhesion assay

To examine the capacity of cells to attach to endothelial monolayers after EV treatment, the static adhesion assay was employed (Lowe and Raj, 2015). According to this method, cells are labelled and allowed to adhere to a monolayer of endothelial cells previously activated by pro-inflammatory cytokines. Non-bound cells are then removed, and the adherent cells are counted.

Preparation of endothelial cells

HUVEC cells were cultured to complete confluence on 13 mm coverslips thickness #1 (Marienfeld), previously sterilised in 70 % ethanol and coated with 0.2% w/v bovine gelatine in

PBS, pH 7.4, in 24-well plates. On the day of the experiment, the endothelial cells were stimulated with 10 ng/ml TNF- α (Sigma-Aldrich®, 10 μ g/ml in PBS stock) for 2 h in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere.

Preparation of cancer cells

T47D cells were cultured in 6-well plates to 60 - 70 % confluence. Then, the cells were washed with PBS and EV depleted medium and the EVs were added for 24 h. After this time, the cells were washed with PBS and stained with 10 μ M CFSE (Sigma-Aldrich®, 18 mM in PBS stock) for 10 min. To remove excess dye, the cells were washed with PBS; then detached from the plate using cell scrapers, pelleted down at 300 x G for 5 min, and resuspended in serum-free medium.

Static adhesion assay

60,000 stained T47D cells were introduced per well of HUVEC cells in serum-free medium (total volume of 1 ml per well) and allowed to adhere to the endothelial layer for 30 min in a humidified tissue culture incubator at 37°C with 5% CO₂ atmosphere. Non-adherent cells were removed by washes with PBS. Cancer cells bound to the endothelial layer were fixed with 4 % PFA, pH 6.9, at room temperature for 20 min. The cells were then washed with PBS and mounted with ProLong™ Diamond Antifade mounting medium.

Imaging and analysis of adhesion

A Zeiss Axio Imager Z2 upright microscope fitted with an ORCA-Flash 4.0 digital CMOS camera was used and 10 images per coverslip were acquired. The Plan-Apochromat 20x/0.8 lens (Zeiss) and the 488 nm laser were used to visualise stained cells. The area occupied by stained cells was measured using the Analyse Particles plugin with default parameter settings in FIJI. The ratio of the area occupied by cancer cells in the treatment group to the 'no EV' control was calculated for each one of the three independent experiments. Data were combined for statistical analysis.

5.3.7 HPA labelling

A fluorescently labelled HPA lectin was used in order to assess the glycosylation changes of cells after incubation with EVs from chemotherapy treated cells (Figure 5.4).

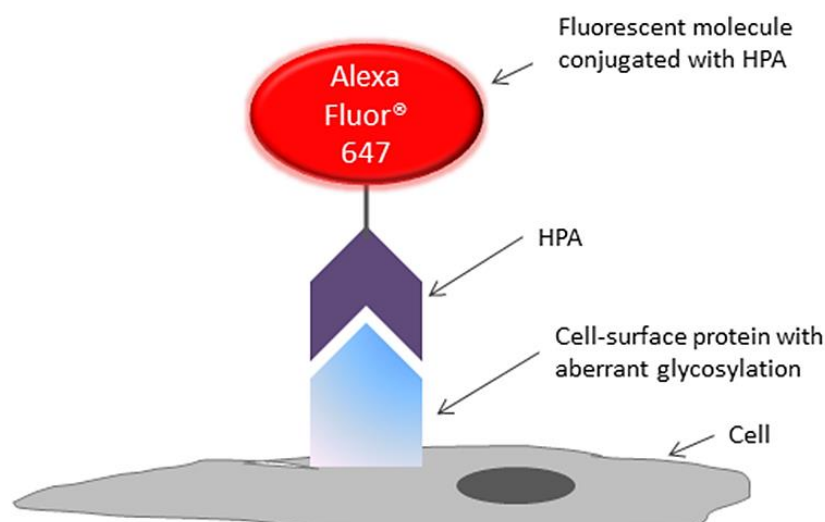


Figure 5.4 Schematic representation of the HPA Alexa Fluor® 647 conjugate used in HPA labelling experiments.

Truncated cell-surface glycoproteins with terminal α -GalNAc are recognised by HPA which is attached to Alexa Fluor® 647, a red fluorescent dye. Objects not in scale.

Preparation of slides

HPA Alexa Fluor® 647 conjugate (red, Thermo Fisher Scientific) was dissolved in 1x TBS (Thermo Fisher Scientific) at 1mg/ml and aliquots were stored at -20°C. Cells were cultured on 13 mm coverslips, thickness #1 previously sterilised in 70 % ethanol, in 24-well plates (Corning™) until they reached approximately 60 - 70 % confluence. Cells were then treated with the appropriate amount of EVs or drug for 24 h, washed with PBS, fixed with 4 % PFA, pH 6.9, at room temperature for 20 min, and then incubated with 5 μ g/ml HPA Alexa Fluor® 647 conjugate in 3% BSA in TBS for 25 min. Cells were then washed with PBS and mounted with ProLong™ Gold Antifade Mountant with DAPI (nuclear counterstain, Thermo Fisher Scientific) on slides for microscopic analysis.

Two types of controls were used in this experiment; a negative and a specificity control. For the negative control, cells were incubated with 3% BSA in TBS instead of 5 μ g/ml HPA Alexa Fluor® 647 conjugate for 25 min. This control established the levels of auto-fluorescence of the cells, which were minimal in all experiments. A specificity control was prepared by mixing 0.1M α -GalNAc (N-acetylgalactosamine, Sigma-Aldrich®) – the monosaccharide to which HPA has the greatest affinity – along with 5 μ g / ml HPA Alexa Fluor® 647 conjugate in 3% BSA in TBS prior to incubation with cells for 25 min. This control should also show no red fluorescence as the lectin should bind to the α -GalNAc in solution.

Imaging and quantification of HPA labelling

Images were acquired using an LSM 880 confocal microscope using the Plan–Apochromat 63x/1.4 oil objective (Zeiss). Excitation and emission wavelengths were 405 and 446 nm for DAPI (laser power 1 – 5 %), and 561 and 668 nm for Alexa Fluor® 647 (laser power 5%). Z-stacks (maximum 5% overlap between slides) of five positions randomly allocated on the slide were acquired for each sample. Two technical replicates per treatment and a total of three independent experiments were done.

In order to quantify the differences in fluorescence labelling amongst treatment groups, projected images were created based on the maximum intensity of each Z-stack using FIJI. On the projected image, a region of interest was drawn around each cell using the free hand tool and parameters such as area, integrated density and mean grey value were measured. The corrected total cell fluorescence (CTCF) = integrated density – (area of selected cell × mean fluorescence of background readings), was calculated in Excel. The ratio of the treatment group to the ‘no EV’ control was calculated for each one of the three independent experiments. Data were combined for statistical analysis.

5.4 Results

5.3.1 Calculating the sensitivity of T47D cells to docetaxel and mitomycin C

In order to calculate the IC₅₀ values (half maximal inhibitory/ lethal concentration) of docetaxel and mitomycin C, T47D cells were treated with increasing concentrations of each of the two drugs for 24 h. Cell viability after drug treatment was assessed by MTT assay. Dose-response curves were produced using the four-parameter regression model and IC₅₀ values were calculated in Graphpad Prism 6 (Figure 5.5). For subsequent experiments the IC₅₀ values were used; 12.5 µM mitomycin C and 40 nM docetaxel.

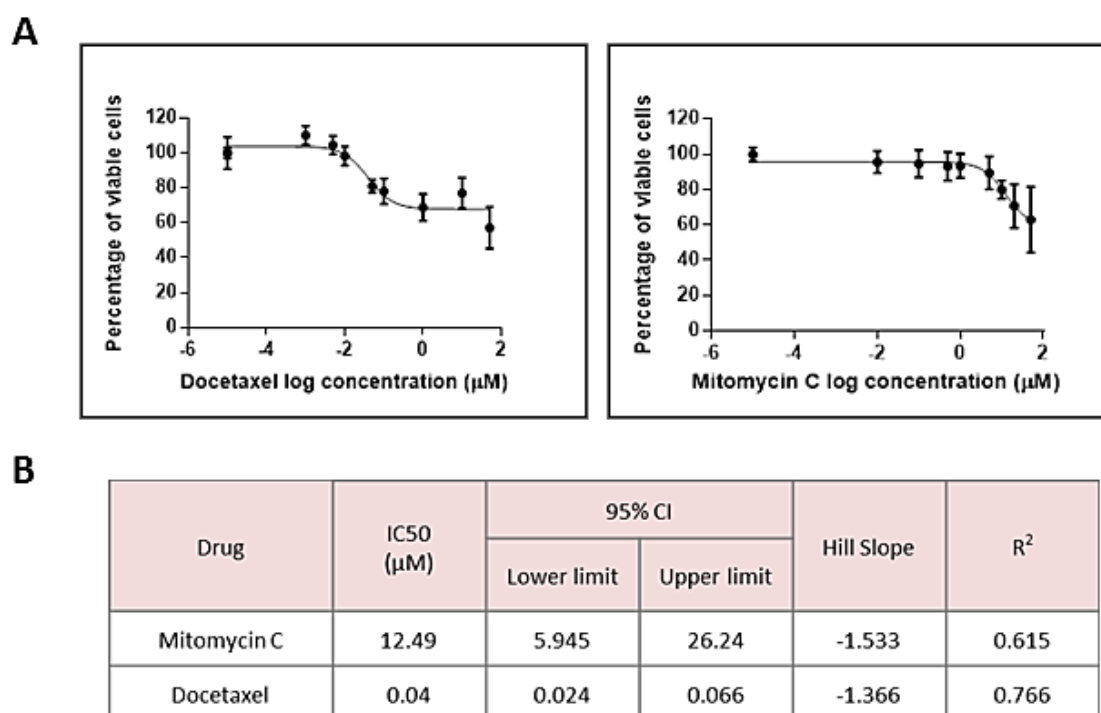


Figure 5.5 Dose-response of T47D cells to docetaxel and mitomycin C.

A) The four-parameter regression model was applied for the production of sigmoidal curves and the calculation of IC₅₀. Y axis: percentage of viable cells as measured by MTT assay, x axis: increasing concentrations of docetaxel and mitomycin C expressed in logarithmic scale. Error bars, 1x SEM. B) The IC₅₀ values for each drug and the goodness of fit of the curves. Two independent experiments were combined, at least 4 technical replicates per treatment.

5.3.2 Characterising the model to study EVs from chemotherapy-treated cells

To study the effect of chemotherapy as communicated by EVs, T47D cells were treated with docetaxel and mitomycin C for 24 h. The flasks were then washed, to remove dead cells and excess of drug, and complete medium was added for 24 h. Then, the flasks were washed again, to ensure cells that died in later time and potential drug remains were removed. EV depleted medium was then added to the flasks and 48 h later EVs were isolated from the conditioned media and characterised by NTA. A schematic representation of the experimental procedure is illustrated in Figure 5.6.

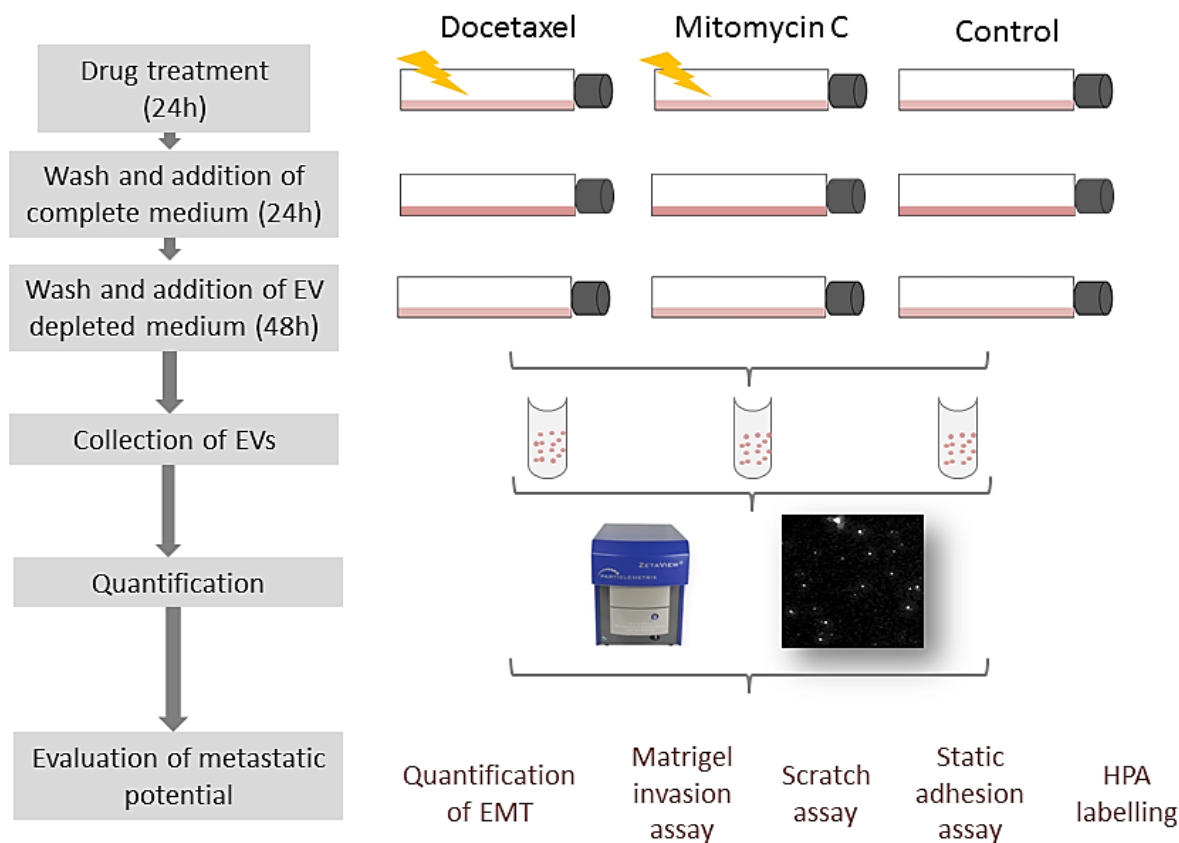


Figure 5.6 Overview of the experimental procedure to study the effect of chemotherapy-derived EVs.

The cells at the moment of EV collection were approximately 60-80% confluent in all treatment groups and no or minimal amounts of dead floating cells were observed (Figure 5.7 A). EV size was consistent across treatments (86-145 nm). EV numbers were calculated per cell (as counted at the end of the 48 h EV collection period). For example, if 10^{11} EVs came from 5×10^7 cells, then the number of EVs per cell would be 2000. Control cells consistently secreted lower amounts of EVs compared with docetaxel- and mitomycin C-treated cells (Figure 5.7 B and C). Statistics using normalised EV numbers show that docetaxel- and mitomycin C-treated cells released statistically significantly higher amounts of EVs compared to non-treated cells ($p=0.037$ in both cases, Mann-Whitney test) (Figure 7.7)

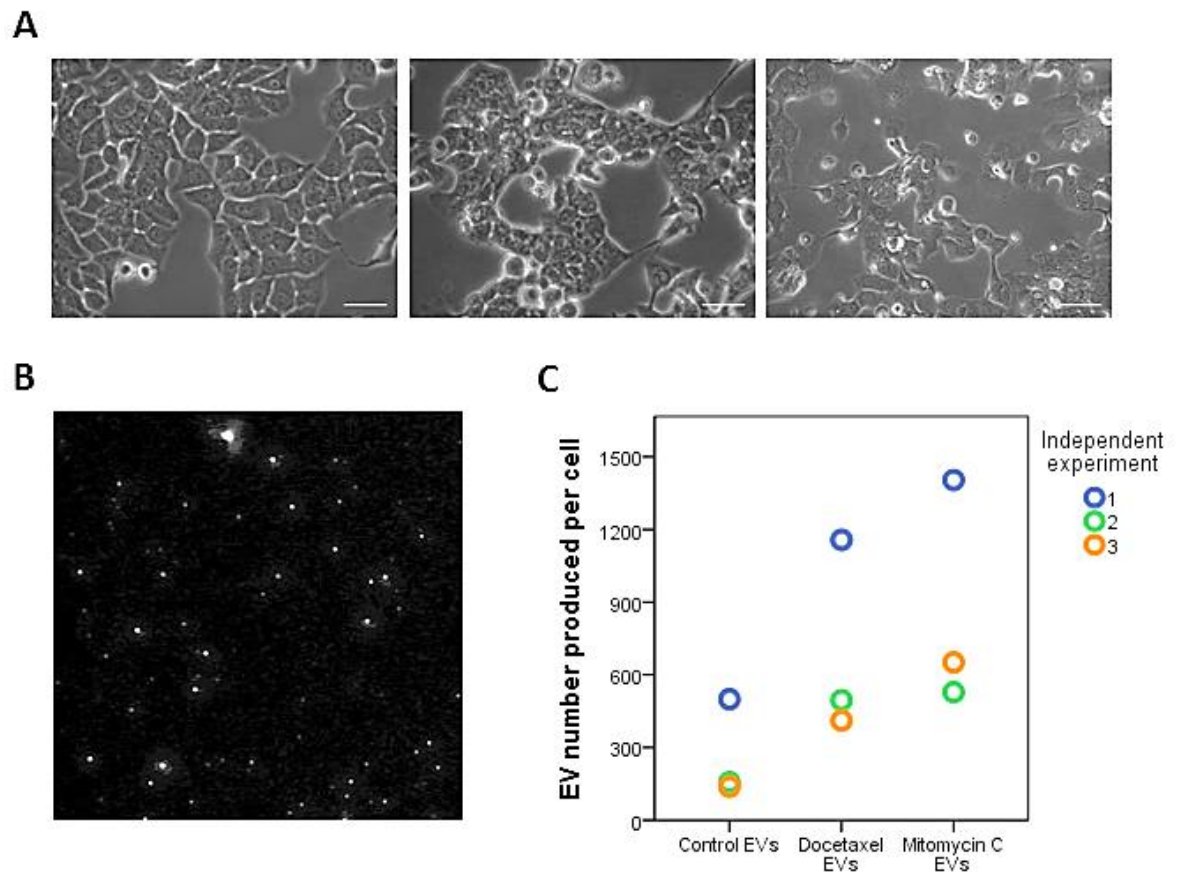


Figure 5.7 EVs from docetaxel and mitomycin C treated cells.

A) From left to right: non-treated, docetaxel-treated and mitomycin C-treated T47D cells at the moment of EV isolation. Scale bar, 20 μ m. B) Representative snapshot of the videos acquired during NTA of EVs isolated from the cells above, for the determination of their size and concentration. C) Non-treated cells secreted significantly lower amounts of EVs (Control EVs) compared with docetaxel- and mitomycin C-treated cells (Docetaxel EVs and Mitomycin C EVs, respectively). EV numbers were not averaged for each experiment to highlight the range of variability within experiments.

5.3.3 Chemotherapy-derived EVs do not affect the expression of EMT markers

To test whether chemotherapy-derived EVs induce EMT in recipient cells, EVs isolated from docetaxel-, mitomycin C-, and control- treated cells were administered to intact T47D cells for 24 h. EV concentration was previously calculated as EVs per cell (see 5.3.2) therefore, the same concentration per seeded recipient cell plus a 10x higher concentration was used for this experiment. Recipient cells were then lysed and the relative expression levels of CDH1, marker of epithelial phenotype and vimentin, marker of mesenchymal phenotype, were measured using qPCR. Expression levels were normalised to the 'no EV' control group (cells that did not receive EVs). No differences in the expression levels of CDH1 or vimentin were observed when cells were treated with the various types of EVs. Moreover, the concentration of EVs (1x and 10x) had no effect on EMT markers. These results suggest that EVs from chemotherapy-treated and non-

treated T47D cells do not trigger EMT in recipient autologous cells and an increase in EV dose does not change this outcome.

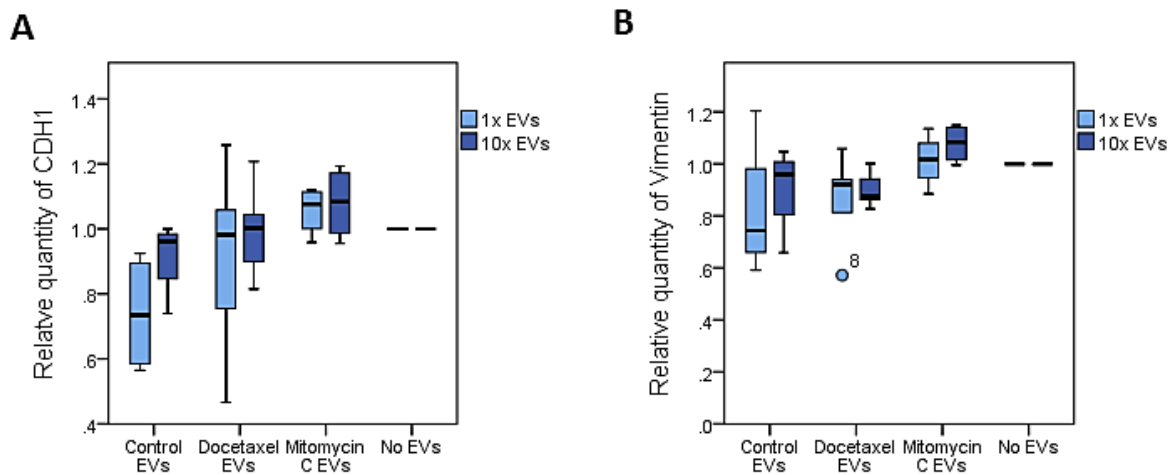


Figure 5.8 The effect of chemotherapy-derived EVs on EMT markers.

T47D cells were treated with control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' for 24 h and the expression levels of A) CDH1 and B) vimentin were assessed by qPCR. ACTB was used as endogenous control. No significant changes were observed in response to EV treatment or number of EVs. The Mann-Whitney test was used to determine the significance of differences. Expression levels were normalised to 'no EV' control. Three independent experiments, with 2 technical replicates each, were combined.

5.3.4 Chemotherapy-derived EVs do not affect the migration rate of recipient cells

To test whether EVs from chemotherapy treated cells can promote migration of intact recipient cells, the 'scratch' assay was performed as described before. Briefly, EVs isolated from docetaxel-, mitomycin C-, and control- treated cells were administered to confluent T47D cells at the moment of 'scratch', and gap closure was quantified using FIJI. To avoid bias in gap quantification due to slight differences in 'scratch' widths, the migration area was calculated as gap size at 6, 24, and 48 h minus gap size at time point 0 h. To confirm that any changes in migration rates are not due to cell proliferation, MTT proliferation tests were performed in parallel with the migration assays. No difference in cell migration or proliferation rates was observed amongst the different EV treatments after 48 and 72 h, respectively (Figure 5.9).

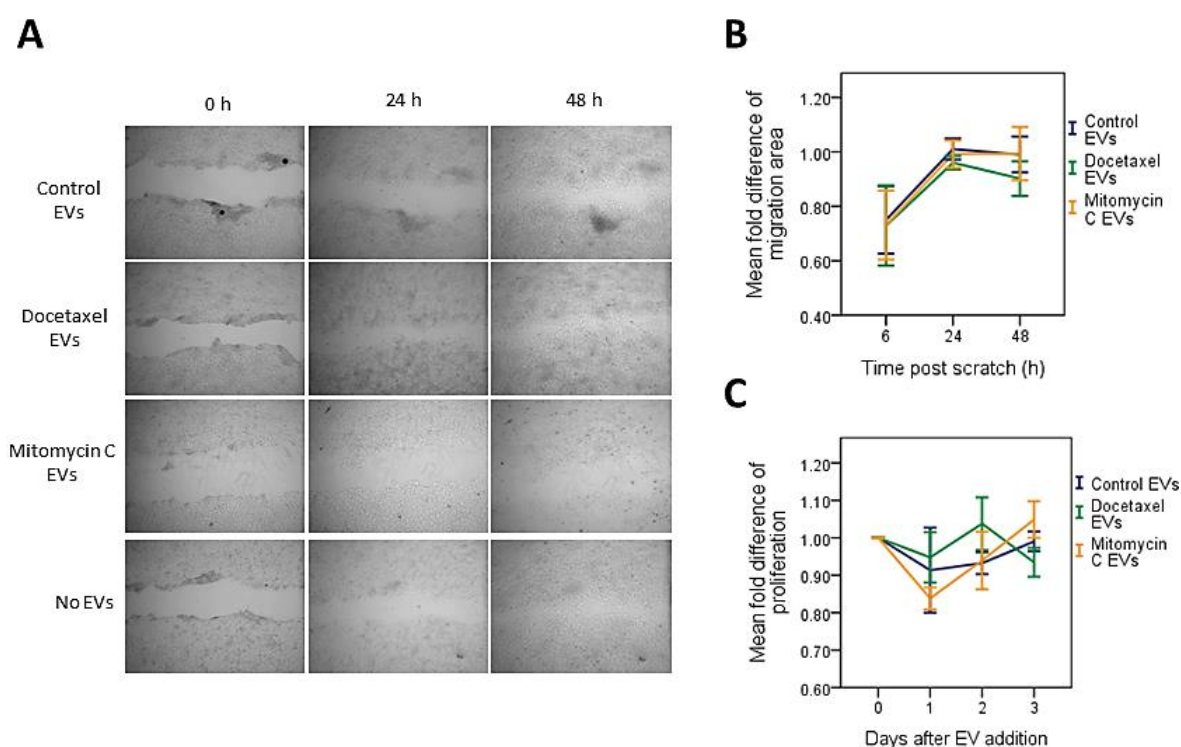


Figure 5.9 The effect of chemotherapy-derived EVs on cell migration.

A) Wound healing assay of T47D cells treated with control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' at the time of 'scratch'. B) The gap closure was monitored and calculated over-time. No difference in cell migration was observed amongst the different EV treatments after 48 h. C) The proliferation rate of cells after EV treatment was also measured, by MTT. No difference in proliferation was observed after EV treatment. Kruskal-Wallis and One-way ANOVA tests were used to determine differences. Error bars, 1x SEM. Migration area and proliferation were normalised to 'no EV' control at time point 0 h. Three independent experiments, with at least 4 technical replicates each, were combined for both migration and proliferation assays.

5.3.5 Chemotherapy-derived EVs do not increase invasiveness of recipient cells

To test whether EVs from chemotherapy treated cells can affect the invasive capability of recipient cells, a Matrigel® invasion assay was employed. Briefly, EVs isolated from docetaxel-, mitomycin C-, and control- treated cells were administered to intact T47D cells at the moment of seeding onto the Matrigel® inserts. Cells were allowed to invade the Matrigel® for 48 h and invaded cells were counted using FIJI (Figure 5.10 A). No statistically significant changes in the invasiveness of cells were observed after treatment with EVs (Figure 5.10 B). These results suggest that EVs from T47D cells, regardless of chemotherapy treatment, cannot induce invasiveness in autologous recipient cells.

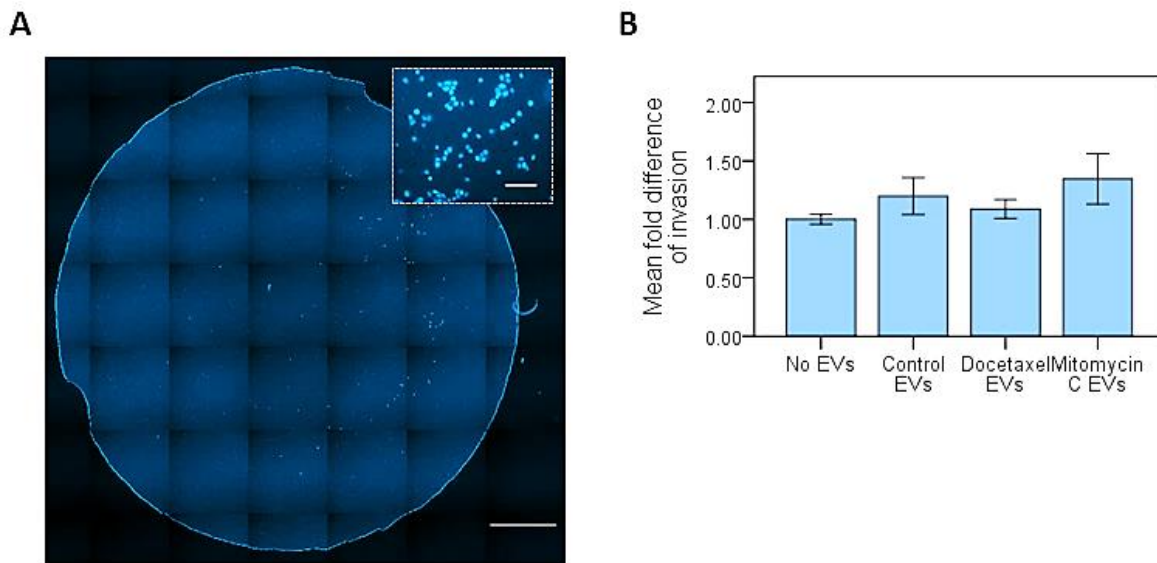


Figure 5.10 The effect of chemotherapy-derived EVs on cell invasion.

Cells were seeded along with EVs and allowed to invade through Matrigel® membranes for 48 h. A) Representative image of whole membranes analysed to assess invasion. Scale bar, 1 μ m. Invaded cells were stained with Hoechst (blue) and counted. Inset shows magnified area. Scale bar, 100 nm. B) No statistically significant differences were observed among EV treatments. Kruskal-Wallis and One-way ANOVA tests were used to determine differences. Error bars, 1x SEM. Invasion was normalized to 'no EV' control of each experiment. At least 3 independent experiments, with 3 technical replicates each, were combined.

5.3.6 Mitomycin C EVs decrease the ability of recipient cells to adhere to endothelial cells

To test whether EVs from chemotherapy treated cells can affect the ability of intact cells to adhere to endothelial cells, a static adhesion assay was employed. Briefly, T47D cells were incubated with control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' for 24 h and then allowed to adhere to a monolayer of HUVEC. Two EV concentrations were used here as previously described for the EMT experiment (section 5.3.3); 1x and 10x. The T47D cells were labelled prior to the experiment therefore, the fluorescent area (area occupied by cells) could be calculated using FIJI (Figure 5.11 A). No statistical differences were observed between 1x and 10x EV concentrations in all treatment groups (Figure 5.11 B), therefore samples were combined for subsequent tests. Mitomycin C EVs significantly decreased the ability of T47D cells to attach to the endothelial monolayer compared with the 'no EV' control (25% decrease, $p = 0.002$, Mann-Whitney test). On the other hand, docetaxel EVs slightly increased the adhesion ability of cells by 14% ($p = 0.065$, Mann-Whitney test) compared with the 'no EV' control. These results suggest that EVs from cells treated with different forms of chemotherapy may affect the cells capacity to adhere to the endothelial cells in a different way.

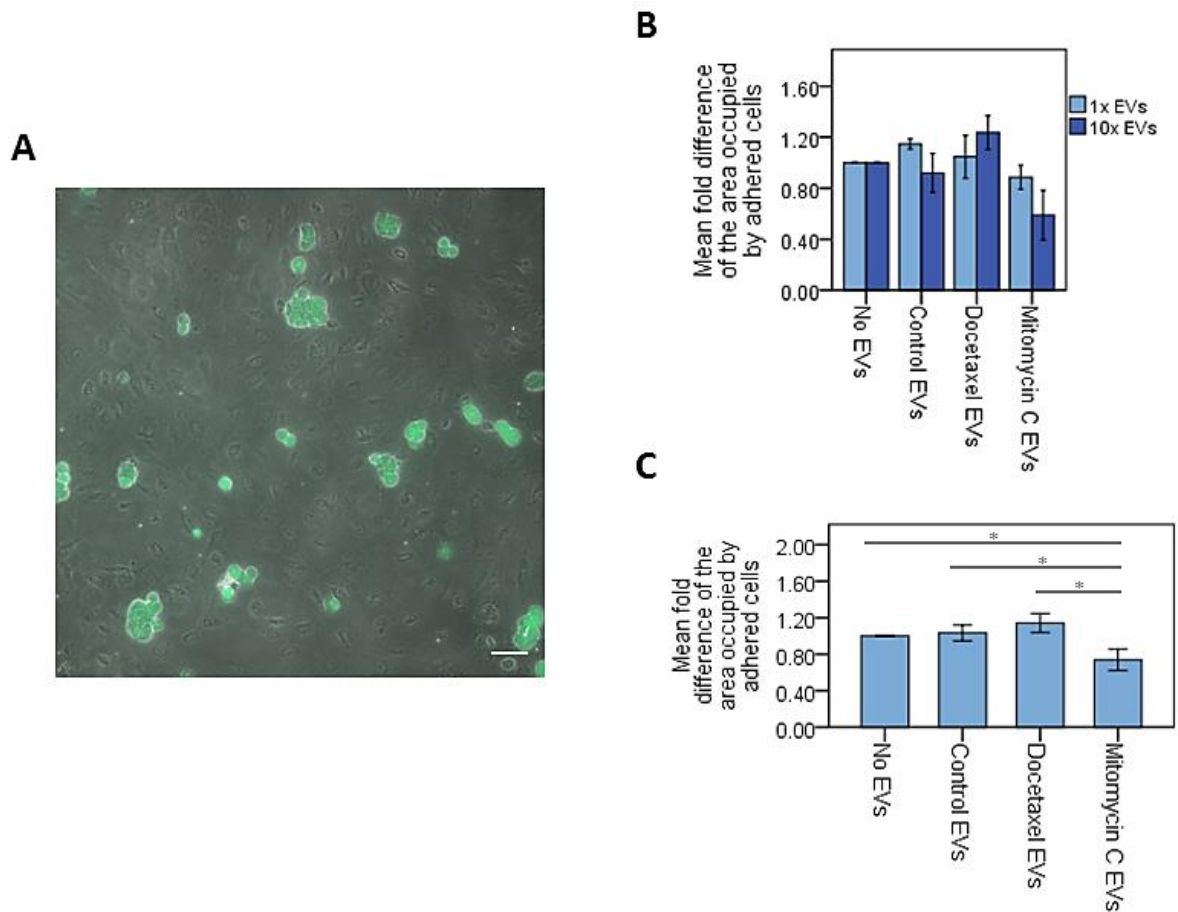


Figure 5.11 The effect of chemotherapy-derived EVs on the cells ability to adhere to the endothelial monolayer.

A static adhesion assay was employed to assess the capacity of T47D cells to adhere to HUVEC cells after treatment with control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' for 24 h prior to the experiment. A) Representative image of CFSE (green) stained T47D cells, attached to HUVEC monolayer. Scale bar, 50 μ m. B) No significant difference in the adhesion capacity was observed when cells were treated with 1x or 10x EVs. Therefore, samples were combined. C) Mitomycin C EVs significantly decreased the ability of T47D cells to attach to the endothelial monolayer. Error bars, 1x SEM. The Mann-Whitney test was used to determine significance of differences. * $P < 0.05$.

5.3.7 Chemotherapy EVs alter HPA labelling levels

In order to examine whether glycosylation patterns change in response to EV treatment, T47D cells were treated with control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' for 24 h and labelled with HPA Alexa Fluor® 647. Total levels of HPA labelling were calculated from the projected images as CTCF using FIJI (Figure 5.12 A). Due to distinct patterns within the same cell population (Figure 5.12 B), HPA labelling levels were categorised as high, intermediate and low. The cut-off value for the high levels was the 66.6th percentile of CTCF values of the 'no EV' control group. The cut-off value for the low levels was the 33.3th percentile of CTCF values of the no EV control group and the in-between values represented intermediate levels of labelling.

Treatment with mitomycin C EVs resulted in reduced levels of HPA labelling in the recipient cells. More specifically, more than 50% of the cells exhibited low HPA levels, while only approximately 20% of the population appeared with high or intermediate levels. The same pattern was observed after treatment with control EVs. These changes in HPA labelling levels were independent of EV numbers. On the contrary, 1x docetaxel EVs increased the HPA high population by 9%, but increased EV dose abrogated this effect. Overall, the levels of HPA labelling were associated with the type of EVs administered to the cells ($p = 2.5 \times 10^{-18}$, Pearson's chi-squared test).

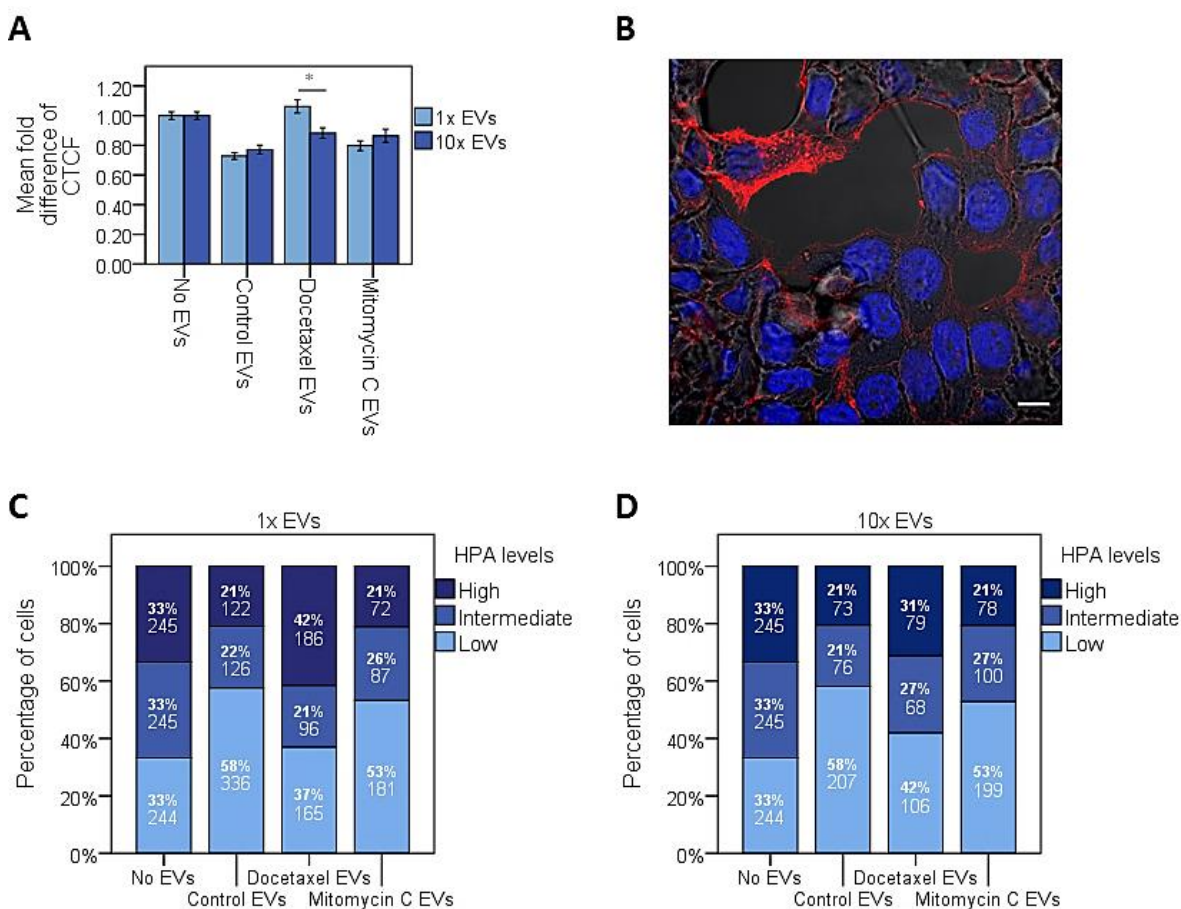


Figure 5.12 The effect of chemotherapy-derived EVs on HPA labelling.

A) The HPA labelling levels were measured as the average CTCF of cells treated with 1x or 10x control EVs, docetaxel EVs, mitomycin C EVs or 'no EVs' for 24 h. Error bars, 1x SEM. B) Representative image of T47D cells labelled with HPA. Distinct patterns of HPA labelling are observed. Scale bar, 5 μ m. C) Percentage and actual numbers of cells exhibiting high, intermediate and low levels of HPA labelling after treatment with control, docetaxel and mitomycin C EVs at 1x EV concentration. D) Percentage and actual numbers of cells exhibiting high, intermediate and low levels of HPA labelling after treatment with control, docetaxel and mitomycin C EVs at 10x EV concentration. At least 3 independent experiments with 2 technical replicates each. The Mann-Whitney test was used to determine the significance of differences. * $P < 0.05$.

5.5 Discussion

The hypothesis tested in this chapter was that EVs from chemotherapy-treated cells can induce a metastatic phenotype to intact autologous cells. This hypothesis was based on the following concepts: a) chemotherapy can promote metastasis and b) EVs as mediators of intercellular communication have been shown to play various roles in favour of metastasis. This hypothesis was further supported by other studies on cancer treatments, such as radiation and heat shock, which have been shown to trigger the release of EVs capable of inducing invasion of intact cells (Bewicke-Copley *et al.*, 2017; Al-Mayah *et al.*, 2012; Arscott *et al.*, 2013). Here, to test this hypothesis, T47D cells, a non-invasive breast cancer cell line was treated with sublethal doses of two chemotherapeutic agents broadly used in the clinic for the treatment of advanced breast cancer: docetaxel and mitomycin C. The EVs released by the surviving cells were collected, counted, and administered to intact T47D cells. The metastatic phenotype of recipient cells was evaluated by testing characteristics required for the individual steps of the metastatic cascade. Finally, changes in the glycosylation patterns of recipient cells were examined using HPA labelling.

EV numbers

Firstly, it was observed that cells treated with docetaxel and mitomycin C secreted higher amounts of EVs, compared with non-treated cells. This effect has been reported before, when Aubertin *et al.* (2016) used photodynamic therapy and doxorubicin, another chemotherapeutic agent – anthracycline, and showed that cells vesiculation due to therapy results in a massive release of EVs *in vitro* and *in vivo*. Moreover, breast cancer patients have significantly higher amounts of EVs in their blood compared with healthy individuals, which further rise after neoadjuvant chemotherapy (Konig *et al.*, 2017). It is still unclear what this high EV secretion after therapy means. For example, it could be the means to carry the drug out of the cell (Safaei *et al.*, 2005) or transmit chemoresistance to recipient cells by carrying signalling molecules (Chen *et al.*, 2014b). In patients, increased levels of circulating EVs after chemotherapy have been associated with therapy failure and disease progression (Konig *et al.*, 2017).

EMT

Epithelial to mesenchymal transition is a key process in cancer metastasis, as it allows cells to change their properties, such as their morphology, in order to travel from the primary tumour to distant sites in the body. To investigate whether EMT occurs after incubation with chemotherapy derived EVs, the expression levels of CDH1 and vimentin were measured by qPCR. The Taqman

(hybridization probes) technology was chosen and the assays were tested in advance to assure specificity of amplification. No change in the expression levels of CDH1 or vimentin was observed when the cells were incubated with chemotherapy EVs or control EVs, even at the higher doses of EVs. This suggests that EMT is not triggered in this case. This result, however, was not consistent with previous literature, showing that tumour derived EVs induce EMT when incubated with autologous cells as evidenced by increased expression of vimentin and decreased expression of CDH1 (You *et al.*, 2015). This might be due to the different type of cancer investigated in the two studies, as You *et al.* (2015) used nasopharyngeal carcinoma cell lines.

To test whether chemotherapy itself was able to induce EMT, two breast cancer cell lines, T47D and MCF7 cells were treated with increasing concentrations of docetaxel for 24 h and then the expression levels of CDH1 and vimentin were measured using qPCR. Even though this experiment was performed once, no considerable changes were observed in the relative quantity of mRNA of these two genes in relation to drug dose. This suggests that docetaxel was not able to induce EMT as observed by the expression of CDH1 and vimentin after 24 h of treatment (Figure 7.7). Perhaps the cells EMT/ transition or mesenchymal phenotype is a prerequisite for the released EVs to be able to further induce EMT in recipient cells. Several studies have demonstrated that macrophages, stroma cells or bone marrow derived mesenchymal cells are able to promote EMT and cancer cell invasion via EVs (Yang *et al.*, 2011; Roccaro *et al.*, 2013; Jossen *et al.*, 2015). However, a recent *in vivo* study by Keklikoglou *et al.* (2018) showed that chemotherapy-elicited EVs from the tumour cells and not the drugs themselves (doxorubicin and paclitaxel) were able to confer the pro-metastatic effects of chemotherapy. Particularly, EVs isolated from the tumour cells excised from chemotherapy-treated mice were able to promote metastasis when injected to immunocompetent mice, while chemotherapy alone had rather prophylactic effects over metastasis (Keklikoglou *et al.*, 2018). It cannot be discarded that more cell types, as found in the body, may act synergistically to induce EMT or that EMT might not definitely be necessary for metastasis (Fischer *et al.*, 2015). Moreover, the amount of EVs administered to recipient cells in each study may differ. This is an important parameter to consider when comparing different studies, as dose-dependent EV effects have been reported before (Schillaci *et al.*, 2017).

Motility and proliferation

As with EMT markers, no difference in the motility or proliferation rates was observed after incubation with chemotherapy or control EVs. The fact that control EVs did not increase the motility rates of autologous cells is contradictory to published research showing that incubation with autologous EVs promotes renal epithelial cell migration *in vitro* (Chen *et al.*, 2014a).

However, this assay utilises a confluent layer of cells and as it is widely accepted, confluent cells do not secrete or uptake EVs the same way as dispersed cells (Gudbergsson *et al.*, 2016). Therefore, another motility assay, such as the transwell assay without the Matrigel® layer, might have produced different results. To confirm that any changes in migration rates would not be due to cell proliferation, MTT proliferation tests were performed in parallel with the migration assays. EVs did not significantly change the proliferation rates of recipient cells. Even though evidence that EVs from breast cancer cells can promote proliferation of non-tumorigenic cells exists (Ozawa *et al.*, 2018), proliferation and invasion are typically but not strictly mutually exclusive processes (Gao *et al.*, 2005).

Invasion

To test the invasive capacity of cells after incubation with chemotherapy, control EVs or 'no EVs', the Matrigel® invasion assay was performed at least thrice. T47D cells are typically non-invasive, therefore only a few cells invaded the Matrigel®. For this reason, the whole membranes were imaged and the total amount of invaded cells was counted, instead of acquiring representative images. Treatment with EVs did not change the invasiveness of recipient cells. A slight increase in the invasion of cells treated with mitomycin C EVs was observed, but these data did not reach statistical significance. Previous studies have shown that autologous EVs but also EVs from drug treated tumours can increase the number of metastatic foci *in vivo* (Zomer *et al.*, 2015; Keklikoglou *et al.*, 2018). *In vitro* studies have also demonstrated that incubation with autologous EVs is sufficient to increase invasiveness of recipient cells (Yu *et al.*, 2017). However, the type of originating cells plays a key role in the EVs ability to confer such effects. For example, mesenchymal-like breast cancer cells, such as MDA-MB231, produce EVs with different characteristics and downstream effects on recipient cells than epithelial cell- (such as MCF7) derived EVs (Pasquier *et al.*, 2014). Also, multiple studies have shown the ability of highly malignant cell-derived EVs to confer metastatic characteristics to less aggressive cells (Lima *et al.*, 2013; O'Brien *et al.*, 2013; Schillaci *et al.*, 2017).

Adhesion to the endothelial monolayer

Finally, to assess whether chemotherapy EVs alter the cells ability to adhere to the endothelial layer (for intra- or extravasation) a static adhesion assay was performed. As previously, the effects observed were not influenced by the amount of EVs administered (1x and 10x), therefore samples were combined and treated as independent biological replicates. Incubation with mitomycin C EVs appeared to reduce the cells ability to adhere to the HUVEC cell layer by 25% compared with 'no EV' control (p=0.002, Mann-Whitney test), while docetaxel EVs slightly

increased it ($p=0.065$, Mann-Whitney test). Acknowledging that more experiments are needed to confirm these results, this was a first indication that different drugs with distinct mechanisms of action may affect metastasis and particularly adhesion to the vascular endothelium in a different way. As similar total numbers of docetaxel and mitomycin C EVs were administered to the cells, (Figure 7.6 shows that docetaxel and mitomycin C treated cells release similar amounts of EVs), the speculation that EVs themselves might mask the cells and therefore prevent their binding to the HUVEC cells is undermined. Control EVs did not have an effect on cell adhesion, which is contradictory to the study by Fu *et al.* (2018), who demonstrated that EVs released from pancreatic cancer cells enhance cell adhesion of the same cells. Of course, the characteristics of each cell line are unique as are the properties of the EVs they produce (Lazaro-Ibanez *et al.*, 2014; Gyorgy *et al.*, 2011).

HPA labelling

HPA binding is a marker of metastatically competent cells. Here, the glycosylation patterns of cells that received EVs were examined using a fluorescently labelled HPA lectin. The aim of these experiments was to assess whether chemotherapy-derived EVs alters HPA binding, and therefore, potentially, metastatic capacity. HPA binding levels were calculated as total cell fluorescence from three dimensional images acquired on a confocal microscope. The values were normalised to the 'no EV' control (T47D cells that did not receive EVs) for every independent experiment. Within each treatment group, labelling was not homogeneous and subpopulations of cells with low, intermediate and high levels of HPA labelling were observed. This is in accordance with observations by Brooks (2000), highlighting that although HPA positivity is linked to metastatic competence of the primary tumour, in all studies a proportion of metastases was HPA negative. Other studies have since confirmed the co-existence of HPA positive and HPA negative cells within the same population *in vitro* (Rambaruth *et al.*, 2012). Unlike previous studies, to define changes in HPA binding, the 'no EV' control was split into three percentiles, representing low, intermediate and high levels of HPA labelling. Changes caused by EV treatment were reflected in changes of the cell proportions in these subpopulations. This approach guaranteed bias free analysis compared with previous studies that were based on judgment of fluorescence intensity by eye (microscopy based methods only)(Brooks *et al.*, 2001; Brooks and Leatham, 1991; Brooks and Wilkinson, 2003).

It was observed that HPA binding levels were associated with the type of EVs administered. Mitomycin C and control EVs increase the population of cells with low HPA labelling by approximately 22%, while reducing both the intermediate and high populations in a non EV dose dependent manner. On the other hand, docetaxel EVs increase the population with high HPA

binding by 9%, but treatment with higher amounts of EVs, abrogated this effect. As already stated above, the speculation that EVs themselves might mask the sugars on the cell surface and therefore prevent HPA binding is undermined, as approximately the same total numbers of EVs were used for the Docetaxel EV and Mitomycin C EV treated cells. Again, this was another indication that drugs with distinct mechanisms of action can affect glycosylation patterns (and probably metastasis) in different ways. However, due to lack of positive results in the previous experiments (EMT, migration, proliferation, and invasion), it is hard to assume the relationship between glycosylation and metastatic stage/potential. It could be hypothesised that docetaxel EVs increase the synthesis of aberrant glycans on the cell surface, allowing the cells to adhere to the endothelium. On the contrary, mitomycin C EVs reduce the cells ability to adhere to the endothelium and also encourage the prevalence of HPA low population. Taken together, aberrant glycosylation, as captured by HPA, might play an important part in the cells ability to extra- or intravasate during metastasis. These results are in agreement with published literature, as it has been shown that the HPA-binding glycans were not involved in invasion (Brooks *et al.*, 2001), but were important for the cells adhesion to the endothelium (Bapu *et al.*, 2016). It might be possible that EVs trigger or hinder the synthesis of such glycans.

Experimental limitations

For the experiments reported in this chapter, one cell line was used as a model of breast cancer metastasis. It was decided that under the time and budget constraints, comprehensively testing the steps of metastasis after treatment with two chemotherapeutic agents in one cell line would be preferential to performing limited analyses in multiple cell lines. It is important that multiple cell lines are used when possible to reflect the complexity of breast cancer, and perhaps different types of cell lines would respond differently in these analyses had they been tested. Here, the T47D cell line was chosen as it is considered a non-invasive line and therefore, its intrinsic characteristics would not mask the effect of chemotherapy, even though most studies that show increased metastatic capacity after EV incubation use cell lines that already exhibit tumourigenic effects (Luga *et al.*, 2012; Chen *et al.*, 2014a). It is also valid that in the body there is constant release and uptake of EVs, while the *in vitro* setting is different, probably not representing physiological conditions. Perhaps a constant flow of EVs and different time-points of incubation would also result in a different outcome. For instance, Josson *et al.* (2015) showed that prostate cancer cells underwent EMT six weeks after incubation with stromal cell media.

For the EMT assessment specifically, more markers should be tested, such as SNAIL, TWIST, beta-catenin, ZO-1 and others. It is well established that not all markers change during EMT and by only testing CDH1 and vimentin, part of the picture might be lost. Furthermore, a more

suitable endogenous control for the qPCRs should be used to assess EMT. Actin, as a structural component of the cytoskeleton, changes with EMT. A ubiquitously expressed RNA, not affected by EMT, such as the 18S ribosomal RNA, might have been a better option for these experiments. Regarding the 'scratch' assay, confluent cells might not incorporate as many EVs as isolated cells, hindering their effect. Another type of assay to investigate changes in cell motility, like a transwell based assay, might have provided different results. Finally, for the adhesion assays, the attachment to endothelium should also be tested under shear forces to resemble physiological blood flow rates. Even though microscopy is a reliable method to assess HPA labelling levels, another method that is time-saving but still unbiased is flow-cytometry. Multiple groups use this method to quickly scan glycosylation profiles in large scale, however, not all laboratories have access to a flow-cytometer (Batisse *et al.*, 2004).

5.6 Key findings

- Docetaxel and Mitomycin C-treated cells secrete significantly higher amounts of EVs compared with non-treated cells.
- T47D EVs (chemotherapy and control-derived) do not trigger EMT in recipient autologous cells.
- T47D EVs (chemotherapy and control-derived) do not affect motility and proliferation rates of intact autologous cells.
- T47D EVs (chemotherapy and control-derived) do not change the invasive capacity of intact autologous cells.
- Mitomycin C EVs lower the ability of recipient autologous cells to adhere to endothelial cells.
- Mitomycin C and control EVs alter the HPA labelling levels of intact recipient cells by reducing the high and increasing the low HPA labelled subpopulations, in a non-dose dependent manner.

Final discussion

Chapter 6

6 Final discussion

6.1 EV labelling

Several methods have been proposed for the labelling of EVs. The ideal labelling method would be 100% specific to EVs, however, due to the lack of specific markers on the surface and in the lumen of EVs, generic labelling methods are frequently employed (de Rond *et al.*, 2018). These include labelling of the EV membrane and cargo, but also the fluorescent tagging of molecules enriched in, or on, EVs. In this project, three of these methods were tested for their suitability to be used in later experiments which aimed to accurately monitor EV uptake by recipient cells.

Firstly, the lipophilic organic dye PKH26 was used to label EVs from two breast cancer cell lines, T47D and MCF7. When labelled EVs were incubated with naïve autologous cells, EVs appeared to be internalised and the uptake pattern observed (Figure 3.3) was similar to that published in multiple studies (Singh *et al.*, 2016; Sento *et al.*, 2016; Bilen *et al.*, 2017). However, the 'no EV' control, which consisted of cells treated with PBS, diluent C, and dye in the same proportions as the mixture used for EV labelling, appeared to give a similar pattern of staining in the naïve cells. After labelling, EVs and 'no EV' control were passed through Zeba™ Spin desalting columns, which are designed to retain molecules smaller than 7 kDa while allowing bigger ones to pass through. The molecular weight of PKH26 is about 1kDa and theoretically, free dye (ie PKH26) should be retained in the column. This method of removing excess dye after EV labelling has been published before (Holder *et al.*, 2016) and was preferred over another ultracentrifugation step, which could reduce the EV yield. To address the problem of non-specific staining, the role of BSA as a quencher of free dye was examined. It was hypothesised that BSA might contribute to increased fluorescent signals in recipient cells, therefore an experiment was conducted where the BSA quenching step was omitted from the protocol. From this, it became apparent that BSA was useful to quench residual dye when comparing the EV preparations. More specifically, equal numbers of labelled EVs were treated and not treated with BSA. The treated group showed reduced fluorescence (in terms of uptake in recipient cells) compared with the non-treated group. Later, newly published work in the field showed that PKH26 can form micelles, spherical structures similar to the size of EVs that could contribute to the non-specific staining (Puzar Dominkus *et al.*, 2018). Indeed, when PKH26-labelled EVs and 'no EV' control samples were imaged using confocal microscopy, similar structures were observed. One way to remove such micelles from sample preparation would be to adopt an isolation method that relies on density rather than size alone, such as sucrose density centrifugation. This method would allow a better

separation of the labelled EVs and the less dense micelles (Puzar Dominkus *et al.*, 2018). A caveat when adapting a protocol originally developed for cell labelling to instead stain EVs may be the concentration of dye used. Here, the same dye concentration (2 μM) was used as in the original protocol for cell labelling, but Takov *et al.* (2017) have used even higher concentrations (3 μM). Using high concentrations of dye might affect the stoichiometry of the reaction between dye and the EV surface, leading to an excess of free dye which in this case can form micelles. Perhaps, during optimisation, different dye concentrations should be used to define the appropriate dye to EVs ratio.

Another generic dye tested in this project was CFSE, which enters the lumen of EVs and binds to the protein load, providing a means of staining EV cargo. Studies have used this type of labelling to monitor EV uptake by flow cytometry (Pospichalova *et al.*, 2015) and by microscopy (Keller *et al.*, 2009). In the experiments reported here, administration of CFSE labelled EVs led to a rapid and homogenous staining of recipient cells, less bright but similar to that of direct cell labelling with CFSE. Unlike PKH26, little evidence exists in the literature regarding the suitability of CFSE for EV labelling. However, comparing published images of uptake of CFSE labelled EVs, it was obvious that cells that incorporated CFSE labelled EVs acquired a rather homogenous staining unlike the punctate pattern observed by PKH26 labelled EVs (Czernek *et al.*, 2015; Keller *et al.*, 2009). Here, the 'no EV' controls were clear of fluorescence, indicating that the desalting columns were able to successfully retain free CFSE dye. A possible explanation for this phenomenon could be the carry-over of free dye in the EV preparation that would result in the homogenous staining of cells; however it is difficult to theorise mechanisms by which this could happen.

Dual labelling of EVs using both CFSE and PKH26 was also explored in this project in order to test whether a higher specificity of EV labelling could be achieved. Such an approach had never been attempted before, and if successful, it could have been used to study cargo dissociation and delivery, as PKH26 labels the membrane, while CFSE the cargo of EVs. Attempts at dual labelling, however, further confirmed the flaws of both dyes in uptake experiments. It was anticipated that cells would acquire both green and red fluorescent labelling simultaneously (due to EV uptake). However, this was not the case, with accumulation of green fluorescence preceding red.

To overcome dye carry-over in EV preparations, a different protocol was also tested. Donor cells were stained with both CFSE and PKH26, and EVs were collected from the stained cells. These EVs exhibited diverse labelling (Figure 3.6) when imaged using confocal microscopy. In addition, this experimental method is very expensive, especially considering the large number of donor cells needed for each EV isolation experiment.

To overcome the limitations of dyes and the cost of repetitive EV labelling, the next step was to use fluorescently tagged EV markers. In such applications, the general exosomal marker CD63 is most frequently used (Heusermann *et al.*, 2016; Suetsugu *et al.*, 2013; Mittelbrunn *et al.*, 2011). In this project, CD81, a tetraspanin predominantly found in small EVs (Kowal *et al.*, 2016), and expressed in the EVs produced by the cell lines used in the current experimental setting (Figure 3.8) was chosen. This was because the plasmid coding for the fusion product of a green fluorescent protein and CD81 was available in the lab. Two cell lines derived from breast cancer (T47D) and normal breast epithelium (hTERT-HME1) were stably transfected to overexpress the fusion protein mEmerald-CD81. EVs isolated from these cells were seen as green fluorescent dots when imaged using confocal microscopy. They appeared slightly smaller than stained EVs (no statistical analysis performed), which might be due to the amount of fluorescent molecules per EV.

When using mEmerald-CD81 EVs in uptake experiments, EVs moved around in the extracellular space and started entering the cells within the first 10 minutes of EV administration. The movement and uptake pattern of this type of EVs were similar to those shown for CD63-GFP EVs (Heusermann *et al.*, 2016). Unlabelled EVs did not produce any fluorescent signals (data not shown). It is worth mentioning that the fluorescent molecule (mEmerald) was in the intraluminal part of the tetraspanin, reducing the possibility of interference with other molecules/moieties important for EV uptake. On the other hand, overexpression of CD81 may alter the properties of EVs. For example, CD81 is an attachment receptor for hepatitis C virus entry in mammalian cells (Petracca *et al.*, 2000) and also participates in EV fusion with the plasma membrane (Andreu and Yanez-Mo, 2014). Similarly, increased expression of CD81 on EVs might alter their uptake characteristics. The effects of overexpressing tetraspanin markers in EVs have not previously been studied, but a suitable method to avoid any potential complications would be the use of the CRISPR-Cas9 system (Hsu *et al.*, 2014). More specifically, this would allow the expression of fluorescent native CD81 proteins, without altering their stoichiometry in the cell. Then, to check whether the addition of mEmerald changes the properties of the CD81 itself, a straightforward experiment would be to test the amount of membrane protrusions in mEmerald-CD81 (created using CRISPR) and parental cells, since CD81 is an important player of this process (Quast *et al.*, 2011).

Going forward in this project, from the range of labelling methods tested, it was decided that mEmerald-CD81 EVs would be the best option to use in subsequent experiments to explore EV uptake dynamics.

6.2 EV docking and uptake

The various mechanisms of EV uptake have been described in section 1.1.3. In the literature, EV uptake routes have been characterised using different cell types and approaches. However, it is still unclear which mechanisms are employed by different cell types under various conditions. In this project, it was hypothesised that cancer cells and normal cells may exhibit different EV uptake behaviours. In addition to T47D and hTERT-HME1 cells, a cervical cancer cell line (HeLa), previously used in EV uptake experiments in our laboratory, was also employed. The inclusion of the cervical cancer cell line would help determine whether there are differences in EV uptake between cancer and normal cells irrespective of their origin. Deciphering the different aspects of EV uptake between cancer and normal cells could have impact on therapeutic interventions and would aid the development of target specific drug delivery tools.

In this project, it became apparent that EV uptake differs across all three cell lines tested and, unexpectedly, patterns were not similar for cancer vs normal cells or cells of the same tissue of origin. The number of studies testing uptake efficiency of different cell lines using the same EV type is limited, however, Horibe *et al.* (2018) showed that EVs are non-selectively incorporated by recipient cells. More specifically, using one lung (A549) and two colon cancer (HCT116 and COLO205) cell lines, they demonstrated that HCT116 cells constitutively internalised more EVs regardless of the donor cell line. Uptake was inhibited by different chemical agents in each cell line, indicating that different uptake mechanisms are employed by different cells (Horibe *et al.*, 2018).

On the other hand, several studies have argued that EV uptake is a highly specific process. For instance, Zech *et al.* (2012) showed that peritoneal exudate cells were able to incorporate pancreatic cancer-derived EVs more efficiently compared to granulocytes and T-cells. Similarly, (Rana *et al.*, 2012) demonstrated that lymph-node stroma EVs were less efficiently incorporated by autologous cells than by endothelial and pancreatic cells, and attributed this specificity to tetraspanins on the EV surface.

Indeed, several molecules have been identified to mediate EV-plasma membrane interactions. Such molecules include tetraspanins, extracellular matrix proteins, integrins, proteoglycans and lectins (reviewed by French *et al.* (2017)). Interaction and docking of EVs on the plasma membrane of the target cell is a crucial step towards EV uptake and signal transduction (Mulcahy *et al.*, 2014; French *et al.*, 2017). In the experiments conducted here, the origin of EVs appeared to affect their docking on the target cells. Interestingly, breast cancer cells accumulated more autologous EVs than EVs from the normal breast cell line, while cervical cancer cells,

accumulated more normal breast-derived EVs than EVs from another type of cancer. It could be argued that (if possible) studying the composition of the plasma membrane in a panel of cells and EVs from different origins could potentially hint at possible interaction schemes. Of course, these predicted interactions should then be validated with functional experiments, such as protein knockouts or lipid depletion, but could be a powerful tool to elucidate specificity of interactions and further uptake.

So far, in the literature, several approaches have been used to modify the membrane properties in order to study EV uptake mechanisms. These include chemical compounds that affect sub-membranous pH, lipid and protein composition, actin and tubulin dynamics, etc. (Mulcahy *et al.*, 2014). In this project, amiloride, dynasore, and cholesterol oxidase were employed to cover a range of active and passive mechanisms of uptake. From high-resolution airy scan images, EVs were observed a) interacting with the plasma membrane, b) enclosed in membrane fragments in the cytoplasm, and c) as single entities in the cytoplasm (Figure 4.2 A). Evidence of single particles in the cytoplasm of recipient cells unaccompanied by membranous fragments led to the assumption that perhaps passive mechanisms, such as changes in membrane permeability, were also involved in EV uptake. Therefore, amiloride and dynasore were used to inhibit active processes, namely micropinocytosis and clathrin- and caveolin-dependent endocytosis, while cholesterol oxidase was used to change membrane fluidity and thus permeability (Koivusalo *et al.*, 2010; Preta *et al.*, 2015a; Kreit and Sampson, 2009). The fact that a drug might affect multiple pathways cannot be overlooked. In this case, dynasore is an inhibitor of dynamin 2, which is involved in clathrin- and caveolin-mediated endocytosis as well as phagocytosis (Feng *et al.*, 2010; Macia *et al.*, 2006). In addition, dynasore reduces cholesterol in the plasma membrane, disrupting lipid rafts, thus making it suitable to block several energy-dependent and -independent mechanisms, but also making it difficult to decipher which specific pathways are relevant (Park *et al.*, 2013). Due to the low number of cells investigated and the variability within the same cell population, the exact mechanism of EV uptake for each cell line was difficult to decode. However, the effect of the aforementioned drugs on the docking of EVs on the membrane in proportion to the internalised EVs was investigated over time. Even though amiloride affected the numbers of EVs found on the membrane of the normal breast cells, it had minimal effects on the other two cell lines tested. Cholesterol oxidase appeared to affect both positively and negatively the amount of EVs found on the membrane of all three cell lines, suggesting a possible selective role of cholesterol in EV- plasma membrane interactions.

To further explore the dynamics of these interactions, movement of EVs on the membrane of cells was investigated using single molecule tracking. Two distinct distributions of EV dynamics

were identified: a) EVs that would interact fast and travel short distances (short range diffusion) and b) EVs that would interact longer and travel further (long range diffusion). Unfortunately, due to experimental/system limitations, complex tracks and behaviours of EVs, such as the 'kiss and run' pattern described by Heusermann *et al.* (2016) for internalised EVs, were not possible to resolve and therefore were not analysed. Instead, the focus was on short interactions and how they were affected by dynasore and cholesterol oxidase in the different cell and EV types. From the data, it is clear that short range diffusion changed among cell types mainly in response to cholesterol oxidase treatment, and less to dynasore. Even though cholesterol has been studied in the context of EV uptake, its role in EV interactions with plasma membrane has not been explored. This project revealed the importance of cholesterol in EV interactions and docking on the plasma membrane. Further studies are needed to confirm these results with other cell and EV types, and also including a higher number of biological replicates. However, considering that EV uptake and viral cell entry processes share characteristics (van Dongen *et al.*, 2016), it is possible that cholesterol may be an important component of the entry mechanism. For example, the human immunodeficiency virus (HIV) has been shown to enter trophoblastic cells in a clathrin-, caveolin- and dynamin- independent way that requires free cholesterol (Vidricaire and Tremblay, 2007).

Overall, there is little agreement in the literature about EV docking and uptake mechanisms. This is perhaps due to the variety of donor and recipient cell types tested, the heterogeneity of EV preparation, the fact that the selected inhibitors might block more than one pathway, the method of labelling and visualisation, and the parameters used during analysis of the data. However, understanding these processes is a key aim of the EV field, as it could lead to major advances in drug delivery systems and personalised medicine.

6.3 The role of EVs in breast cancer metastasis after chemotherapy

Some cancer treatment schemes include chemotherapy, radiation and the not so widely available hyperthermia (Duchemann and Zelek, 2017; van der Zee, 2002). EVs released by cells that have been treated with radiation or hyperthermia have been shown to increase invasion *in vitro* (Bewicke-Copley *et al.*, 2017; Arscott *et al.*, 2013). In this project, acknowledging the possible effects of chemotherapy in metastasis (described in section 1.3.1) it was hypothesised that EVs from chemotherapy-treated cells may confer metastatic properties to non-treated autologous cells. A recent *in vivo* study in mouse models of breast cancer has shown that the EVs from chemotherapy-treated cells, and not the chemotherapy itself, is responsible for the

augmented metastatic phenotype in breast cancer (Keklikoglou *et al.*, 2018), further supporting this hypothesis.

Here, a non-invasive breast cancer cell line (T47D) was used for the majority of the experiments. Docetaxel and mitomycin C, two chemotherapeutic agents used in the clinic for the treatment of breast cancer were employed. Cells were treated with sub-lethal doses of the drugs, EVs were collected from the cells that survived treatment and incubated with naïve autologous cells.

An increased release of EVs was observed by the cells that had been treated with both drugs. This finding is in agreement with other studies that have also reported enhanced release of EVs in response to various chemotherapeutic agents, such as doxorubicin (Aubertin *et al.*, 2016) and paclitaxel (Keklikoglou *et al.*, 2018). On the contrary, Samuel *et al.* (2018) showed that cisplatin treatment did not affect the amount of EVs secreted by ovarian cancer cells. Expanding to other types of treatment, tunicamycin, a glycosylation inhibitor (Collett *et al.*, 2018), radiation (Arscott *et al.*, 2013), and photodynamic therapy (Aubertin *et al.*, 2016) resulted in enhanced release of EVs, while hyperthermia did not affect EV numbers (Bewicke-Copley *et al.*, 2017). These controversies in the literature might reflect the diversity in the mechanisms of action of each treatment, or could possibly indicate the need of standardised protocols in the study of EVs (Witwer *et al.*, 2017).

The next step was to assess whether EVs from chemotherapy-treated cells can confer metastasis-related properties to naïve autologous cells. EMT, as indicated by the expression levels of CDH1 and vimentin, was not observed in cells that received docetaxel or mitomycin C EVs, neither control EVs from non-treated cells. Similarly, no change was observed in cell motility or capacity to invade through Matrigel® after treatment with the aforementioned EVs. On the other hand, mitomycin C EVs appeared to reduce the ability of recipient cells to attach to endothelial cells, perhaps implying an anti-metastatic effect; however more experiments are needed to further validate these results.

The role of EVs after chemotherapy with docetaxel or mitomycin C in cancer has not been studied before. Studies on other anti-cancer therapy regimes exist, showing that EVs from radiation or heat shock treatment can increase the invasiveness of recipient cells (Al-Mayah *et al.*, 2012; Bewicke-Copley *et al.*, 2017). EVs from cisplatin (another cancer chemotherapy drug) treated cells have also been shown to increase invasion in naïve autologous ovarian cancer cells (Samuel *et al.*, 2018). In breast cancer, taxanes and anthracyclines have been shown to impart cancer-derived EVs with augmented pro-metastatic activity in mouse mammary tumour models (Keklikoglou *et al.*, 2018). In the present study, none of the chemotherapeutic agents appeared

to promote EVs with pro-metastatic activity. In addition, incubation with EVs from non-treated cells did not confer any effects. Other studies have shown that incubation with self-derived EVs enhances the tumourigenic characteristics of cells (Menck *et al.*, 2015; Raimondo *et al.*, 2015; You *et al.*, 2015). However, this might depend on the type of cells used in each study, as the content of EVs released by cells with a metastatic phenotype differs from that of non-metastatic cells (Jeppesen *et al.*, 2014). Many studies have focused on transfer of EVs from highly metastatic cells to non-malignant or less invasive cells (Rodriguez *et al.*, 2015; Lima *et al.*, 2013; Zomer *et al.*, 2015). Perhaps, in future experiments, the metastatic phenotype of the EV donor cells (cells that survived chemotherapy) could be assessed before the EV collection. Moreover, other factors like the EV amount, or time of incubation, might have contributed to the differences observed between this project and other studies reported in the literature. Under physiological conditions, there is a constant release and uptake of EVs in the body (Yanez-Mo *et al.*, 2015), which is not easy to simulate in the lab.

Lastly, the effect of chemotherapy derived EVs on the glycosylation patterns of recipient cells, as revealed by HPA labelling, was assessed. It was observed that EVs from mitomycin C treated cells and control non-treated cells increased the population of cells with low HPA labelling, irrespective of EV dose. Docetaxel derived EVs had minimal effects, slightly increasing the HPA positive cell population. HPA-positivity of tumour biopsies is well characterised and associated with metastatic competence (Leathem and Brooks, 1987; Noguchi *et al.*, 1993; Brooks and Leathem, 1991). But, both HPA positive and negative cell populations are found within tumours and interestingly, HPA positive tumours can result in both HPA positive and negative metastatic foci (Schumacher *et al.*, 1992; Brooks, 2000). This highlights the plasticity of glycosylation expressed as HPA-positivity in these studies. Indeed, glycosylation changes in cancer is a complex subject, extensively described by Pinho and Reis (2015), but is not the focus of this project. Yet, HPA labelling was assessed as a means of evaluating pro-metastatic behaviour. HPA labelling cannot directly be linked to the metastatic properties of the cells, as this would require further functional experiments. However, had the previous experiments (regarding invasion and migration) in this section been successful/positive, this project would be able to provide a link between HPA labelling levels and pro-metastatic properties.

6.4 Future directions

This research could be extended to identify the optimal EV labelling methods according to the downstream applications. For example, EVs from body fluids could not be labelled using the mEmerald-CD81 method described here. Optimization of the generic dye protocols, perhaps

testing different clean-up methods, could provide a meaningful tool for researchers in the EV field and help prevent conflicting results as seen in different studies. Understanding the limitations of each method and how these could affect uptake, would also be beneficial to researchers allowing them to choose the best tool according to the scientific question being asked.

EV docking and uptake experiments could be expanded to incorporate a greater number of cells, and further biological replicates, in order to validate the preliminary results presented here. More cell types should be included in the study to more accurately represent the diversity across cell lines and EVs. A wider range of uptake inhibitors and other drugs should be tested in order to decipher the different pathways involved in the various combinations of cells and EVs. Given the cross-reactivity of most uptake inhibitors, perhaps knock-down experiments of important effectors of the pathway tested could also be conducted. To elucidate the role of cholesterol in EV-plasma membrane interactions and thus uptake, more cholesterol reducing agents should be tested including cyclodextrins, which completely deplete cholesterol from the cell membrane. To be able to spatiotemporally track EVs and accurately calculate their diffusion rate (reflective of active or passive uptake mechanisms), advanced microscopy methods could be applied. Some of these include camera-based systems, Airyscan fast, and TIRF. Alternatively, fast confocal scanning imaging could still provide a lot of information, but tracking in three dimensions with enough time resolution for EV movement would be difficult to achieve. Overall, these experiments could provide insights on the different mechanisms each cell type employs to interact with and uptake EVs and could in the long run be used to design specific drug delivery tools.

Regarding the role of EVs after chemotherapy, these experiments should be repeated using more cell lines. Understandably, breast cancer is a complex disease and cannot be represented by one cell line. Moreover, different concentrations of EVs should be tested to explore whether the lack of effect regarding migration, invasion and EMT observed in this study, was due to inadequate amounts of EVs. Combining with results from the previous section, T47D cells incorporated relatively low numbers of EVs within 100 min of administration. This may mean that these cells need longer incubation for the EVs to get internalised and further confer their effect. On the other hand, incubation with mitomycin C-derived EVs and control EVs changed the glycosylation profile of recipient cells. To further investigate what this change means and which glycans are involved, a lectin, glycan or expression microarray could be used to explore the glycans on the cell surface and the expression of glycosyltransferases and other molecules (e.g. signalling pathway effectors) in the cells exhibiting different HPA labelling levels. These

experiments could give insights into the changes conferred by EVs at a molecular level but also relate these with changes to the cell surface molecules.

Overall, combining the different aspects of this project, interactions and uptake mechanisms of EVs from chemotherapy treated and non-treated cells could illuminate potential mechanisms of metastasis after treatment and further lead towards new developments towards blocking this process, which accounts for most deaths from cancer. *In vivo* experiments would provide a physiological environment to further support these ideas and pave the way to their application in humans in the future.

6.5 Concluding remarks

This project has highlighted the variability amongst different EV labelling methods, explored the EV specificity in cellular docking and uptake, and investigated the role of chemotherapy-derived EVs in mechanisms of breast cancer metastasis. More specifically, PKH26 labelling may result in false-positive signals as the dye itself forms micelles. The use of BSA is important to reduce, but does not eliminate, non-specific staining. Non-specific, rapid, and homogenous staining of recipient cells was observed when CFSE labelled EVs were used. Dual labelling with PKH26 and CFSE resulted in EV populations with heterogeneous labelling. These findings led to the conclusion that cell labelling with generic dyes might not be ideal for uptake experiments and optimisation of free dye clean-up is required. EVs bearing the fusion protein mEmerald-CD81 showed a clearer uptake pattern than the stained EVs and therefore were preferential for subsequent experiments. It was shown that EV uptake differs across cell lines and the EV origin affects docking on the plasma membrane of target cells. Using a range of uptake inhibitors, it was shown that cholesterol, and further lipid condensation, may be involved in the early interactions of EVs with the plasma membrane affecting EV uptake. Single molecule tracking revealed two patterns of EV behaviour: short and fast, and long and slow interactions with the plasma membrane. Chemotherapy (docetaxel and mitomycin C) treated cells released significantly higher amounts of EVs compared to non-treated cells. EVs from chemotherapy-treated cells did not induce any pro-metastatic effects (increased motility and invasiveness, EMT, and ability to adhere to endothelial cells) in naïve autologous cells. EVs from mitomycin C treated cells and non-treated cells were able to confer glycosylation changes to recipient cells, as revealed by HPA labelling.

Appendices

7 Appendices

Appendix 1: Supplementary material for chapter 2

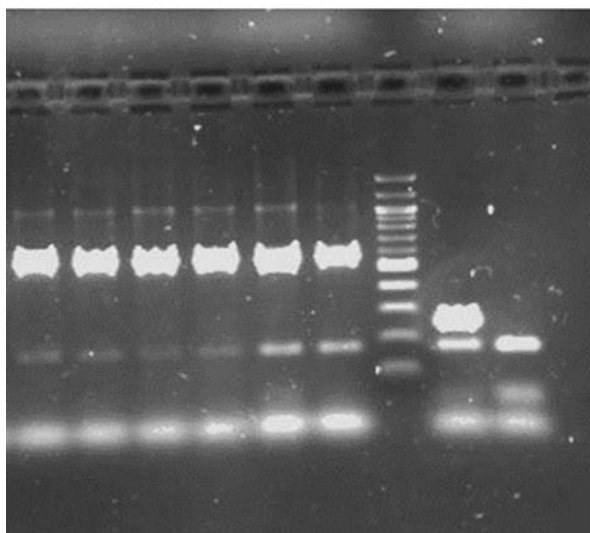


Figure 7.1 Mycoplasma testing.

PCR product analysis of the mycoplasma test in the cell lines under investigation. From left to right: HeLa, T47D, MCF7, hTERT-HME1, mEmerald-CD81 T47D, and mEmerald-CD81 hTERT-HME1, 100 bp DNA ladder, positive control, negative control. The 160 bp band serves as internal control and is the amplification product of TNFa, while the 260 bp band indicates mycoplasma contamination. The 570 bp band serves as a sample control and is the amplification product of a human/ mammalian-specific gene. All cell lines tested lack the 260 bp band, indicating absence of mycoplasma infection.

Table 7.1 Authentication of the cell lines used in this project.

	Amelogenin	CSF1PO	D13S317	D16S539	D5S818	D7S820	THO1	TPOX	vWA
T47D ATCC info	X	11,13	12	10	12	11	6	11	14
T47D test result	X	11,13	12	5, 10	12	11	6	11	14
MCF7 ATCC info	X	10	11	11, 12	11, 12	8, 9	6	9, 12	14, 15
MCF7 test result	X	10	11	11, 12	11, 12	8, 9	6	9, 12	14, 15
hTERT-HME1 ATCC info	X	10	11,12	11,12	11	7,12	7,8	10,12	15,16
hTERT-HME1 test result	X	10	11,12	11,12	11	7,12	7,8	10,12	15,16
HeLa ATCC info	X	9,10	12,13.3	9,10	11,12	8,12	7	8,12	16,18
HeLa test result	X	9,10	12,14	9,10	11,12	8,12	7	8,12	16,18

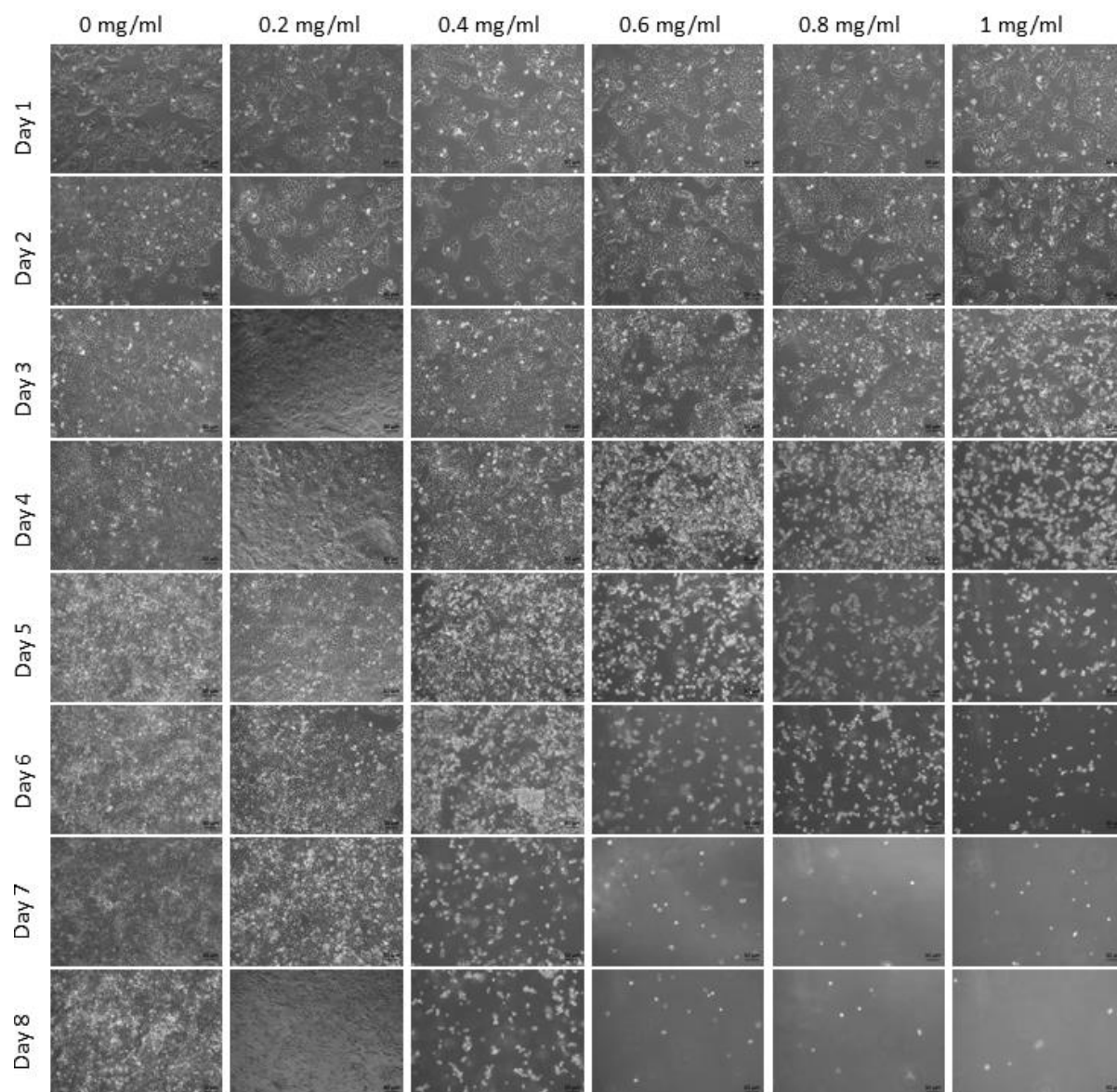


Figure 7.2 Estimating the optimal concentration of the selective antibiotic.

The response of T47D cells over a period of 8 days to increasing concentrations of geneticin. The concentration of drug able to kill the un-transfected cells within 7 days was used in the production of stably transfected cell lines. Images taken using a cell culture microscope.

Appendix 2: Supplementary material for chapter 4

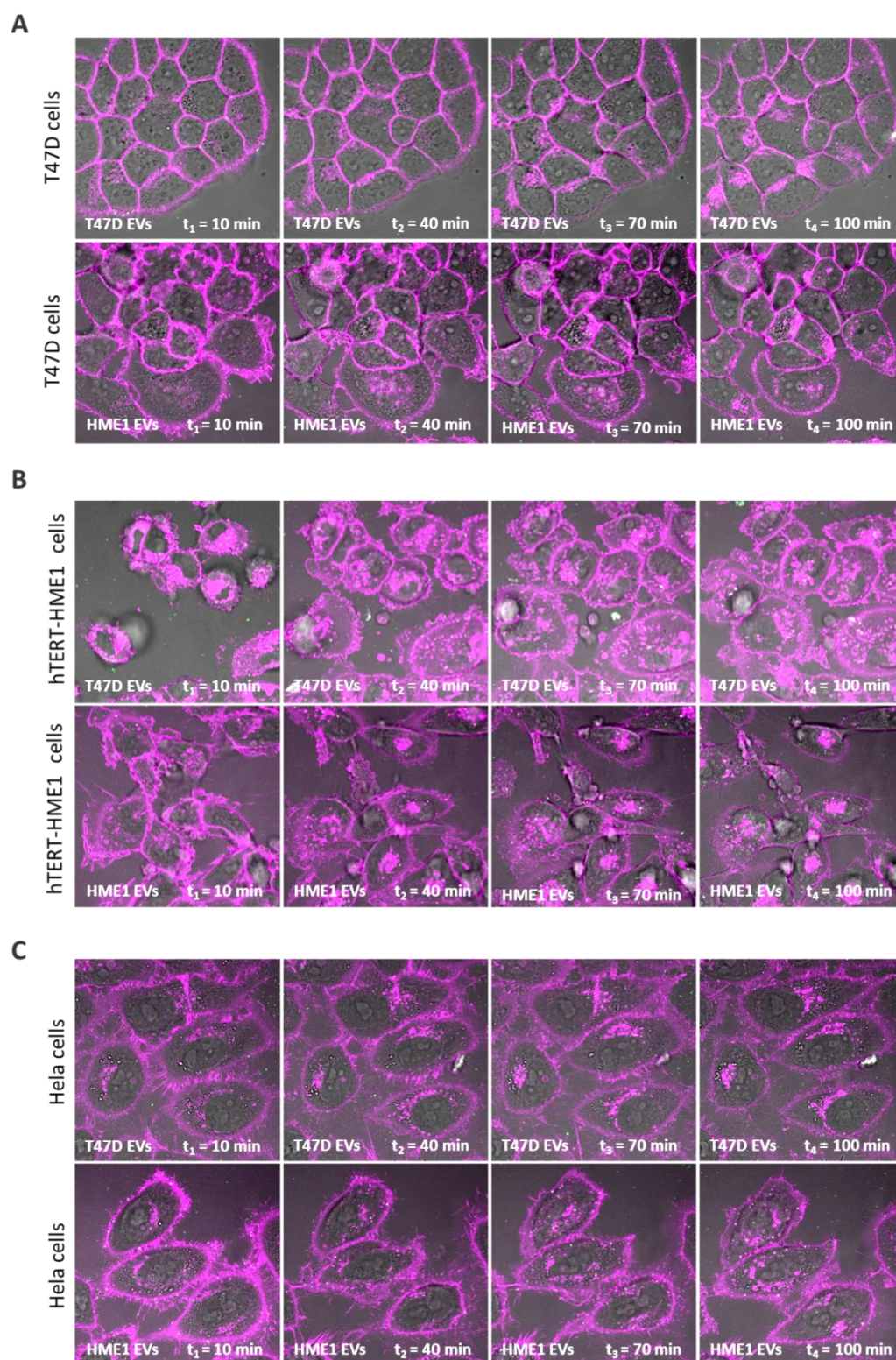


Figure 7.3 Live cell confocal time lapse imaging of EV uptake.

A) T47D cells, B) hTERT-HME1 cells, and C) HeLa cells were incubated with mEmerald-CD81 T47D EVs (upper panels) and mEmerald-CD81 T47D EVs (lower panels) and EV uptake was monitored over time.

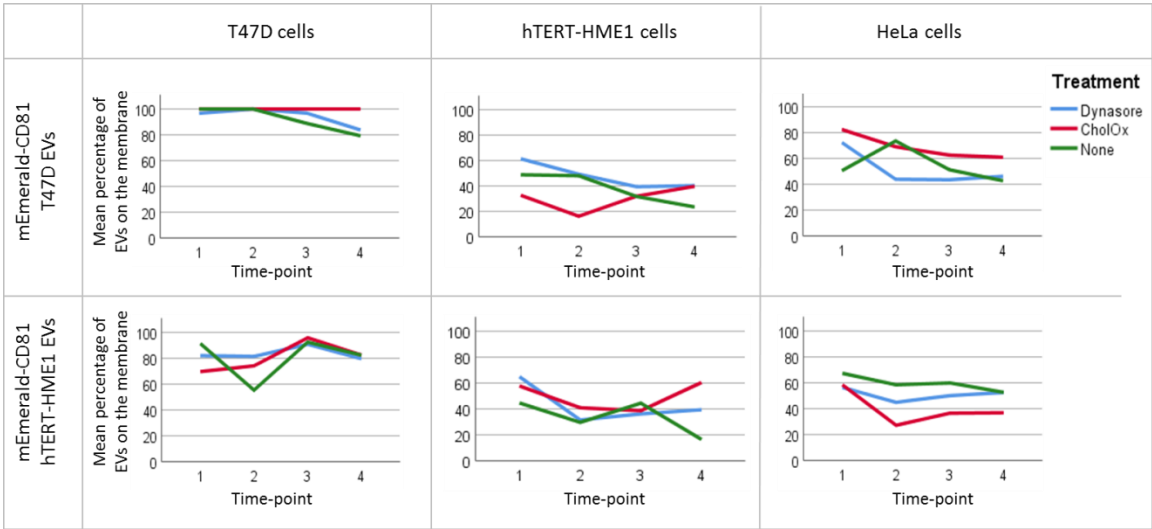


Figure 7.4 Proportion of EVs on the membrane and in the cytoplasm over time.

The amount of EVs on the membrane and in the cytoplasm was expressed as a percentage of the total amount of EVs counted in that cell. Changes in the proportion of membrane EVs are depicted over-time and in response to dynasore, cholesterol oxidase, or no treatment. Upper panel: mEmerald-CD81 T47D EVs. Lower panel: mEmerald-CD81 hTERT-HME1 EVs.

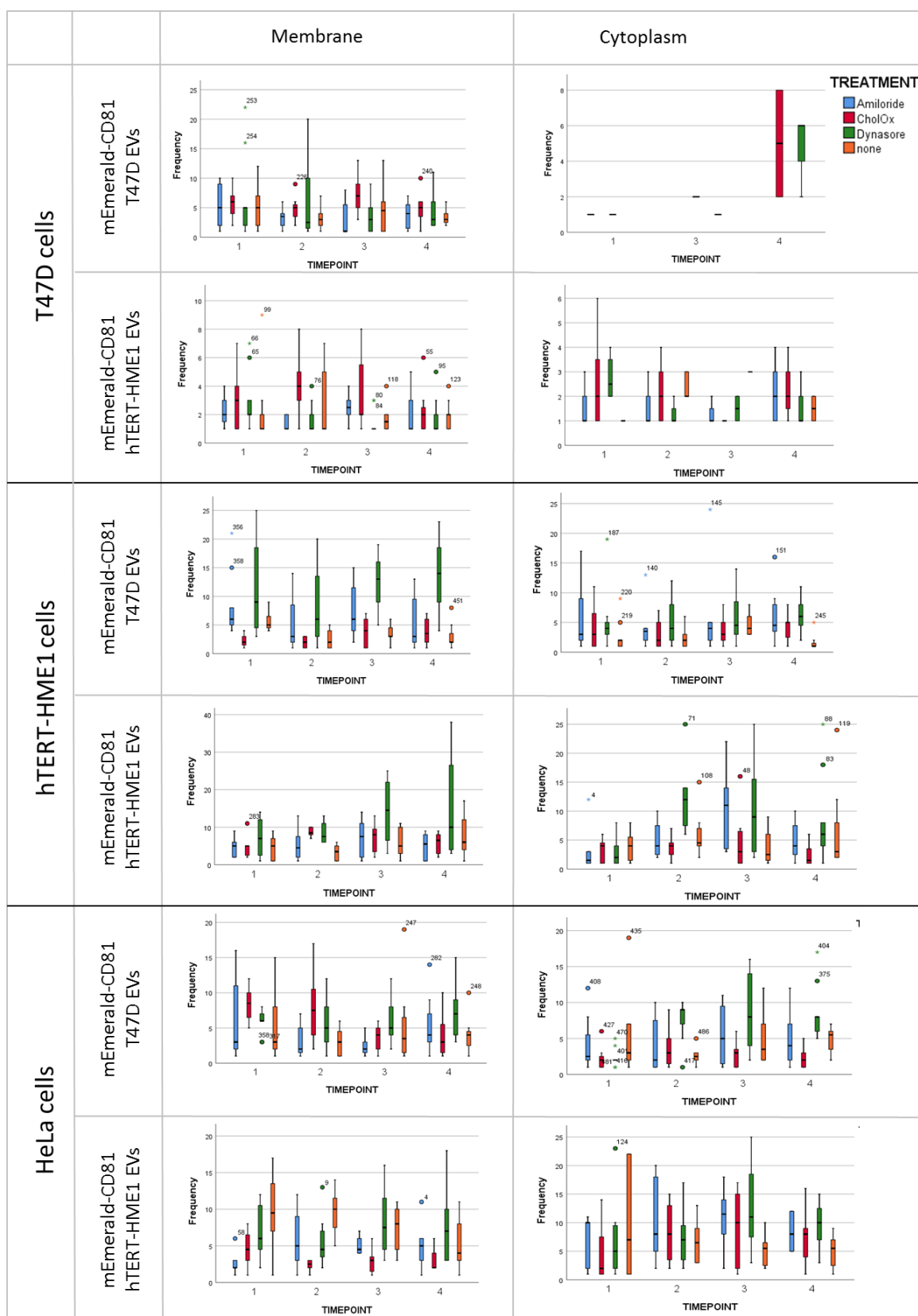


Figure 7.5 Raw numbers of EVs on the membrane and in the cytoplasm.

Box plots of the actual numbers of EVs counted in each of the 8-10 cells counted per condition for each EV and cell type over time.

Appendix 3: Supplementary material for chapter 5

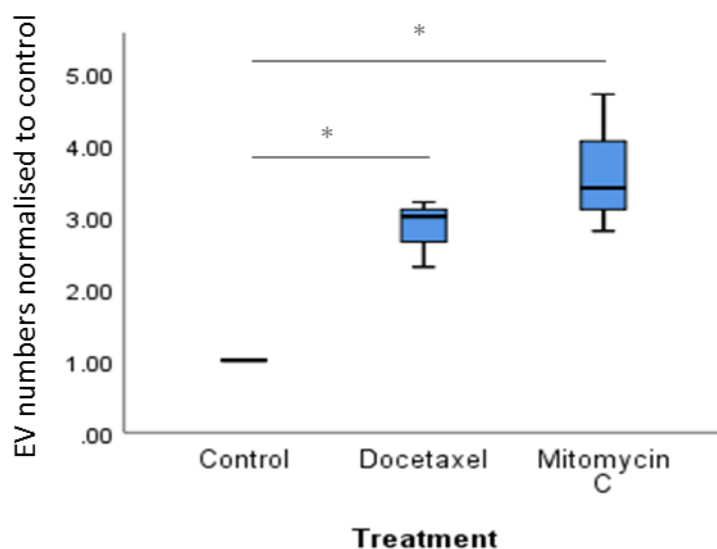


Figure 7.6 EV release by chemotherapy-treated and control cells.

Box plots of EV numbers normalised to control. The Mann-Whitney test was used to determine the significance of differences. *, $P < 0.05$. $N=3$ independent experiments.

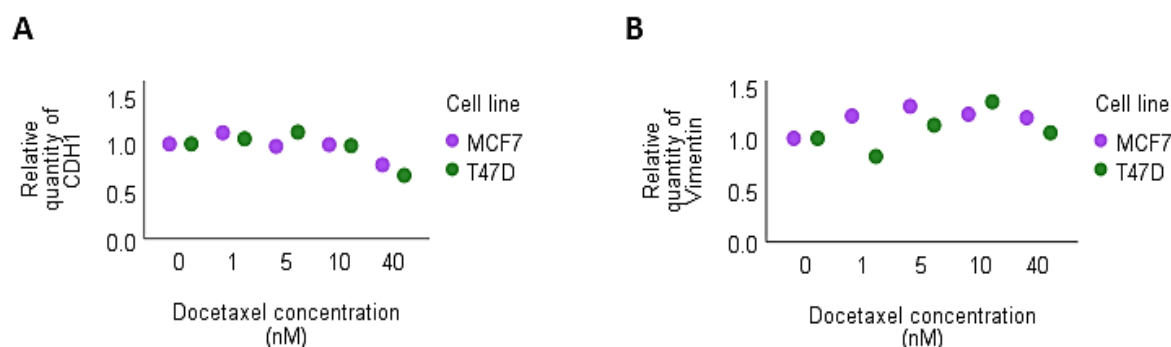


Figure 7.7 The effect of chemotherapy on EMT markers.

T47D and MCF7 cells were treated with increasing concentrations of docetaxel for 24 h and the expression levels of A) CDH1, marker of epithelial phenotype, and B) Vimentin, marker of mesenchymal phenotype, were assessed by qPCR. Single experiment, 2 technical replicates. Expression levels were normalised to 1.

References

8 References

- Al-Mayah, A. H., Irons, S. L., Pink, R. C., Carter, D. R. and Kadhim, M. A. (2012) 'Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation', *Radiat Res*, 177(5), pp. 539-45.
- Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C. and Rak, J. (2009) 'Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR', *Proc Natl Acad Sci U S A*, 106(10), pp. 3794-9. doi: 10.1073/pnas.0804543106.
- Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A. and Rak, J. (2008) 'Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells', *Nat Cell Biol*, 10(5), pp. 619-24. doi: 10.1038/ncb1725.
- Aliotta, J. M., Pereira, M., Sears, E. H., Dooner, M. S., Wen, S., Goldberg, L. R. and Quesenberry, P. J. (2015) 'Lung-derived exosome uptake into and epigenetic modulation of marrow progenitor/stem and differentiated cells', *J Extracell Vesicles*, 4, pp. 26166. doi: 10.3402/jev.v4.26166.
- Anderson, R. G. (1998) 'The caveolae membrane system', *Annu Rev Biochem*, 67, pp. 199-225. doi: 10.1146/annurev.biochem.67.1.199.
- Andre, F., Schartz, N. E., Movassagh, M., Flament, C., Pautier, P., Morice, P., Pomel, C., Lhomme, C., Escudier, B., Le Chevalier, T., Tursz, T., Amigorena, S., Raposo, G., Angevin, E. and Zitvogel, L. (2002) 'Malignant effusions and immunogenic tumour-derived exosomes', *Lancet*, 360(9329), pp. 295-305. doi: 10.1016/S0140-6736(02)09552-1.
- Andreu, Z. and Yanez-Mo, M. (2014) 'Tetraspanins in extracellular vesicle formation and function', *Front Immunol*, 5, pp. 442. doi: 10.3389/fimmu.2014.00442.
- Arscott, W. T., Tandle, A. T., Zhao, S., Shabason, J. E., Gordon, I. K., Schlaff, C. D., Zhang, G., Tofilon, P. J. and Camphausen, K. A. (2013) 'Ionizing radiation and glioblastoma exosomes: implications in tumor biology and cell migration', *Transl Oncol*, 6(6), pp. 638-48.
- Arya, M., Shergill, I. S., Williamson, M., Gommersall, L., Arya, N. and Patel, H. R. (2005) 'Basic principles of real-time quantitative PCR', *Expert Rev Mol Diagn*, 5(2), pp. 209-19. doi: 10.1586/14737159.5.2.209.
- Aubertin, K., Silva, A. K., Luciani, N., Espinosa, A., Djemat, A., Charue, D., Gallet, F., Blanc-Brude, O. and Wilhelm, C. (2016) 'Massive release of extracellular vesicles from cancer cells after photodynamic treatment or chemotherapy', *Sci Rep*, 6, pp. 35376. doi: 10.1038/srep35376.
- Banizs, A. B., Huang, T., Nakamoto, R. K., Shi, W. and He, J. (2018) 'Endocytosis Pathways of Endothelial Cell Derived Exosomes', *Mol Pharm*. doi: 10.1021/acs.molpharmaceut.8b00765.
- Bapu, D., Runions, J., Kadhim, M. and Brooks, S. A. (2016) 'N-acetylgalactosamine glycans function in cancer cell adhesion to endothelial cells: A role for truncated O-glycans in metastatic mechanisms', *Cancer Lett*, 375(2), pp. 367-374. doi: 10.1016/j.canlet.2016.03.019.
- Barres, C., Blanc, L., Bette-Bobillo, P., Andre, S., Mamoun, R., Gabius, H. J. and Vidal, M. (2010) 'Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages', *Blood*, 115(3), pp. 696-705. doi: 10.1182/blood-2009-07-231449.
- Baselga, J., Semiglazov, V., van Dam, P., Manikhas, A., Bellet, M., Mayordomo, J., Campone, M., Kubista, E., Greil, R., Bianchi, G., Steinseifer, J., Molloy, B., Tokaji, E., Gardner, H., Phillips, P., Stumm, M., Lane, H. A., Dixon, J. M., Jonat, W. and Rugo, H. S. (2009) 'Phase II randomized study of neoadjuvant everolimus plus letrozole compared with placebo plus letrozole in patients with estrogen receptor-positive breast cancer', *J Clin Oncol*, 27(16), pp. 2630-7. doi: 10.1200/JCO.2008.18.8391.

- Batisse, C., Marquet, J., Greffard, A., Fleury-Feith, J., Jaurand, M. C. and Pilatte, Y. (2004) 'Lectin-based three-color flow cytometric approach for studying cell surface glycosylation changes that occur during apoptosis', *Cytometry A*, 62(2), pp. 81-8. doi: 10.1002/cyto.a.20094.
- Batista, B. S., Eng, W. S., Pilobello, K. T., Hendricks-Munoz, K. D. and Mahal, L. K. (2011) 'Identification of a conserved glycan signature for microvesicles', *J Proteome Res*, 10(10), pp. 4624-33. doi: 10.1021/pr200434y.
- Becker, A., Thakur, B. K., Weiss, J. M., Kim, H. S., Peinado, H. and Lyden, D. (2016) 'Extracellular Vesicles in Cancer: Cell-to-Cell Mediators of Metastasis', *Cancer Cell*, 30(6), pp. 836-848. doi: 10.1016/j.ccell.2016.10.009.
- Bergers, G. and Benjamin, L. E. (2003) 'Tumorigenesis and the angiogenic switch', *Nat Rev Cancer*, 3(6), pp. 401-10. doi: 10.1038/nrc1093.
- Bewicke-Copley, F., Mulcahy, L. A., Jacobs, L. A., Samuel, P., Akbar, N., Pink, R. C. and Carter, D. R. F. (2017) 'Extracellular vesicles released following heat stress induce bystander effect in unstressed populations', *J Extracell Vesicles*, 6(1), pp. 1340746. doi: 10.1080/20013078.2017.1340746.
- Bilen, M. A., Pan, T., Lee, Y. C., Lin, S. C., Yu, G., Pan, J., Hawke, D. H., Pan, B. F., Vykoukal, J., Gray, K., Satcher, R. L., Gallick, G. E., Yu-Lee, L. Y. and Lin, S. H. (2017) 'Proteomics profiling of exosomes from primary mouse osteoblasts under proliferation versus mineralization conditions and characterization of their uptake into prostate cancer cells', *J Proteome Res*. doi: 10.1021/acs.jproteome.6b00981.
- Bissig, C. and Gruenberg, J. (2014) 'ALIX and the multivesicular endosome: ALIX in Wonderland', *Trends Cell Biol*, 24(1), pp. 19-25. doi: 10.1016/j.tcb.2013.10.009.
- Boyden, S. (1962) 'The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes', *J Exp Med*, 115, pp. 453-66.
- Brenton, J. D., Carey, L. A., Ahmed, A. A. and Caldas, C. (2005) 'Molecular classification and molecular forecasting of breast cancer: ready for clinical application?', *J Clin Oncol*, 23(29), pp. 7350-60. doi: 10.1200/JCO.2005.03.3845.
- Brooks, S. A. (2000) 'The involvement of Helix pomatia lectin (HPA) binding N-acetylgalactosamine glycans in cancer progression', *Histol Histopathol*, 15(1), pp. 143-58.
- Brooks, S. A. (2001) 'In vitro invasion assay using matrigel(R)', *Methods Mol Med*, 58, pp. 61-70. doi: 10.1385/1-59259-137-X:061.
- Brooks, S. A., Hall, D. M. and Buley, I. (2001) 'GalNAc glycoprotein expression by breast cell lines, primary breast cancer and normal breast epithelial membrane', *Br J Cancer*, 85(7), pp. 1014-22. doi: 10.1038/sj.bjc.6692028.
- Brooks, S. A. and Leatham, A. J. (1991) 'Prediction of lymph node involvement in breast cancer by detection of altered glycosylation in the primary tumour', *Lancet*, 338(8759), pp. 71-4.
- Brooks, S. A., Lomax-Browne, H. J., Carter, T. M., Kinch, C. E. and Hall, D. M. (2010) 'Molecular interactions in cancer cell metastasis', *Acta Histochem*, 112(1), pp. 3-25. doi: 10.1016/j.acthis.2008.11.022.
- Brooks, S. A. and Wilkinson, D. (2003) 'Validation of a simple avidin-biotin detection method for Helix pomatia lectin (HPA) binding as a prognostic marker in cancer', *Acta Histochem*, 105(3), pp. 205-12. doi: 10.1078/0065-1281-00705.
- Brown, C. M., Dalal, R. B., Hebert, B., Digman, M. A., Horwitz, A. R. and Gratton, E. (2008) 'Raster image correlation spectroscopy (RICS) for measuring fast protein dynamics and concentrations with a commercial laser scanning confocal microscope', *J Microsc*, 229(Pt 1), pp. 78-91. doi: 10.1111/j.1365-2818.2007.01871.x.
- Byers, S. W., Sommers, C. L., Hoxter, B., Mercurio, A. M. and Tozeren, A. (1995) 'Role of E-cadherin in the response of tumor cell aggregates to lymphatic, venous and arterial flow: measurement of cell-cell adhesion strength', *J Cell Sci*, 108 (Pt 5), pp. 2053-64.
- Caby, M. P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G. and Bonnerot, C. (2005) 'Exosomal-like vesicles are present in human blood plasma', *Int Immunol*, 17(7), pp. 879-87. doi: 10.1093/intimm/dxh267.

- Cai, J., Han, Y., Ren, H., Chen, C., He, D., Zhou, L., Eisner, G. M., Asico, L. D., Jose, P. A. and Zeng, C. (2013) 'Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells', *J Mol Cell Biol*, 5(4), pp. 227-38. doi: 10.1093/jmcb/mjt011.
- Cancer Research UK 2018. Available at: <http://www.cancerresearchuk.org/health-professional/cancer-statistics> (Accessed: 10/12/2018).
- Castro, B. M., Torreno-Pina, J. A., van Zanten, T. S. and Gracia-Parajo, M. F. (2013) 'Biochemical and imaging methods to study receptor membrane organization and association with lipid rafts', *Methods Cell Biol*, 117, pp. 105-22. doi: 10.1016/B978-0-12-408143-7.00006-2.
- Chambers, A. F., Groom, A. C. and MacDonald, I. C. (2002) 'Dissemination and growth of cancer cells in metastatic sites', *Nat Rev Cancer*, 2(8), pp. 563-72. doi: 10.1038/nrc865.
- Chang, J. and Erler, J. (2014) 'Hypoxia-mediated metastasis', *Adv Exp Med Biol*, 772, pp. 55-81. doi: 10.1007/978-1-4614-5915-6_3.
- Chang, Y. and Finnemann, S. C. (2007) 'Tetraspanin CD81 is required for the alpha v beta5-integrin-dependent particle-binding step of RPE phagocytosis', *J Cell Sci*, 120(Pt 17), pp. 3053-63. doi: 10.1242/jcs.006361.
- Chavez, K. J., Garimella, S. V. and Lipkowitz, S. (2010) 'Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer', *Breast Dis*, 32(1-2), pp. 35-48. doi: 10.3233/BD-2010-0307.
- Chen, C., Zong, S., Wang, Z., Lu, J., Zhu, D., Zhang, Y. and Cui, Y. (2016) 'Imaging and Intracellular Tracking of Cancer-Derived Exosomes Using Single-Molecule Localization-Based Super-Resolution Microscope', *ACS Appl Mater Interfaces*, 8(39), pp. 25825-25833. doi: 10.1021/acsami.6b09442.
- Chen, G., Zhang, Y. and Wu, X. (2014a) '786-O Renal cancer cell line-derived exosomes promote 786-O cell migration and invasion in vitro', *Oncol Lett*, 7(5), pp. 1576-1580. doi: 10.3892/ol.2014.1962.
- Chen, W. X., Liu, X. M., Lv, M. M., Chen, L., Zhao, J. H., Zhong, S. L., Ji, M. H., Hu, Q., Luo, Z., Wu, J. Z. and Tang, J. H. (2014b) 'Exosomes from drug-resistant breast cancer cells transmit chemoresistance by a horizontal transfer of microRNAs', *PLoS One*, 9(4), pp. e95240. doi: 10.1371/journal.pone.0095240.
- Chin, A. R. and Wang, S. E. (2016) 'Cancer-derived extracellular vesicles: the 'soil conditioner' in breast cancer metastasis?', *Cancer Metastasis Rev*, 35(4), pp. 669-676. doi: 10.1007/s10555-016-9639-8.
- Christianson, H. C., Svensson, K. J., van Kuppevelt, T. H., Li, J. P. and Belting, M. (2013) 'Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity', *Proc Natl Acad Sci U S A*, 110(43), pp. 17380-5. doi: 10.1073/pnas.1304266110.
- Collett, G. P., Redman, C. W., Sargent, I. L. and Vatish, M. (2018) 'Endoplasmic reticulum stress stimulates the release of extracellular vesicles carrying danger-associated molecular pattern (DAMP) molecules', *Oncotarget*, 9(6), pp. 6707-6717. doi: 10.18632/oncotarget.24158.
- Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L. F., Thery, C. and Raposo, G. (2013) 'Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles', *J Cell Sci*, 126(Pt 24), pp. 5553-65. doi: 10.1242/jcs.128868.
- Colombo, M., Raposo, G. and Thery, C. (2014) 'Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles', *Annu Rev Cell Dev Biol*, 30, pp. 255-89. doi: 10.1146/annurev-cellbio-101512-122326.
- Cossetti, C., Iraci, N., Mercer, T. R., Leonardi, T., Alpi, E., Drago, D., Alfaro-Cervello, C., Saini, H. K., Davis, M. P., Schaeffer, J., Vega, B., Stefanini, M., Zhao, C., Muller, W., Garcia-Verdugo, J. M., Mathivanan, S., Bachi, A., Enright, A. J., Mattick, J. S. and Pluchino, S. (2014) 'Extracellular vesicles from neural stem cells transfer IFN-gamma via Ifngr1 to activate

- Stat1 signaling in target cells', *Mol Cell*, 56(2), pp. 193-204. doi: 10.1016/j.molcel.2014.08.020.
- 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., Garcia-Santos, G., Williams, C., Ararso, Y., Huang, Y., Rodrigues, G., Shen, T. L., Labori, K. J., Lothe, I. M., Kure, E. H., Hernandez, J., Doussot, A., Ebbesen, S. H., Grandgenett, P. M., Hollingsworth, M. A., Jain, M., Mallya, K., Batra, S. K., Jarnagin, W. R., Schwartz, R. E., Matei, I., Peinado, H., Stanger, B. Z., Bromberg, J. and Lyden, D. (2015) 'Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver', *Nat Cell Biol*, 17(6), pp. 816-26. doi: 10.1038/ncb3169.
- Costa Verdera, H., Gitz-Francois, J. J., Schiffelers, R. M. and Vader, P. (2017) 'Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis', *J Control Release*, 266, pp. 100-108. doi: 10.1016/j.jconrel.2017.09.019.
- Crown, J., O'Leary, M. and Ooi, W. S. (2004) 'Docetaxel and paclitaxel in the treatment of breast cancer: a review of clinical experience', *Oncologist*, 9 Suppl 2, pp. 24-32.
- Czernek, L., Chworos, A. and Duechler, M. (2015) 'The uptake of extracellular vesicles is affected by the differentiation status of myeloid cells', *Scand J Immunol*, 82(6), pp. 506-14. doi: 10.1111/sji.12371.
- Daenen, L. G., Roodhart, J. M., van Amersfoort, M., Dehnad, M., Roessingh, W., Ulfman, L. H., Derksen, P. W. and Voest, E. E. (2011) 'Chemotherapy enhances metastasis formation via VEGFR-1-expressing endothelial cells', *Cancer Res*, 71(22), pp. 6976-85. doi: 10.1158/0008-5472.CAN-11-0627.
- Dawood, S. (2010) 'Triple-negative breast cancer: epidemiology and management options', *Drugs*, 70(17), pp. 2247-58. doi: 10.2165/11538150-000000000-00000.
- Dawood, S., Broglio, K., Buzdar, A. U., Hortobagyi, G. N. and Giordano, S. H. (2010) 'Prognosis of women with metastatic breast cancer by HER2 status and trastuzumab treatment: an institutional-based review', *J Clin Oncol*, 28(1), pp. 92-8. doi: 10.1200/JCO.2008.19.9844.
- de Rond, L., van der Pol, E., Hau, C. M., Varga, Z., Sturk, A., van Leeuwen, T. G., Nieuwland, R. and Coumans, F. A. W. (2018) 'Comparison of generic fluorescent markers for detection of extracellular vesicles by flow cytometry', *Clin Chem*, 64(4), pp. 680-689. doi: 10.1373/clinchem.2017.278978.
- Deng, G., Qu, J., Zhang, Y., Che, X., Cheng, Y., Fan, Y., Zhang, S., Na, D., Liu, Y. and Qu, X. (2017) 'Gastric cancer-derived exosomes promote peritoneal metastasis by destroying the mesothelial barrier', *FEBS Lett*, 591(14), pp. 2167-2179. doi: 10.1002/1873-3468.12722.
- Denzer, K., van Eijk, M., Kleijmeer, M. J., Jakobson, E., de Groot, C. and Geuze, H. J. (2000) 'Follicular dendritic cells carry MHC class II-expressing microvesicles at their surface', *J Immunol*, 165(3), pp. 1259-65.
- Donnarumma, E., Fiore, D., Nappa, M., Roscigno, G., Adamo, A., Iaboni, M., Russo, V., Affinito, A., Puoti, I., Quintavalle, C., Rienzo, A., Piscuoglio, S., Thomas, R. and Condorelli, G. (2017) 'Cancer-associated fibroblasts release exosomal microRNAs that dictate an aggressive phenotype in breast cancer', *Oncotarget*, 8(12), pp. 19592-19608. doi: 10.18632/oncotarget.14752.
- Dörsam, B., Bösl, T., Reiners, K. S., Barnert, S., Schubert, R., Shatnyeva, O., Zigrino, P., Engert, A., Hansen, H. P. and von Strandmann, E. P. (2018) 'Hodgkin lymphoma-derived extracellular vesicles change the secretome of fibroblasts toward a CAF phenotype', *Frontiers in Immunology*, 9(1358). doi: 10.3389/fimmu.2018.01358.
- Drexler, H. G., Dirks, W. G. and MacLeod, R. A. (1999) 'False human hematopoietic cell lines: cross-contaminations and misinterpretations', *Leukemia*, 13(10), pp. 1601-7.
- Drexler, H. G. and Uphoff, C. C. (2002) 'Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention', *Cytotechnology*, 39(2), pp. 75-90. doi: 10.1023/A:1022913015916.

- Duchemann, B. and Zelek, L. (2017) 'Cancer treatment. Surgery, radiotherapy, medical treatments (chemotherapy, targeted therapies, immunotherapy). Multidisciplinary therapeutic decision and patient information', *Rev Prat*, 67(10), pp. e499-e509.
- Dumic, J., Dabelic, S. and Fogel, M. (2006) 'Galectin-3: an open-ended story', *Biochim Biophys Acta*, 1760(4), pp. 616-35. doi: 10.1016/j.bbagen.2005.12.020.
- Dunnwald, L. K., Rossing, M. A. and Li, C. I. (2007) 'Hormone receptor status, tumor characteristics, and prognosis: a prospective cohort of breast cancer patients', *Breast Cancer Res*, 9(1), pp. R6. doi: 10.1186/bcr1639.
- Eisenhauer, E. A. and Vermorken, J. B. (1998) 'The taxoids. Comparative clinical pharmacology and therapeutic potential', *Drugs*, 55(1), pp. 5-30. doi: 10.2165/00003495-199855010-00002.
- El-Andaloussi, S., Breakefield, X. O. and Wood, M. J. (2013) 'Extracellular vesicles: biology and emerging therapeutic opportunities', *Nat Rev Drug Discov*, 12(5), pp. 347-57. doi: 10.1038/nrd3978.
- Escrevente, C., Keller, S., Altevogt, P. and Costa, J. (2011) 'Interaction and uptake of exosomes by ovarian cancer cells', *BMC Cancer*, 11, pp. 108. doi: 10.1186/1471-2407-11-108.
- Esteva, F. J. (2002) 'The current status of docetaxel for metastatic breast cancer', *Oncology (Williston Park)*, 16(6 Suppl 6), pp. 17-26.
- Fabbri, M., Paone, A., Calore, F., Galli, R., Gaudio, E., Santhanam, R., Lovat, F., Fadda, P., Mao, C., Nuovo, G. J., Zanesi, N., Crawford, M., Ozer, G. H., Wernicke, D., Alder, H., Caligiuri, M. A., Nana-Sinkam, P., Perrotti, D. and Croce, C. M. (2012) 'MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response', *Proc Natl Acad Sci U S A*, 109(31), pp. E2110-6. doi: 10.1073/pnas.1209414109.
- Feng, D., Zhao, W. L., Ye, Y. Y., Bai, X. C., Liu, R. Q., Chang, L. F., Zhou, Q. and Sui, S. F. (2010) 'Cellular internalization of exosomes occurs through phagocytosis', *Traffic*, 11(5), pp. 675-87. doi: 10.1111/j.1600-0854.2010.01041.x.
- Ferlay, J., Steliarova-Foucher, E., Lortet-Tieulent, J., Rosso, S., Coebergh, J. W., Comber, H., Forman, D. and Bray, F. (2013) 'Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012', *Eur J Cancer*, 49(6), pp. 1374-403. doi: 10.1016/j.ejca.2012.12.027.
- Fischer, K. R., Durrans, A., Lee, S., Sheng, J., Li, F., Wong, S. T., Choi, H., El Rayes, T., Ryu, S., Troeger, J., Schwabe, R. F., Vahdat, L. T., Altorki, N. K., Mittal, V. and Gao, D. (2015) 'Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance', *Nature*, 527(7579), pp. 472-6. doi: 10.1038/nature15748.
- Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hanisch, U. K. and Simons, M. (2011) 'Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis', *J Cell Sci*, 124(Pt 3), pp. 447-58. doi: 10.1242/jcs.074088.
- Franzen, C. A., Simms, P. E., Van Huis, A. F., Foreman, K. E., Kuo, P. C. and Gupta, G. N. (2014) 'Characterization of uptake and internalization of exosomes by bladder cancer cells', *Biomed Res Int*, 2014, pp. 619829. doi: 10.1155/2014/619829.
- French, K. C., Antonyak, M. A. and Cerione, R. A. (2017) 'Extracellular vesicle docking at the cellular port: Extracellular vesicle binding and uptake', *Semin Cell Dev Biol*, 67, pp. 48-55. doi: 10.1016/j.semcdb.2017.01.002.
- Frey, B. and Gaip, U. S. (2011) 'The immune functions of phosphatidylserine in membranes of dying cells and microvesicles', *Semin Immunopathol*, 33(5), pp. 497-516. doi: 10.1007/s00281-010-0228-6.
- Frick, M., Bright, N. A., Riento, K., Bray, A., Merrified, C. and Nichols, B. J. (2007) 'Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding', *Curr Biol*, 17(13), pp. 1151-6. doi: 10.1016/j.cub.2007.05.078.
- Friedl, P. and Wolf, K. (2010) 'Plasticity of cell migration: a multiscale tuning model', *J Cell Biol*, 188(1), pp. 11-9. doi: 10.1083/jcb.200909003.

- Frisch, S. M., Schaller, M. and Cieply, B. (2013) 'Mechanisms that link the oncogenic epithelial-mesenchymal transition to suppression of anoikis', *J Cell Sci*, 126(Pt 1), pp. 21-9. doi: 10.1242/jcs.120907.
- Fu, Q., Zhang, Q., Lou, Y., Yang, J., Nie, G., Chen, Q., Chen, Y., Zhang, J., Wang, J., Wei, T., Qin, H., Dang, X., Bai, X. and Liang, T. (2018) 'Primary tumor-derived exosomes facilitate metastasis by regulating adhesion of circulating tumor cells via SMAD3 in liver cancer', *Oncogene*. doi: 10.1038/s41388-018-0391-0.
- Galindo-Hernandez, O., Serna-Marquez, N., Castillo-Sanchez, R. and Salazar, E. P. (2014) 'Extracellular vesicles from MDA-MB-231 breast cancer cells stimulated with linoleic acid promote an EMT-like process in MCF10A cells', *Prostaglandins Leukot Essent Fatty Acids*, 91(6), pp. 299-310. doi: 10.1016/j.plefa.2014.09.002.
- Gao, C. F., Xie, Q., Su, Y. L., Koeman, J., Khoo, S. K., Gustafson, M., Knudsen, B. S., Hay, R., Shinomiya, N. and Vande Woude, G. F. (2005) 'Proliferation and invasion: plasticity in tumor cells', *Proc Natl Acad Sci U S A*, 102(30), pp. 10528-33. doi: 10.1073/pnas.0504367102.
- Gay, L. J. and Felding-Habermann, B. (2011) 'Contribution of platelets to tumour metastasis', *Nat Rev Cancer*, 11(2), pp. 123-34. doi: 10.1038/nrc3004.
- Geminard, C., De Gassart, A., Blanc, L. and Vidal, M. (2004) 'Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes', *Traffic*, 5(3), pp. 181-93. doi: 10.1111/j.1600-0854.2004.0167.x.
- Glebov, O. O., Bright, N. A. and Nichols, B. J. (2006) 'Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells', *Nat Cell Biol*, 8(1), pp. 46-54. doi: 10.1038/ncb1342.
- Goicoechea, S. M., Bednarski, B., Garcia-Mata, R., Prentice-Dunn, H., Kim, H. J. and Otey, C. A. (2009) 'Palladin contributes to invasive motility in human breast cancer cells', *Oncogene*, 28(4), pp. 587-98. doi: 10.1038/onc.2008.408.
- Gold, E. S., Underhill, D. M., Morrisette, N. S., Guo, J., McNiven, M. A. and Aderem, A. (1999) 'Dynamin 2 is required for phagocytosis in macrophages', *J Exp Med*, 190(12), pp. 1849-56.
- Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. and Sharon, N. (1980) 'What should be called a lectin?', *Nature*, 285(5760), pp. 66. doi: 10.1038/285066b0.
- Graner, M. W., Alzate, O., Dechkovskaia, A. M., Keene, J. D., Sampson, J. H., Mitchell, D. A. and Bigner, D. D. (2009) 'Proteomic and immunologic analyses of brain tumor exosomes', *FASEB J*, 23(5), pp. 1541-57. doi: 10.1096/fj.08-122184.
- Grange, C., Tapparo, M., Collino, F., Vitillo, L., Damasco, C., Deregibus, M. C., Tetta, C., Bussolati, B. and Camussi, G. (2011) 'Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche', *Cancer Res*, 71(15), pp. 5346-56. doi: 10.1158/0008-5472.CAN-11-0241.
- Graves, L. E., Ariztia, E. V., Navari, J. R., Matzel, H. J., Stack, M. S. and Fishman, D. A. (2004) 'Proinvasive properties of ovarian cancer ascites-derived membrane vesicles', *Cancer Res*, 64(19), pp. 7045-9. doi: 10.1158/0008-5472.CAN-04-1800.
- Gray, W. D., Mitchell, A. J. and Searles, C. D. (2015) 'An accurate, precise method for general labeling of extracellular vesicles', *MethodsX*, 2, pp. 360-7. doi: 10.1016/j.mex.2015.08.002.
- Green, T. M., Alpaugh, M. L., Barsky, S. H., Rappa, G. and Lorico, A. (2015) 'Breast Cancer-Derived Extracellular Vesicles: Characterization and Contribution to the Metastatic Phenotype', *Biomed Res Int*, 2015, pp. 634865. doi: 10.1155/2015/634865.
- Grimolizzi, F., Monaco, F., Leoni, F., Bracci, M., Staffolani, S., Bersaglieri, C., Gaetani, S., Valentino, M., Amati, M., Rubini, C., Saccucci, F., Neuzil, J., Tomasetti, M. and Santarelli, L. (2017) 'Exosomal miR-126 as a circulating biomarker in non-small-cell lung cancer regulating cancer progression', *Sci Rep*, 7(1), pp. 15277. doi: 10.1038/s41598-017-15475-6.

- Gudbergsson, J. M., Johnsen, K. B., Skov, M. N. and Duroux, M. (2016) 'Systematic review of factors influencing extracellular vesicle yield from cell cultures', *Cytotechnology*, 68(4), pp. 579-92. doi: 10.1007/s10616-015-9913-6.
- Gyorgy, B., Szabo, T. G., Pasztoi, M., Pal, Z., Misjak, P., Aradi, B., Laszlo, V., Pallinger, E., Pap, E., Kittel, A., Nagy, G., Falus, A. and Buzas, E. I. (2011) 'Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles', *Cell Mol Life Sci*, 68(16), pp. 2667-88. doi: 10.1007/s00018-011-0689-3.
- Haraszti, R. A., Didiot, M. C., Sapp, E., Leszyk, J., Shaffer, S. A., Rockwell, H. E., Gao, F., Narain, N. R., DiFiglia, M., Kiebish, M. A., Aronin, N. and Khvorova, A. (2016) 'High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources', *J Extracell Vesicles*, 5, pp. 32570. doi: 10.3402/jev.v5.32570.
- Hay, E. D. (1995) 'An overview of epithelio-mesenchymal transformation', *Acta Anat (Basel)*, 154(1), pp. 8-20.
- Heijnen, H. F., Schiel, A. E., Fijnheer, R., Geuze, H. J. and Sixma, J. J. (1999) 'Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules', *Blood*, 94(11), pp. 3791-9.
- Heusermann, W., Hean, J., Trojer, D., Steib, E., von Bueren, S., Graff-Meyer, A., Genoud, C., Martin, K., Pizzato, N., Voshol, J., Morrissey, D. V., Andaloussi, S. E., Wood, M. J. and Meisner-Kober, N. C. (2016) 'Exosomes surf on filopodia to enter cells at endocytic hot spots, traffic within endosomes, and are targeted to the ER', *J Cell Biol*, 213(2), pp. 173-84. doi: 10.1083/jcb.201506084.
- Hilakivi-Clarke, L. (2000) 'Estrogens, BRCA1, and breast cancer', *Cancer Res*, 60(18), pp. 4993-5001.
- Hinshaw, J. E. (2000) 'Dynamin and its role in membrane fission', *Annu Rev Cell Dev Biol*, 16, pp. 483-519. doi: 10.1146/annurev.cellbio.16.1.483.
- Holder, B., Jones, T., Sancho Shimizu, V., Rice, T. F., Donaldson, B., Bouqueau, M., Forbes, K. and Kampmann, B. (2016) 'Macrophage exosomes induce placental inflammatory cytokines: A novel mode of maternal-placental messaging', *Traffic*, 17(2), pp. 168-78. doi: 10.1111/tra.12352.
- Horibe, S., Tanahashi, T., Kawauchi, S., Murakami, Y. and Rikitake, Y. (2018) 'Mechanism of recipient cell-dependent differences in exosome uptake', *BMC Cancer*, 18(1), pp. 47. doi: 10.1186/s12885-017-3958-1.
- Hoshi, H. and McKeenan, W. L. (1984) 'Brain- and liver cell-derived factors are required for growth of human endothelial cells in serum-free culture', *Proc Natl Acad Sci U S A*, 81(20), pp. 6413-7.
- Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., Singh, S., Williams, C., Soplop, N., Uryu, K., Pharmed, L., King, T., Bojmar, L., Davies, A. E., Ararso, Y., Zhang, T., Zhang, H., Hernandez, J., Weiss, J. M., Dumont-Cole, V. D., Kramer, K., Wexler, L. H., Narendran, A., Schwartz, G. K., Healey, J. H., Sandstrom, P., Labori, K. J., Kure, E. H., Grandgenett, P. M., Hollingsworth, M. A., de Sousa, M., Kaur, S., Jain, M., Mallya, K., Batra, S. K., Jarnagin, W. R., Brady, M. S., Fodstad, O., Muller, V., Pantel, K., Minn, A. J., Bissell, M. J., Garcia, B. A., Kang, Y., Rajasekhar, V. K., Ghajar, C. M., Matei, I., Peinado, H., Bromberg, J. and Lyden, D. (2015) 'Tumour exosome integrins determine organotropic metastasis', *Nature*, 527(7578), pp. 329-35. doi: 10.1038/nature15756.
- Hoshino, D., Kirkbride, K. C., Costello, K., Clark, E. S., Sinha, S., Grega-Larson, N., Tyska, M. J. and Weaver, A. M. (2013) 'Exosome secretion is enhanced by invadopodia and drives invasive behavior', *Cell Rep*, 5(5), pp. 1159-68. doi: 10.1016/j.celrep.2013.10.050.
- Hsu, C., Morohashi, Y., Yoshimura, S., Manrique-Hoyos, N., Jung, S., Lauterbach, M. A., Bakhti, M., Gronborg, M., Mobius, W., Rhee, J., Barr, F. A. and Simons, M. (2010) 'Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C', *J Cell Biol*, 189(2), pp. 223-32. doi: 10.1083/jcb.200911018.

- Hsu, P. D., Lander, E. S. and Zhang, F. (2014) 'Development and applications of CRISPR-Cas9 for genome engineering', *Cell*, 157(6), pp. 1262-78. doi: 10.1016/j.cell.2014.05.010.
- Hsu, Y. L., Hung, J. Y., Chang, W. A., Lin, Y. S., Pan, Y. C., Tsai, P. H., Wu, C. Y. and Kuo, P. L. (2017) 'Hypoxic lung cancer-secreted exosomal miR-23a increased angiogenesis and vascular permeability by targeting prolyl hydroxylase and tight junction protein ZO-1', *Oncogene*, 36(34), pp. 4929-4942. doi: 10.1038/onc.2017.105.
- Hupalowska, A. and Miaczynska, M. (2012) 'The new faces of endocytosis in signaling', *Traffic*, 13(1), pp. 9-18. doi: 10.1111/j.1600-0854.2011.01249.x.
- Hutchinson, L. (2010) 'Breast cancer: challenges, controversies, breakthroughs', *Nat Rev Clin Oncol*, 7(12), pp. 669-70. doi: 10.1038/nrclinonc.2010.192.
- Hwang, I., Shen, X. and Sprent, J. (2003) 'Direct stimulation of naive T cells by membrane vesicles from antigen-presenting cells: distinct roles for CD54 and B7 molecules', *Proc Natl Acad Sci U S A*, 100(11), pp. 6670-5. doi: 10.1073/pnas.1131852100.
- Inder, K. L., Ruelcke, J. E., Petelin, L., Moon, H., Choi, E., Rae, J., Blumenthal, A., Hutmacher, D., Saunders, N. A., Stow, J. L., Parton, R. G. and Hill, M. M. (2014) 'Cavin-1/PTRF alters prostate cancer cell-derived extracellular vesicle content and internalization to attenuate extracellular vesicle-mediated osteoclastogenesis and osteoblast proliferation', *J Extracell Vesicles*, 3. doi: 10.3402/jev.v3.23784.
- Jahn, R. and Scheller, R. H. (2006) 'SNAREs--engines for membrane fusion', *Nat Rev Mol Cell Biol*, 7(9), pp. 631-43. doi: 10.1038/nrm2002.
- Janowska-Wieczorek, A., Wysoczynski, M., Kijowski, J., Marquez-Curtis, L., Machalinski, B., Ratajczak, J. and Ratajczak, M. Z. (2005) 'Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer', *Int J Cancer*, 113(5), pp. 752-60. doi: 10.1002/ijc.20657.
- Jeppesen, D. K., Nawrocki, A., Jensen, S. G., Thorsen, K., Whitehead, B., Howard, K. A., Dyrskjot, L., Orntoft, T. F., Larsen, M. R. and Ostensfeld, M. S. (2014) 'Quantitative proteomics of fractionated membrane and lumen exosome proteins from isogenic metastatic and nonmetastatic bladder cancer cells reveal differential expression of EMT factors', *Proteomics*, 14(6), pp. 699-712. doi: 10.1002/pmic.201300452.
- Ji, H., Greening, D. W., Barnes, T. W., Lim, J. W., Tauro, B. J., Rai, A., Xu, R., Adda, C., Mathivanan, S., Zhao, W., Xue, Y., Xu, T., Zhu, H. J. and Simpson, R. J. (2013) 'Proteome profiling of exosomes derived from human primary and metastatic colorectal cancer cells reveal differential expression of key metastatic factors and signal transduction components', *Proteomics*, 13(10-11), pp. 1672-86. doi: 10.1002/pmic.201200562.
- Jones, H. W., Jr., McKusick, V. A., Harper, P. S. and Wu, K. D. (1971) 'George Otto Gey. (1899-1970). The HeLa cell and a reappraisal of its origin', *Obstet Gynecol*, 38(6), pp. 945-9.
- Josson, S., Gururajan, M., Sung, S. Y., Hu, P., Shao, C., Zhau, H. E., Liu, C., Lichterman, J., Duan, P., Li, Q., Rogatko, A., Posadas, E. M., Haga, C. L. and Chung, L. W. (2015) 'Stromal fibroblast-derived miR-409 promotes epithelial-to-mesenchymal transition and prostate tumorigenesis', *Oncogene*, 34(21), pp. 2690-9. doi: 10.1038/onc.2014.212.
- 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., Rafii, S. and Lyden, D. (2005) 'VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche', *Nature*, 438(7069), pp. 820-7. doi: 10.1038/nature04186.
- Karagiannis, G. S., Condeelis, J. S. and Oktay, M. H. (2018) 'Chemotherapy-induced metastasis: mechanisms and translational opportunities', *Clin Exp Metastasis*, 35(4), pp. 269-284. doi: 10.1007/s10585-017-9870-x.
- Keklikoglou, I., Cianciaruso, C., Guc, E., Squadrito, M. L., Spring, L. M., Tazzyman, S., Lambein, L., Poissonnier, A., Ferraro, G. B., Baer, C., Cassara, A., Guichard, A., Iruela-Arispe, M. L., Lewis, C. E., Coussens, L. M., Bardia, A., Jain, R. K., Pollard, J. W. and De Palma, M. (2018) 'Chemotherapy elicits pro-metastatic extracellular vesicles in breast cancer models', *Nat Cell Biol*. doi: 10.1038/s41556-018-0256-3.

- Keller, S., Konig, A. K., Marme, F., Runz, S., Wolterink, S., Koensgen, D., Mustea, A., Sehouli, J. and Altevogt, P. (2009) 'Systemic presence and tumor-growth promoting effect of ovarian carcinoma released exosomes', *Cancer Lett*, 278(1), pp. 73-81. doi: 10.1016/j.canlet.2008.12.028.
- Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delarea, J., Radu, M., Chaitcik, S. and Brenner, H. J. (1979) 'Establishment and characterization of a cell line of human breast carcinoma origin', *Eur J Cancer*, 15(5), pp. 659-70.
- Kim, J. W., Wieckowski, E., Taylor, D. D., Reichert, T. E., Watkins, S. and Whiteside, T. L. (2005) 'Fas ligand-positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes', *Clin Cancer Res*, 11(3), pp. 1010-20.
- Kim, Y., Edwards, N. and Fenselau, C. (2016) 'Extracellular vesicle proteomes reflect developmental phases of *Bacillus subtilis*', *Clin Proteomics*, 13, pp. 6. doi: 10.1186/s12014-016-9107-z.
- Kirchhausen, T. (2000) 'Clathrin', *Annu Rev Biochem*, 69, pp. 699-727. doi: 10.1146/annurev.biochem.69.1.699.
- Kitai, Y., Kawasaki, T., Sueyoshi, T., Kobiyama, K., Ishii, K. J., Zou, J., Akira, S., Matsuda, T. and Kawai, T. (2017) 'DNA-Containing Exosomes Derived from Cancer Cells Treated with Topotecan Activate a STING-Dependent Pathway and Reinforce Antitumor Immunity', *J Immunol*, 198(4), pp. 1649-1659. doi: 10.4049/jimmunol.1601694.
- Koivusalo, M., Welch, C., Hayashi, H., Scott, C. C., Kim, M., Alexander, T., Touret, N., Hahn, K. M. and Grinstein, S. (2010) 'Amiloride inhibits macropinocytosis by lowering submembranous pH and preventing Rac1 and Cdc42 signaling', *J Cell Biol*, 188(4), pp. 547-63. doi: 10.1083/jcb.200908086.
- Konig, L., Kasimir-Bauer, S., Bittner, A. K., Hoffmann, O., Wagner, B., Santos Manvailer, L. F., Kimmig, R., Horn, P. A. and Rebmann, V. (2017) 'Elevated levels of extracellular vesicles are associated with therapy failure and disease progression in breast cancer patients undergoing neoadjuvant chemotherapy', *Oncoimmunology*, 7(1), pp. e1376153. doi: 10.1080/2162402X.2017.1376153.
- Koumangoye, R. B., Sakwe, A. M., Goodwin, J. S., Patel, T. and Ochieng, J. (2011) 'Detachment of breast tumor cells induces rapid secretion of exosomes which subsequently mediate cellular adhesion and spreading', *PLoS One*, 6(9), pp. e24234. doi: 10.1371/journal.pone.0024234.
- Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J. P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M. and Thery, C. (2016) 'Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes', *Proc Natl Acad Sci U S A*, 113(8), pp. E968-77. doi: 10.1073/pnas.1521230113.
- Kowal, J., Tkach, M. and Thery, C. (2014) 'Biogenesis and secretion of exosomes', *Curr Opin Cell Biol*, 29, pp. 116-25. doi: 10.1016/j.ceb.2014.05.004.
- Kramer, R. H. and Nicolson, G. L. (1979) 'Interactions of tumor cells with vascular endothelial cell monolayers: a model for metastatic invasion', *Proc Natl Acad Sci U S A*, 76(11), pp. 5704-8.
- Kraus, L. A., Samuel, S. K., Schmid, S. M., Dykes, D. J., Waud, W. R. and Bissery, M. C. (2003) 'The mechanism of action of docetaxel (Taxotere) in xenograft models is not limited to bcl-2 phosphorylation', *Invest New Drugs*, 21(3), pp. 259-68.
- Kreit, J. and Sampson, N. S. (2009) 'Cholesterol oxidase: physiological functions', *FEBS J*, 276(23), pp. 6844-56. doi: 10.1111/j.1742-4658.2009.07378.x.
- Lai, C. P., Kim, E. Y., Badr, C. E., Weissleder, R., Mempel, T. R., Tannous, B. A. and Breakefield, X. O. (2015) 'Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters', *Nat Commun*, 6, pp. 7029. doi: 10.1038/ncomms8029.
- Lai, C. P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C., Chen, J. W., Tannous, B. A. and Breakefield, X. O. (2014) 'Dynamic biodistribution of extracellular vesicles in vivo using a multimodal imaging reporter', *ACS Nano*, 8(1), pp. 483-494. doi: 10.1021/nn404945r.

- Lazaro-Ibanez, E., Sanz-Garcia, A., Visakorpi, T., Escobedo-Lucea, C., Siljander, P., Ayuso-Sacido, A. and Yliperttula, M. (2014) 'Different gDNA content in the subpopulations of prostate cancer extracellular vesicles: apoptotic bodies, microvesicles, and exosomes', *Prostate*, 74(14), pp. 1379-90. doi: 10.1002/pros.22853.
- Leathem, A. J. and Brooks, S. A. (1987) 'Predictive value of lectin binding on breast-cancer recurrence and survival', *Lancet*, 1(8541), pp. 1054-6.
- Li, K., Chen, Y., Li, A., Tan, C. and Liu, X. (2018) 'Exosomes play roles in sequential processes of tumor metastasis', *Int J Cancer*. doi: 10.1002/ijc.31774.
- Li, P., Kaslan, M., Lee, S. H., Yao, J. and Gao, Z. (2017) 'Progress in Exosome Isolation Techniques', *Theranostics*, 7(3), pp. 789-804. doi: 10.7150/thno.18133.
- Liang, C. C., Park, A. Y. and Guan, J. L. (2007) 'In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro', *Nat Protoc*, 2(2), pp. 329-33. doi: 10.1038/nprot.2007.30.
- Lim, J. P. and Gleeson, P. A. (2011) 'Macropinocytosis: an endocytic pathway for internalising large gulps', *Immunol Cell Biol*, 89(8), pp. 836-43. doi: 10.1038/icb.2011.20.
- Lima, L. G., Leal, A. C., Vargas, G., Porto-Carreiro, I. and Monteiro, R. Q. (2013) 'Intercellular transfer of tissue factor via the uptake of tumor-derived microvesicles', *Thromb Res*, 132(4), pp. 450-6. doi: 10.1016/j.thromres.2013.07.026.
- Lindoso, R. S., Collino, F. and Camussi, G. (2015) 'Extracellular vesicles derived from renal cancer stem cells induce a pro-tumorigenic phenotype in mesenchymal stromal cells', *Oncotarget*, 6(10), pp. 7959-69. doi: 10.18632/oncotarget.3503.
- Liu, G., Chen, Y., Qi, F., Jia, L., Lu, X. A., He, T., Fu, Y., Li, L. and Luo, Y. (2015) 'Specific chemotherapeutic agents induce metastatic behaviour through stromal- and tumour-derived cytokine and angiogenic factor signalling', *J Pathol*, 237(2), pp. 190-202. doi: 10.1002/path.4564.
- Lo Cicero, A., Stahl, P. D. and Raposo, G. (2015) 'Extracellular vesicles shuffling intercellular messages: for good or for bad', *Curr Opin Cell Biol*, 35, pp. 69-77. doi: 10.1016/j.ceb.2015.04.013.
- Lopatina, T., Grange, C., Fonsato, V., Tapparo, M., Brossa, A., Fallo, S., Pitino, A., Herrera-Sanchez, M. B., Kholia, S., Camussi, G. and Bussolati, B. (2018) 'Extracellular vesicles from human liver stem cells inhibit tumor angiogenesis', *Int J Cancer*. doi: 10.1002/ijc.31796.
- Lowe, D. J. and Raj, K. (2015) 'Quantitation of Endothelial Cell Adhesiveness In Vitro', *J Vis Exp*, (100), pp. e52924. doi: 10.3791/52924.
- Lu, J., Liu, Q. H., Wang, F., Tan, J. J., Deng, Y. Q., Peng, X. H., Liu, X., Zhang, B., Xu, X. and Li, X. P. (2018) 'Exosomal miR-9 inhibits angiogenesis by targeting MDK and regulating PDK/AKT pathway in nasopharyngeal carcinoma', *J Exp Clin Cancer Res*, 37(1), pp. 147. doi: 10.1186/s13046-018-0814-3.
- Luga, V. and Wrana, J. L. (2013) 'Tumor-stroma interaction: Revealing fibroblast-secreted exosomes as potent regulators of Wnt-planar cell polarity signaling in cancer metastasis', *Cancer Res*, 73(23), pp. 6843-7. doi: 10.1158/0008-5472.CAN-13-1791.
- Luga, V., Zhang, L., Vilorio-Petit, A. M., Ogunjimi, A. A., Inanlou, M. R., Chiu, E., Buchanan, M., Hosein, A. N., Basik, M. and Wrana, J. L. (2012) 'Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration', *Cell*, 151(7), pp. 1542-56. doi: 10.1016/j.cell.2012.11.024.
- Lynch, S., Santos, S. G., Campbell, E. C., Nimmo, A. M., Botting, C., Prescott, A., Antoniou, A. N. and Powis, S. J. (2009) 'Novel MHC class I structures on exosomes', *J Immunol*, 183(3), pp. 1884-91. doi: 10.4049/jimmunol.0900798.
- Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C. and Kirchhausen, T. (2006) 'Dynasore, a cell-permeable inhibitor of dynamin', *Dev Cell*, 10(6), pp. 839-50. doi: 10.1016/j.devcel.2006.04.002.
- Maji, S., Chaudhary, P., Akopova, I., Nguyen, P. M., Hare, R. J., Gryczynski, I. and Vishwanatha, J. K. (2017) 'Exosomal Annexin II promotes angiogenesis and breast cancer metastasis', *Mol Cancer Res*, 15(1), pp. 93-105. doi: 10.1158/1541-7786.MCR-16-0163.

- Malone, K. E., Daling, J. R., Thompson, J. D., O'Brien, C. A., Francisco, L. V. and Ostrander, E. A. (1998) 'BRCA1 mutations and breast cancer in the general population: analyses in women before age 35 years and in women before age 45 years with first-degree family history', *JAMA*, 279(12), pp. 922-9.
- McCarty, O. J., Mousa, S. A., Bray, P. F. and Konstantopoulos, K. (2000) 'Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions', *Blood*, 96(5), pp. 1789-97.
- McKelvey, J. K., Powell, L. K., Ashton, W. A., Morris, M. J. and McCracken, A. S. (2015) 'Exosomes: mechanisms of uptake', *Journal of Circulating Biomarkers*, 4(7).
- Meehan, K. and Vella, L. J. (2016) 'The contribution of tumour-derived exosomes to the hallmarks of cancer', *Crit Rev Clin Lab Sci*, 53(2), pp. 121-31. doi: 10.3109/10408363.2015.1092496.
- Menck, K., Scharf, C., Bleckmann, A., Dyck, L., Rost, U., Wenzel, D., Dhople, V. M., Siam, L., Pukrop, T., Binder, C. and Klemm, F. (2015) 'Tumor-derived microvesicles mediate human breast cancer invasion through differentially glycosylated EMMPRIN', *J Mol Cell Biol*, 7(2), pp. 143-53. doi: 10.1093/jmcb/mju047.
- Mierke, C. T., Zitterbart, D. P., Kollmannsberger, P., Raupach, C., Schlotzer-Schrehardt, U., Goecke, T. W., Behrens, J. and Fabry, B. (2008) 'Breakdown of the endothelial barrier function in tumor cell transmigration', *Biophys J*, 94(7), pp. 2832-46. doi: 10.1529/biophysj.107.113613.
- Miranda, A. M., Lasiecka, Z. M., Xu, Y., Neufeld, J., Shahriar, S., Simoes, S., Chan, R. B., Oliveira, T. G., Small, S. A. and Di Paolo, G. (2018) 'Neuronal lysosomal dysfunction releases exosomes harboring APP C-terminal fragments and unique lipid signatures', *Nat Commun*, 9(1), pp. 291. doi: 10.1038/s41467-017-02533-w.
- Mittelbrunn, M., Gutierrez-Vazquez, C., Villarroya-Beltri, C., Gonzalez, S., Sanchez-Cabo, F., Gonzalez, M. A., Bernad, A. and Sanchez-Madrid, F. (2011) 'Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells', *Nat Commun*, 2, pp. 282. doi: 10.1038/ncomms1285.
- Monopoli, M. P., Zendrini, A., Wu, D., Cheung, S., Sampedro, G., Ffrench, B., Nolan, J., Piskareva, O., Stalings, R. L., Ducoli, S., Bergese, P. and O'Shea, D. F. (2018) 'Endogenous exosome labelling with an amphiphilic NIR-fluorescent probe', *Chem Commun (Camb)*, 54(52), pp. 7219-7222. doi: 10.1039/c8cc02135j.
- Montecalvo, A., Larregina, A. T., Shufesky, W. J., Stolz, D. B., Sullivan, M. L., Karlsson, J. M., Baty, C. J., Gibson, G. A., Erdos, G., Wang, Z., Milosevic, J., Tkacheva, O. A., Divito, S. J., Jordan, R., Lyons-Weiler, J., Watkins, S. C. and Morelli, A. E. (2012) 'Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes', *Blood*, 119(3), pp. 756-66. doi: 10.1182/blood-2011-02-338004.
- Morelli, A. E., Larregina, A. T., Shufesky, W. J., Sullivan, M. L., Stolz, D. B., Papworth, G. D., Zahorchak, A. F., Logar, A. J., Wang, Z., Watkins, S. C., Falo, L. D., Jr. and Thomson, A. W. (2004) 'Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells', *Blood*, 104(10), pp. 3257-66. doi: 10.1182/blood-2004-03-0824.
- Moreno-Bueno, G., Portillo, F. and Cano, A. (2008) 'Transcriptional regulation of cell polarity in EMT and cancer', *Oncogene*, 27(55), pp. 6958-69. doi: 10.1038/onc.2008.346.
- Mulcahy, L. A., Pink, R. C. and Carter, D. R. (2014) 'Routes and mechanisms of extracellular vesicle uptake', *J Extracell Vesicles*, 3. doi: 10.3402/jev.v3.24641.
- Nabi, I. R. and Le, P. U. (2003) 'Caveolae/raft-dependent endocytosis', *J Cell Biol*, 161(4), pp. 673-7. doi: 10.1083/jcb.200302028.
- Nakajima, S., Doi, R., Toyoda, E., Tsuji, S., Wada, M., Koizumi, M., Tulachan, S. S., Ito, D., Kami, K., Mori, T., Kawaguchi, Y., Fujimoto, K., Hosotani, R. and Imamura, M. (2004) 'N-cadherin expression and epithelial-mesenchymal transition in pancreatic carcinoma', *Clin Cancer Res*, 10(12 Pt 1), pp. 4125-33. doi: 10.1158/1078-0432.CCR-0578-03.
- Nakase, I., Kobayashi, N. B., Takatani-Nakase, T. and Yoshida, T. (2015) 'Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras

- expression potentiates cellular uptake efficacy of exosomes', *Sci Rep*, 5, pp. 10300. doi: 10.1038/srep10300.
- Nanbo, A., Kawanishi, E., Yoshida, R. and Yoshiyama, H. (2013) 'Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells', *J Virol*, 87(18), pp. 10334-47. doi: 10.1128/JVI.01310-13.
- Naslund, T. I., Paquin-Proulx, D., Paredes, P. T., Vallhov, H., Sandberg, J. K. and Gabrielsson, S. (2014) 'Exosomes from breast milk inhibit HIV-1 infection of dendritic cells and subsequent viral transfer to CD4+ T cells', *AIDS*, 28(2), pp. 171-80. doi: 10.1097/QAD.000000000000159.
- Newton, A. J., Kirchhausen, T. and Murthy, V. N. (2006) 'Inhibition of dynamin completely blocks compensatory synaptic vesicle endocytosis', *Proc Natl Acad Sci U S A*, 103(47), pp. 17955-60. doi: 10.1073/pnas.0606212103.
- Nicolson, G. L. (2015) 'Cell membrane fluid-mosaic structure and cancer metastasis', *Cancer Res*, 75(7), pp. 1169-76. doi: 10.1158/0008-5472.CAN-14-3216.
- Nierodzik, M. L., Plotkin, A., Kajumo, F. and Karparkin, S. (1991) 'Thrombin stimulates tumor-platelet adhesion in vitro and metastasis in vivo', *J Clin Invest*, 87(1), pp. 229-36. doi: 10.1172/JCI114976.
- Nishida-Aoki, N., Tominaga, N., Takeshita, F., Sonoda, H., Yoshioka, Y. and Ochiya, T. (2017) 'Disruption of Circulating Extracellular Vesicles as a Novel Therapeutic Strategy against Cancer Metastasis', *Mol Ther*, 25(1), pp. 181-191. doi: 10.1016/j.ymthe.2016.10.009.
- Noguchi, M., Thomas, M., Kitagawa, H., Kinoshita, K., Ohta, N., Nagamori, M. and Miyazaki, I. (1993) 'Further analysis of predictive value of Helix pomatia lectin binding to primary breast cancer for axillary and internal mammary lymph node metastases', *Br J Cancer*, 67(6), pp. 1368-71.
- O'Brien, K., Rani, S., Corcoran, C., Wallace, R., Hughes, L., Friel, A. M., McDonnell, S., Crown, J., Radomski, M. W. and O'Driscoll, L. (2013) 'Exosomes from triple-negative breast cancer cells can transfer phenotypic traits representing their cells of origin to secondary cells', *Eur J Cancer*, 49(8), pp. 1845-59. doi: 10.1016/j.ejca.2013.01.017.
- Ozawa, P. M. M., Alkhilaiwi, F., Cavalli, I. J., Malheiros, D., de Souza Fonseca Ribeiro, E. M. and Cavalli, L. R. (2018) 'Extracellular vesicles from triple-negative breast cancer cells promote proliferation and drug resistance in non-tumorigenic breast cells', *Breast Cancer Res Treat*, 172(3), pp. 713-723. doi: 10.1007/s10549-018-4925-5.
- Pacheca-Palomar, M. T. (2009) *MVP: Mitomycin C / Vinblastine / Cisplatin in Advanced Breast Cancer*. Available at: [http://www.londoncanceralliance.nhs.uk/media/37102/Breast_MVP_\(PALLIATIVE\)_protocol_V2.0.pdf](http://www.londoncanceralliance.nhs.uk/media/37102/Breast_MVP_(PALLIATIVE)_protocol_V2.0.pdf) (Accessed: 02/12/2018).
- Pang, M. F., Georgoudaki, A. M., Lambut, L., Johansson, J., Tabor, V., Hagikura, K., Jin, Y., Jansson, M., Alexander, J. S., Nelson, C. M., Jakobsson, L., Betsholtz, C., Sund, M., Karlsson, M. C. and Fuxe, J. (2016) 'TGF-beta1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis', *Oncogene*, 35(6), pp. 748-60. doi: 10.1038/ncr.2015.133.
- Park, R. J., Shen, H., Liu, L., Liu, X., Ferguson, S. M. and De Camilli, P. (2013) 'Dynamin triple knockout cells reveal off target effects of commonly used dynamin inhibitors', *J Cell Sci*, 126(Pt 22), pp. 5305-12. doi: 10.1242/jcs.138578.
- Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A., Colone, M., Tatti, M., Sargiacomo, M. and Fais, S. (2009) 'Microenvironmental pH is a key factor for exosome traffic in tumor cells', *J Biol Chem*, 284(49), pp. 34211-22. doi: 10.1074/jbc.M109.041152.
- Pasquier, J., Thawadi, H. A., Ghiabi, P., Abu-Kaoud, N., Maleki, M., Guerrouahen, B. S., Vidal, F., Courderc, B., Ferron, G., Martinez, A., Al Sulaiti, H., Gupta, R., Rafii, S. and Rafii, A. (2014) 'Microparticles mediated cross-talk between tumoral and endothelial cells promote the

- constitution of a pro-metastatic vascular niche through Arf6 up regulation', *Cancer Microenviron*, 7(1-2), pp. 41-59. doi: 10.1007/s12307-013-0142-2.
- Paul, C. D., Mistriotis, P. and Konstantopoulos, K. (2017) 'Cancer cell motility: lessons from migration in confined spaces', *Nat Rev Cancer*, 17(2), pp. 131-140. doi: 10.1038/nrc.2016.123.
- Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B. A., Callahan, M. K., Yuan, J., Martins, V. R., Skog, J., Kaplan, R. N., Brady, M. S., Wolchok, J. D., Chapman, P. B., Kang, Y., Bromberg, J. and Lyden, D. (2012) 'Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET', *Nat Med*, 18(6), pp. 883-91. doi: 10.1038/nm.2753.
- Perez-Boza, J., Lion, M. and Struman, I. (2018) 'Exploring the RNA landscape of endothelial exosomes', *RNA*, 24(3), pp. 423-435. doi: 10.1261/rna.064352.117.
- Perez-Hernandez, D., Gutierrez-Vazquez, C., Jorge, I., Lopez-Martin, S., Ursa, A., Sanchez-Madrid, F., Vazquez, J. and Yanez-Mo, M. (2013) 'The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes', *J Biol Chem*, 288(17), pp. 11649-61. doi: 10.1074/jbc.M112.445304.
- Petracca, R., Falugi, F., Galli, G., Norais, N., Rosa, D., Campagnoli, S., Burgio, V., Di Stasio, E., Giardina, B., Houghton, M., Abrignani, S. and Grandi, G. (2000) 'Structure-function analysis of hepatitis C virus envelope-CD81 binding', *J Virol*, 74(10), pp. 4824-30.
- Pinho, S. S. and Reis, C. A. (2015) 'Glycosylation in cancer: mechanisms and clinical implications', *Nat Rev Cancer*, 15(9), pp. 540-55. doi: 10.1038/nrc3982.
- Pisitkun, T., Shen, R. F. and Knepper, M. A. (2004) 'Identification and proteomic profiling of exosomes in human urine', *Proc Natl Acad Sci U S A*, 101(36), pp. 13368-73. doi: 10.1073/pnas.0403453101.
- Pospichalova, V., Svoboda, J., Dave, Z., Kotrbova, A., Kaiser, K., Klemova, D., Ilkovic, L., Hampl, A., Crha, I., Jandakova, E., Minar, L., Weinberger, V. and Bryja, V. (2015) 'Simplified protocol for flow cytometry analysis of fluorescently labeled exosomes and microvesicles using dedicated flow cytometer', *J Extracell Vesicles*, 4, pp. 25530. doi: 10.3402/jev.v4.25530.
- Poste, G. and Fidler, I. J. (1980) 'The pathogenesis of cancer metastasis', *Nature*, 283(5743), pp. 139-46.
- Prabhat, P., Ram, S., Ward, E. S. and Ober, R. J. (2004) 'Simultaneous imaging of different focal planes in fluorescence microscopy for the study of cellular dynamics in three dimensions', *IEEE Trans Nanobioscience*, 3(4), pp. 237-42.
- Prada, I., Amin, L., Furlan, R., Legname, G., Verderio, C. and Cojoc, D. (2016) 'A new approach to follow a single extracellular vesicle-cell interaction using optical tweezers', *Biotechniques*, 60(1), pp. 35-41. doi: 10.2144/000114371.
- Preta, G., Cronin, J. G. and Sheldon, I. M. (2015a) 'Dynasore - not just a dynamin inhibitor', *Cell Commun Signal*, 13, pp. 24. doi: 10.1186/s12964-015-0102-1.
- Preta, G., Lotti, V., Cronin, J. G. and Sheldon, I. M. (2015b) 'Protective role of the dynamin inhibitor Dynasore against the cholesterol-dependent cytolysis of *Trueperella pyogenes*', *FASEB J*, 29(4), pp. 1516-28. doi: 10.1096/fj.14-265207.
- Purushothaman, A., Bandari, S. K., Liu, J., Mobley, J. A., Brown, E. E. and Sanderson, R. D. (2016) 'Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions', *J Biol Chem*, 291(4), pp. 1652-63. doi: 10.1074/jbc.M115.686295.
- Puzar Dominkus, P., Stenovec, M., Sitar, S., Lasic, E., Zorec, R., Plemenitas, A., Zagar, E., Kreft, M. and Lenassi, M. (2018) 'PKH26 labeling of extracellular vesicles: Characterization and cellular internalization of contaminating PKH26 nanoparticles', *Biochim Biophys Acta Biomembr*, 1860(6), pp. 1350-1361. doi: 10.1016/j.bbamem.2018.03.013.
- Pyzer, A. R., Cole, L., Rosenblatt, J. and Avigan, D. E. (2016) 'Myeloid-derived suppressor cells as effectors of immune suppression in cancer', *Int J Cancer*, 139(9), pp. 1915-26. doi: 10.1002/ijc.30232.

- Quast, T., Eppler, F., Semmling, V., Schild, C., Homsy, Y., Levy, S., Lang, T., Kurts, C. and Kolanus, W. (2011) 'CD81 is essential for the formation of membrane protrusions and regulates Rac1-activation in adhesion-dependent immune cell migration', *Blood*, 118(7), pp. 1818-27. doi: 10.1182/blood-2010-12-326595.
- Raimondo, S., Saieva, L., Corrado, C., Fontana, S., Flugy, A., Rizzo, A., De Leo, G. and Alessandro, R. (2015) 'Chronic myeloid leukemia-derived exosomes promote tumor growth through an autocrine mechanism', *Cell Commun Signal*, 13, pp. 8. doi: 10.1186/s12964-015-0086-x.
- Rambaruth, N. D., Greenwell, P. and Dwek, M. V. (2012) 'The lectin Helix pomatia agglutinin recognizes O-GlcNAc containing glycoproteins in human breast cancer', *Glycobiology*, 22(6), pp. 839-48. doi: 10.1093/glycob/cws051.
- Ramteke, A., Ting, H., Agarwal, C., Mateen, S., Somasagara, R., Hussain, A., Graner, M., Frederick, B., Agarwal, R. and Deep, G. (2015) 'Exosomes secreted under hypoxia enhance invasiveness and stemness of prostate cancer cells by targeting adherens junction molecules', *Mol Carcinog*, 54(7), pp. 554-65. doi: 10.1002/mc.22124.
- Rana, S., Claas, C., Kretz, C. C., Nazarenko, I. and Zoeller, M. (2011) 'Activation-induced internalization differs for the tetraspanins CD9 and Tspan8: Impact on tumor cell motility', *Int J Biochem Cell Biol*, 43(1), pp. 106-19. doi: 10.1016/j.biocel.2010.10.002.
- Rana, S., Yue, S., Stadel, D. and Zoller, M. (2012) 'Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection', *Int J Biochem Cell Biol*, 44(9), pp. 1574-84. doi: 10.1016/j.biocel.2012.06.018.
- Raposo, G. and Stoorvogel, W. (2013) 'Extracellular vesicles: exosomes, microvesicles, and friends', *J Cell Biol*, 200(4), pp. 373-83. doi: 10.1083/jcb.201211138.
- Ratajczak, M. Z., Jadczyk, T., Schneider, G., Kakar, S. S. and Kucia, M. (2013) 'Induction of a tumor-metastasis-receptive microenvironment as an unwanted and underestimated side effect of treatment by chemotherapy or radiotherapy', *J Ovarian Res*, 6(1), pp. 95. doi: 10.1186/1757-2215-6-95.
- Ren, Y., Zhou, X., Yang, J. J., Liu, X., Zhao, X. H., Wang, Q. X., Han, L., Song, X., Zhu, Z. Y., Tian, W. P., Zhang, L., Mei, M. and Kang, C. S. (2015) 'AC1MMYR2 impairs high dose paclitaxel-induced tumor metastasis by targeting miR-21/CDK5 axis', *Cancer Lett*, 362(2), pp. 174-82. doi: 10.1016/j.canlet.2015.03.038.
- Riches, A., Campbell, E., Borger, E. and Powis, S. (2014) 'Regulation of exosome release from mammary epithelial and breast cancer cells - a new regulatory pathway', *Eur J Cancer*, 50(5), pp. 1025-34. doi: 10.1016/j.ejca.2013.12.019.
- Robert, A., Hookway, C. and Gelfand, V. I. (2016) 'Intermediate filament dynamics: What we can see now and why it matters', *Bioessays*, 38(3), pp. 232-43. doi: 10.1002/bies.201500142.
- Roberts-Dalton, H. D., Cocks, A., Falcon-Perez, J. M., Sayers, E. J., Webber, J. P., Watson, P., Clayton, A. and Jones, A. T. (2017) 'Fluorescence labelling of extracellular vesicles using a novel thiol-based strategy for quantitative analysis of cellular delivery and intracellular traffic', *Nanoscale*, 9(36), pp. 13693-13706. doi: 10.1039/c7nr04128d.
- Roccaro, A. M., Sacco, A., Maiso, P., Azab, A. K., Tai, Y. T., Reagan, M., Azab, F., Flores, L. M., Campigotto, F., Weller, E., Anderson, K. C., Scadden, D. T. and Ghobrial, I. M. (2013) 'BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression', *J Clin Invest*, 123(4), pp. 1542-55. doi: 10.1172/JCI66517.
- Rodriguez, M., Silva, J., Herrera, A., Herrera, M., Pena, C., Martin, P., Gil-Calderon, B., Larriba, M. J., Coronado, M. J., Soldevilla, B., Turrion, V. S., Provencio, M., Sanchez, A., Bonilla, F. and Garcia-Barberan, V. (2015) 'Exosomes enriched in stemness/metastatic-related mRNAs promote oncogenic potential in breast cancer', *Oncotarget*, 6(38), pp. 40575-87. doi: 10.18632/oncotarget.5818.
- Rogers-Broadway, K. R. and Karteris, E. (2015) 'Amplification efficiency and thermal stability of qPCR instrumentation: Current landscape and future perspectives', *Exp Ther Med*, 10(4), pp. 1261-1264. doi: 10.3892/etm.2015.2712.

- Safaei, R., Larson, B. J., Cheng, T. C., Gibson, M. A., Otani, S., Naerdemann, W. and Howell, S. B. (2005) 'Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells', *Mol Cancer Ther*, 4(10), pp. 1595-604. doi: 10.1158/1535-7163.MCT-05-0102.
- Salamone, M., Siragusa, M., Nasca, M., Pitarresi, L., Vittorelli, M. L., Chen, W. T. and Gherzi, G. (2006) 'Type-II transmembrane prolyl dipeptidases and matrix metalloproteinases in membrane vesicles of active endothelial cells', *Adv Exp Med Biol*, 575, pp. 207-12. doi: 10.1007/0-387-32824-6_22.
- Samuel, P., Mulcahy, L. A., Furlong, F., McCarthy, H. O., Brooks, S. A., Fabbri, M., Pink, R. C. and Carter, D. R. F. (2018) 'Cisplatin induces the release of extracellular vesicles from ovarian cancer cells that can induce invasiveness and drug resistance in bystander cells', *Philos Trans R Soc Lond B Biol Sci*, 373(1737). doi: 10.1098/rstb.2017.0065.
- Sanchez, J. F., Lescar, J., Chazalet, V., Audfray, A., Gagnon, J., Alvarez, R., Breton, C., Imberty, A. and Mitchell, E. P. (2006) 'Biochemical and structural analysis of Helix pomatia agglutinin. A hexameric lectin with a novel fold', *J Biol Chem*, 281(29), pp. 20171-80. doi: 10.1074/jbc.M603452200.
- Savagner, P. (2001) 'Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition', *Bioessays*, 23(10), pp. 912-23. doi: 10.1002/bies.1132.
- Sceneay, J., Chow, M. T., Chen, A., Halse, H. M., Wong, C. S., Andrews, D. M., Sloan, E. K., Parker, B. S., Bowtell, D. D., Smyth, M. J. and Moller, A. (2012) 'Primary tumor hypoxia recruits CD11b⁺/Ly6C^{med}/Ly6G⁺ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche', *Cancer Res*, 72(16), pp. 3906-11. doi: 10.1158/0008-5472.CAN-11-3873.
- Schillaci, O., Fontana, S., Monteleone, F., Taverna, S., Di Bella, M. A., Di Vizio, D. and Alessandro, R. (2017) 'Exosomes from metastatic cancer cells transfer amoeboid phenotype to non-metastatic cells and increase endothelial permeability: their emerging role in tumor heterogeneity', *Sci Rep*, 7(1), pp. 4711. doi: 10.1038/s41598-017-05002-y.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012) 'Fiji: an open-source platform for biological-image analysis', *Nat Methods*, 9(7), pp. 676-82. doi: 10.1038/nmeth.2019.
- Schneider, P. and Tschopp, J. (2000) 'Apoptosis induced by death receptors', *Pharm Acta Helv*, 74(2-3), pp. 281-6.
- Schumacher, U., Kretzschmar, H., Brooks, S. and Leatham, A. (1992) 'Helix pomatia lectin binding pattern of brain metastases originating from breast cancers', *Pathol Res Pract*, 188(3), pp. 284-6.
- Senkus, E., Kyriakides, S., Penault-Llorca, F., Poortmans, P., Thompson, A., Zackrisson, S., Cardoso, F. and Group, E. G. W. (2013) 'Primary breast cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up', *Ann Oncol*, 24 Suppl 6, pp. vi7-23. doi: 10.1093/annonc/mdt284.
- Sento, S., Sasabe, E. and Yamamoto, T. (2016) 'Application of a Persistent Heparin Treatment Inhibits the Malignant Potential of Oral Squamous Carcinoma Cells Induced by Tumor Cell-Derived Exosomes', *PLoS One*, 11(2), pp. e0148454. doi: 10.1371/journal.pone.0148454.
- Singh, A., Fedeles, C., Lu, H., Nevalainen, M. T., Keen, J. H. and Languino, L. R. (2016) 'Exosome-mediated transfer of alpha-v beta-3 integrin from tumorigenic to nontumorigenic cells promotes a migratory phenotype', *Mol Cancer Res*, 14(11), pp. 1136-1146. doi: 10.1158/1541-7786.MCR-16-0058.
- Skotland, T., Sandvig, K. and Llorente, A. (2017) 'Lipids in exosomes: Current knowledge and the way forward', *Prog Lipid Res*, 66, pp. 30-41. doi: 10.1016/j.plipres.2017.03.001.
- Slamon, D. J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., Baselga, J. and Norton, L. (2001) 'Use of

- chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2', *N Engl J Med*, 344(11), pp. 783-92. doi: 10.1056/NEJM200103153441101.
- Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Thorsen, T., Quist, H., Matese, J. C., Brown, P. O., Botstein, D., Lonning, P. E. and Borresen-Dale, A. L. (2001) 'Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications', *Proc Natl Acad Sci U S A*, 98(19), pp. 10869-74. doi: 10.1073/pnas.191367098.
- Soule, H. D., Vazquez, J., Long, A., Albert, S. and Brennan, M. (1973) 'A human cell line from a pleural effusion derived from a breast carcinoma', *J Natl Cancer Inst*, 51(5), pp. 1409-16.
- Stoner, S. A., Duggan, E., Condello, D., Guerrero, A., Turk, J. R., Narayanan, P. K. and Nolan, J. P. (2016) 'High sensitivity flow cytometry of membrane vesicles', *Cytometry A*, 89(2), pp. 196-206. doi: 10.1002/cyto.a.22787.
- Stoorvogel, W., Strous, G. J., Geuze, H. J., Oorschot, V. and Schwartz, A. L. (1991) 'Late endosomes derive from early endosomes by maturation', *Cell*, 65(3), pp. 417-27.
- Suarez, H., Gamez-Valero, A., Reyes, R., Lopez-Martin, S., Rodriguez, M. J., Carrascosa, J. L., Cabanas, C., Borrás, F. E. and Yanez-Mo, M. (2017) 'A bead-assisted flow cytometry method for the semi-quantitative analysis of Extracellular Vesicles', *Sci Rep*, 7(1), pp. 11271. doi: 10.1038/s41598-017-11249-2.
- Suchorska, W. M. and Lach, M. S. (2016) 'The role of exosomes in tumor progression and metastasis (Review)', *Oncol Rep*, 35(3), pp. 1237-44. doi: 10.3892/or.2015.4507.
- Suetsugu, A., Honma, K., Saji, S., Moriwaki, H., Ochiya, T. and Hoffman, R. M. (2013) 'Imaging exosome transfer from breast cancer cells to stroma at metastatic sites in orthotopic nude-mouse models', *Adv Drug Deliv Rev*, 65(3), pp. 383-90. doi: 10.1016/j.addr.2012.08.007.
- Svensson, K. J., Christianson, H. C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L. M., Morgelin, M. and Belting, M. (2013) 'Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1', *J Biol Chem*, 288(24), pp. 17713-24. doi: 10.1074/jbc.M112.445403.
- Szybalski, W. and Iyer, V. N. (1964) 'Crosslinking of DNA by Enzymatically or Chemically Activated Mitomycins and Porfiromycins, Bifunctionally "Alkylating" Antibiotics', *Fed Proc*, 23, pp. 946-57.
- Takov, K., Yellon, D. M. and Davidson, S. M. (2017) 'Confounding factors in vesicle uptake studies using fluorescent lipophilic membrane dyes', *J Extracell Vesicles*, 6(1), pp. 1388731. doi: 10.1080/20013078.2017.1388731.
- Taraboletti, G., D'Ascenzo, S., Borsotti, P., Giavazzi, R., Pavan, A. and Dolo, V. (2002) 'Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells', *Am J Pathol*, 160(2), pp. 673-80. doi: 10.1016/S0002-9440(10)64887-0.
- Thery, C., Amigorena, S., Raposo, G. and Clayton, A. (2006) 'Isolation and characterization of exosomes from cell culture supernatants and biological fluids', *Curr Protoc Cell Biol*, Chapter 3, pp. Unit 3 22. doi: 10.1002/0471143030.cb0322s30.
- Thery, C., Zitvogel, L. and Amigorena, S. (2002) 'Exosomes: composition, biogenesis and function', *Nat Rev Immunol*, 2(8), pp. 569-79. doi: 10.1038/nri855.
- Tian, T., Wang, Y., Wang, H., Zhu, Z. and Xiao, Z. (2010) 'Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy', *J Cell Biochem*, 111(2), pp. 488-96. doi: 10.1002/jcb.22733.
- Tian, T., Zhu, Y. L., Zhou, Y. Y., Liang, G. F., Wang, Y. Y., Hu, F. H. and Xiao, Z. D. (2014) 'Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery', *J Biol Chem*, 289(32), pp. 22258-67. doi: 10.1074/jbc.M114.588046.
- Tinevez, J. Y., Perry, N., Schindelin, J., Hoopes, G. M., Reynolds, G. D., Laplantine, E., Bednarek, S. Y., Shorte, S. L. and Eliceiri, K. W. (2017) 'TrackMate: An open and extensible platform for single-particle tracking', *Methods*, 115, pp. 80-90. doi: 10.1016/j.ymeth.2016.09.016.

- Tomaskovic-Crook, E., Thompson, E. W. and Thiery, J. P. (2009) 'Epithelial to mesenchymal transition and breast cancer', *Breast Cancer Res*, 11(6), pp. 213. doi: 10.1186/bcr2416.
- Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B. and Simons, M. (2008) 'Ceramide triggers budding of exosome vesicles into multivesicular endosomes', *Science*, 319(5867), pp. 1244-7. doi: 10.1126/science.1153124.
- Tremblay, P. L., Huot, J. and Auger, F. A. (2008) 'Mechanisms by which E-selectin regulates diapedesis of colon cancer cells under flow conditions', *Cancer Res*, 68(13), pp. 5167-76. doi: 10.1158/0008-5472.CAN-08-1229.
- Tsien, R. Y. (1998) 'The green fluorescent protein', *Annu Rev Biochem*, 67, pp. 509-44. doi: 10.1146/annurev.biochem.67.1.509.
- Umez, T., Tadokoro, H., Azuma, K., Yoshizawa, S., Ohyashiki, K. and Ohyashiki, J. H. (2014) 'Exosomal miR-135b shed from hypoxic multiple myeloma cells enhances angiogenesis by targeting factor-inhibiting HIF-1', *Blood*, 124(25), pp. 3748-57. doi: 10.1182/blood-2014-05-576116.
- Valenti, R., Huber, V., Filipazzi, P., Pilla, L., Sovenia, G., Villa, A., Corbelli, A., Fais, S., Parmiani, G. and Rivoltini, L. (2006) 'Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-beta-mediated suppressive activity on T lymphocytes', *Cancer Res*, 66(18), pp. 9290-8. doi: 10.1158/0008-5472.CAN-06-1819.
- Valentiner, U., Hall, D. M., Brooks, S. A. and Schumacher, U. (2005) 'HPA binding and metastasis formation of human breast cancer cell lines transplanted into severe combined immunodeficient (scid) mice', *Cancer Lett*, 219(2), pp. 233-42. doi: 10.1016/j.canlet.2004.07.046.
- Van der Haegen, B. A. and Shay, J. W. (1993) 'Immortalization of human mammary epithelial cells by SV40 large T-antigen involves a two step mechanism', *In Vitro Cell Dev Biol*, 29A(3 Pt 1), pp. 180-2.
- van der Zee, J. (2002) 'Heating the patient: a promising approach?', *Ann Oncol*, 13(8), pp. 1173-84.
- van Dongen, H. M., Masoumi, N., Witwer, K. W. and Pegtel, D. M. (2016) 'Extracellular vesicles exploit viral entry routes for cargo delivery', *Microbiol Mol Biol Rev*, 80(2), pp. 369-86. doi: 10.1128/MMBR.00063-15.
- van Niel, G., Charrin, S., Simoes, S., Romao, M., Rochin, L., Saftig, P., Marks, M. S., Rubinstein, E. and Raposo, G. (2011) 'The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis', *Dev Cell*, 21(4), pp. 708-21. doi: 10.1016/j.devcel.2011.08.019.
- Verweij, F. J., Bebelman, M. P., Jimenez, C. R., Garcia-Vallejo, J. J., Janssen, H., Neefjes, J., Knol, J. C., de Goeij-de Haas, R., Piersma, S. R., Baglio, S. R., Verhage, M., Middeldorp, J. M., Zomer, A., van Rheenen, J., Coppolino, M. G., Hurbain, I., Raposo, G., Smit, M. J., Toonen, R. F. G., van Niel, G. and Pegtel, D. M. (2018) 'Quantifying exosome secretion from single cells reveals a modulatory role for GPCR signaling', *J Cell Biol*, 217(3), pp. 1129-1142. doi: 10.1083/jcb.201703206.
- Vidricaire, G. and Tremblay, M. J. (2007) 'A clathrin, caveolae, and dynamin-independent endocytic pathway requiring free membrane cholesterol drives HIV-1 internalization and infection in polarized trophoblastic cells', *J Mol Biol*, 368(5), pp. 1267-83. doi: 10.1016/j.jmb.2007.03.003.
- Villarroya-Beltri, C., Baixauli, F., Gutierrez-Vazquez, C., Sanchez-Madrid, F. and Mittelbrunn, M. (2014) 'Sorting it out: regulation of exosome loading', *Semin Cancer Biol*, 28, pp. 3-13. doi: 10.1016/j.semcancer.2014.04.009.
- Volk-Draper, L., Hall, K., Griggs, C., Rajput, S., Kohio, P., DeNardo, D. and Ran, S. (2014) 'Paclitaxel therapy promotes breast cancer metastasis in a TLR4-dependent manner', *Cancer Res*, 74(19), pp. 5421-34. doi: 10.1158/0008-5472.CAN-14-0067.

- Vyas, D., Laput, G. and Vyas, A. K. (2014) 'Chemotherapy-enhanced inflammation may lead to the failure of therapy and metastasis', *Onco Targets Ther*, 7, pp. 1015-23. doi: 10.2147/OTT.S60114.
- Warren, A. J., Mustra, D. J. and Hamilton, J. W. (2001) 'Detection of mitomycin C-DNA adducts in human breast cancer cells grown in culture, as xenografted tumors in nude mice, and in biopsies of human breast cancer patient tumors as determined by (32)P-postlabeling', *Clin Cancer Res*, 7(4), pp. 1033-42.
- Webber, J., Steadman, R., Mason, M. D., Tabi, Z. and Clayton, A. (2010) 'Cancer exosomes trigger fibroblast to myofibroblast differentiation', *Cancer Res*, 70(23), pp. 9621-30. doi: 10.1158/0008-5472.CAN-10-1722.
- Weigelt, B., Ng, C. K., Shen, R., Popova, T., Schizas, M., Natrajan, R., Mariani, O., Stern, M. H., Norton, L., Vincent-Salomon, A. and Reis-Filho, J. S. (2015) 'Metaplastic breast carcinomas display genomic and transcriptomic heterogeneity', *Mod Pathol*, 28(3), pp. 340-51. doi: 10.1038/modpathol.2014.142.
- Welton, J. L., Khanna, S., Giles, P. J., Brennan, P., Brewis, I. A., Staffurth, J., Mason, M. D. and Clayton, A. (2010) 'Proteomics analysis of bladder cancer exosomes', *Mol Cell Proteomics*, 9(6), pp. 1324-38. doi: 10.1074/mcp.M000063-MCP201.
- Whiteside, T. L. (2016) 'Exosomes and tumor-mediated immune suppression', *J Clin Invest*, 126(4), pp. 1216-23. doi: 10.1172/JCI81136.
- WHO *World Health Organization Model Formulary 2008*. Available at: <http://apps.who.int/medicinedocs/documents/s16879e/s16879e.pdf> (Accessed: 05/01/2019).
- Wieckowski, E. U., Visus, C., Szajnik, M., Szczepanski, M. J., Storkus, W. J. and Whiteside, T. L. (2009) 'Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes', *J Immunol*, 183(6), pp. 3720-30. doi: 10.4049/jimmunol.0900970.
- Witwer, K. W., Soekmadji, C., Hill, A. F., Wauben, M. H., Buzas, E. I., Di Vizio, D., Falcon-Perez, J. M., Gardiner, C., Hochberg, F., Kurochkin, I. V., Lotvall, J., Mathivanan, S., Nieuwland, R., Sahoo, S., Tahara, H., Torrecilhas, A. C., Weaver, A. M., Yin, H., Zheng, L., Gho, Y. S., Quesenberry, P. and Thery, C. (2017) 'Updating the MISEV minimal requirements for extracellular vesicle studies: building bridges to reproducibility', *J Extracell Vesicles*, 6(1), pp. 1396823. doi: 10.1080/20013078.2017.1396823.
- Wysoczynski, M. and Ratajczak, M. Z. (2009) 'Lung cancer secreted microvesicles: underappreciated modulators of microenvironment in expanding tumors', *Int J Cancer*, 125(7), pp. 1595-603. doi: 10.1002/ijc.24479.
- Xiao, X., Yu, S., Li, S., Wu, J., Ma, R., Cao, H., Zhu, Y. and Feng, J. (2014) 'Exosomes: decreased sensitivity of lung cancer A549 cells to cisplatin', *PLoS One*, 9(2), pp. e89534. doi: 10.1371/journal.pone.0089534.
- Yanez-Mo, M., Siljander, P. R., Andreu, Z., Zavec, A. B., Borrás, F. E., Buzas, E. I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., Colás, E., Cordeiro-da Silva, A., Fais, S., Falcon-Perez, J. M., Ghobrial, I. M., Giebel, B., Gimona, M., Graner, M., Gursel, I., Gursel, M., Heegaard, N. H., Hendrix, A., Kierulf, P., Kokubun, K., Kosanovic, M., Kralj-Iglic, V., Kramer-Albers, E. M., Laitinen, S., Lasser, C., Lener, T., Ligeti, E., Line, A., Lipps, G., Llorente, A., Lotvall, J., Mancek-Keber, M., Marcilla, A., Mittelbrunn, M., Nazarenko, I., Nolte-'t Hoen, E. N., Nyman, T. A., O'Driscoll, L., Olivan, M., Oliveira, C., Pallinger, E., Del Portillo, H. A., Reventos, J., Rigau, M., Rohde, E., Sammar, M., Sanchez-Madrid, F., Santarem, N., Schallmoser, K., Ostendorf, M. S., Stoorvogel, W., Stukelj, R., Van der Grein, S. G., Vasconcelos, M. H., Wauben, M. H. and De Wever, O. (2015) 'Biological properties of extracellular vesicles and their physiological functions', *J Extracell Vesicles*, 4, pp. 27066. doi: 10.3402/jev.v4.27066.
- Yang, M., Chen, J., Su, F., Yu, B., Su, F., Lin, L., Liu, Y., Huang, J. D. and Song, E. (2011) 'Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells', *Mol Cancer*, 10, pp. 117. doi: 10.1186/1476-4598-10-117.

- Yao, D., Dai, C. and Peng, S. (2011) 'Mechanism of the mesenchymal-epithelial transition and its relationship with metastatic tumor formation', *Mol Cancer Res*, 9(12), pp. 1608-20. doi: 10.1158/1541-7786.MCR-10-0568.
- Yilmaz, M. and Christofori, G. (2010) 'Mechanisms of motility in metastasizing cells', *Mol Cancer Res*, 8(5), pp. 629-42. doi: 10.1158/1541-7786.MCR-10-0139.
- You, Y., Shan, Y., Chen, J., Yue, H., You, B., Shi, S., Li, X. and Cao, X. (2015) 'Matrix metalloproteinase 13-containing exosomes promote nasopharyngeal carcinoma metastasis', *Cancer Sci*, 106(12), pp. 1669-77. doi: 10.1111/cas.12818.
- Yu, Z., Zhao, S., Ren, L., Wang, L., Chen, Z., Hoffman, R. M. and Zhou, J. (2017) 'Pancreatic cancer-derived exosomes promote tumor metastasis and liver pre-metastatic niche formation', *Oncotarget*, 8(38), pp. 63461-63483. doi: 10.18632/oncotarget.18831.
- Yuana, Y., Sturk, A. and Nieuwland, R. (2013) 'Extracellular vesicles in physiological and pathological conditions', *Blood Rev*, 27(1), pp. 31-9. doi: 10.1016/j.blre.2012.12.002.
- Zech, D., Rana, S., Buchler, M. W. and Zoller, M. (2012) 'Tumor-exosomes and leukocyte activation: an ambivalent crosstalk', *Cell Commun Signal*, 10(1), pp. 37. doi: 10.1186/1478-811X-10-37.
- Zhou, W., Fong, M. Y., Min, Y., Somlo, G., Liu, L., Palomares, M. R., Yu, Y., Chow, A., O'Connor, S. T., Chin, A. R., Yen, Y., Wang, Y., Marcusson, E. G., Chu, P., Wu, J., Wu, X., Li, A. X., Li, Z., Gao, H., Ren, X., Boldin, M. P., Lin, P. C. and Wang, S. E. (2014) 'Cancer-secreted miR-105 destroys vascular endothelial barriers to promote metastasis', *Cancer Cell*, 25(4), pp. 501-15. doi: 10.1016/j.ccr.2014.03.007.
- Zomer, A., Maynard, C., Verweij, F. J., Kamermans, A., Schafer, R., Beerling, E., Schiffelers, R. M., de Wit, E., Berenguer, J., Ellenbroek, S. I. J., Wurdinger, T., Pegtel, D. M. and van Rheenen, J. (2015) 'In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior', *Cell*, 161(5), pp. 1046-1057. doi: 10.1016/j.cell.2015.04.042.
- Zomer, A., Steenbeek, S. C., Maynard, C. and van Rheenen, J. (2016) 'Studying extracellular vesicle transfer by a Cre-loxP method', *Nat Protoc*, 11(1), pp. 87-101. doi: 10.1038/nprot.2015.138.