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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications referred to by the Roman numerals I–III.

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II. Kuldkepp, A.; Karakai, M.; Toomsoo, E.; **Reinsalu, O.**; Kurg, R. Cancer-Testis Antigens MAGEA Proteins Are Incorporated into Extracellular Vesicles Released by Cells. *Oncotarget* **2019**, *10*, 3694–3708, https://doi.org/10.18632/oncotarget.26979.

III. **Reinsalu, O.**; Samel, A.; Niemeister, E.; Kurg, R. MAGEA4 Coated Extracellular Vesicles Are Stable and Can Be Assembled In Vitro. *Int. J. Mol. Sci.* **2021**, *22*, 5208, https://doi.org/10.3390/ijms22105208.

The author's contribution to each article is as follows:

I. Carried out most of the experiments and analysed the data.

II. Participated in MAGE-A4-related extracellular vesicle isolation and characterisation experiments and analysed the data.

III. Participated in experimental design, carried out all the experiments, analysed the data, prepared the figures and wrote the manuscript.

LIST OF ABBREVIATIONS

2K EVs	Extracellular vesicles sedimented by centrifugation at 2000 × g
16K EVs	Extracellular vesicles sedimented by centrifugation at 16 000 × g
120K EVs	Extracellular vesicles sedimented by centrifugation at 120 000 × g
AB	Apoptotic bodies
ARF6	ADP-ribosylation factor 6
BAGE	B melanoma antigen family
CTA	Cancer-testis antigen
CT-X	Cancer testis antigens with genes in the X chromosome
DLS	Dynamic light scattering
ERK	Extracellular signal-regulated kinase
ESCRT	Endosomal sorting complex required for transport
EV	Extracellular vesicle
GAGE	G antigen 1 family
IDP	Intrinsically disordered protein
ILV	Intraluminal vesicle
LAGE	L antigen family
MAGE	Melanoma antigen gene family
MAGE-A4-EV	MAGE-A4 carrying extracellular vesicle
MHD	MAGE homology domain
MLCK	Myosin light chain kinase
MLV	Moloney leukemia virus
MV	Microvesicle
MVB	Multivesicular body
MYLP	Myosin light chain phosphatase
Non-X CT	Cancer-testis antigens with genes in the autosomes
NTA	Nanoparticle tracking analysis
NY-ESO-1	New York oesophageal squamous cell carcinoma 1
PCNA	Proliferating cell nuclear antigen
PDI	Polydispersity index
PLD	Phospholipase D
PS	Phosphatidylserine
RING	Really Interesting New Gene; an E3 ubiquitin ligase
RLP	Retroviral-like particle
SEC	Size-exclusion chromatography
SSX	Synovial sarcoma, X breakpoint family
SSXRD	SSX repression domain
TEM	Transmission electron microscopy

TPTE	Transmembrane phosphatase with tensin homology
VLP	Virus-like particle
WASH	Wiskott-Aldrich syndrome protein and scar homolog
WH	Winged-helix motif

INTRODUCTION

Cancer is currently one of the deadliest diseases. According to the International Agency for Research on Cancer, there were approximately 19.3 million new cases of cancer and 10 million deaths in 2020 worldwide [1]. Thus, cancer has been considered one of the highest priorities among medical and scientific efforts. Nevertheless, standard cancer treatments still impose risks, as side effects may be severe. In addition to surgery, typical treatments include chemotherapy and radiation therapy, which may damage normal tissue as well as the tumour itself. More precise approaches have been developed and are already being applied, including targeted therapy and immunotherapy. However, these therapies, especially contemporary immunotherapeutic approaches, are not administered as frequently, as these treatments often presume that the specific conditions and characteristics of cancer are met. The essential prerequisite is the presence of biomarkers unique to cancer, for example, nucleic acids, proteins, or metabolomic profiles. Therefore, the identification and detection of biomarkers for more effective and safer anticancer therapies and cancer diagnostics are vital.

Cancer-testis antigens (CTAs) are considered good candidates for cancer theranostics. These proteins have a highly tumour-specific expression profile, as they are mainly normally expressed in testis and aberrantly expressed in numerous cancer types, and induce anticancer immune responses. CTAs have been shown to contribute to tumorigenic processes, suggesting that they are potential targets for novel anticancer therapeutic technologies.

Extracellular vesicles (EVs) are a diverse group of nanosized particles released by all cells into the surrounding extracellular space. EVs are present in all body fluids, such as blood, urine, and saliva. Limited by a phospholipid bilayer, EVs contain different types of bioactive cargo acquired from the parental cells. With this cargo, EVs function as messengers in intercellular communication when they are delivered from one cell to another and are involved in most physiological and pathological processes in the human body, including cancer. EVs have been shown to play an important role in cancer development but also anticancer immunity induction and have a high potential for being vessels of anticancer therapeutics and noninvasive diagnosis.

CTAs and EVs, two different cancer research subfields, have received close attention from the scientific community for a few decades. Interestingly, only a few crossroads between the two areas have been reported in the scientific literature.

MAGE-A4 is a CTA that has been shown to have tumorigenic and antitumor properties. Its expression in different cancer tissues is correlated with adverse patient outcomes and advanced cancer development. Here, MAGE-A4 is incorporated into EVs by the cells that express it. The knowledge acquired might support cancer research aiming to determine its biological context and provide possibilities for noninvasive cancer diagnostics, prognostics and therapeutic approaches.

1. LITERATURE REVIEW

1.1 Cancer-testis antigens

Approximately three decades ago, a set of three genes was identified as being expressed only in different tumour cell lines and was shown to be part of gene families called melanoma antigen gene (MAGE) [2], B melanoma antigen (BAGE) [3] and G antigen 1 (GAGE) [4]. Discoveries of other such gene families, e.g., Synovial sarcoma, X breakpoint (SSX) [5], and L Antigen Family (LAGE), also known as New York Oesophageal Squamous Cell Carcinoma 1 (NY-ESO-1) [6], followed soon afterwards. These gene families have a common trait of being normally expressed in germline tissues, mostly in testis, and aberrantly in different types of tumour tissues [7–9]. Additionally, the proteins expressed by the genes often elicit an immunogenic response in the form of T-cell cytotoxicity or antibody recognition [2,6]. Based on these features, the group of proteins was named cancer-testis antigens (CTAs). Currently, more than 200 genes in approximately 100 gene families belonging to the CTA group have been identified [10]. CTAs immediately received attention as potential targets for anticancer therapies and diagnostics due to their strictly restricted expression pattern, often in immune-privileged sites such as the testis and placenta [11,12], and relatively high immunogenicity.

1.1.1 Characteristics of CTA genes

CTAs are divided into two large subgroups, CT-X and non-X CT antigens, depending on their localization on the X-chromosome [13]. CT-X antigens are mainly grouped into gene clusters with direct and reverse repeats of different copy numbers [14,15]. CTAs on autosomal chromosomes are typically single-copy genes [13,16]. Based on an in silico analysis of the members of the human CTA group, the majority of the genes are evolutionarily novel and are present only in humans [17]. The superfamilies of the CTA group commonly exhibit high intra- and interspecies homologies between their members. For example, human SSX family genes have conserved exon–intron structures, and the similarity of protein and cDNA sequences are 73–92% and 87–96%, respectively [18]. The identity of SSX orthologues between human and mouse genes has been shown to be up to 35% [19].

1.1.2 Characteristics of CTA proteins

The proteins encoded by the CTA genes form a highly heterogeneous group. Some CTA protein superfamilies have highly conserved domains that are unique to the family, such as the MAGE homology domain (MHD) in MAGE or SSX repression domain (SSXRD) in the SSX family [18,20]. Some of the other CTAs

share known functional domains, e.g., the KRAB zinc-finger domain or nuclear receptor domains AF-1, LBD and DBD [21,22]. Nevertheless, many CTAs do not have a protein classification based on protein sequence homology. According to a bioinformatics study on CTA protein structures, at least 90% of CTAs may be internally disordered proteins (IDPs) [23]. As IDPs, these proteins may lack rigid 3D structures either along their entire length or in localized regions under physiological conditions but still perform important biological tasks [24]. Structural flexibility and plasticity might enable them to interact with a much broader range of binding partners than regular proteins [25].

1.1.3 CTA functions in normal and cancerous tissues

The general knowledge of the biological functions of CTAs in normal tissues is relatively low. As they are primarily expressed in the testis, they have an important role in the development of germ cells and fertility. Most X-CTs are expressed during an earlier stage of spermatogenesis, in which they play various essential roles. For example, X-CTs are involved in meiotic recombination [26,27], RNA regulation [28] or cancelling imprinting in the final round of mitosis [29]. Other CTAs, characteristically non-X CTs, are expressed during later stages of gametogenesis and in fully developed sperm, where they may have different functions, e.g., regulating sperm metabolism and motility [30,31].

Numerous reports describe different cancer supportive functions for CTAs, and they are therefore implicated as hallmarks of cancer. Some CTAs sustain proliferative signalling, resist cell death, or support migration and invasion [32–35]. Others may promote angiogenesis, regulate tumour metabolism or induce epigenetic modifications [36–39]. Researchers have proposed that CTAs may cause genomic instability by inducing DNA damage [40] or interfere with homologous recombination in somatic cells if misexpressed [41].

1.1.4 Expression of CTAs in normal and tumour tissues

Although CTAs are normally expressed at high levels in the testis, some other tissues may also express specific CTAs, although often at less than 1% of the expression level in the testis [42]. Most notably, CTAs have been detected in trophoblasts, female gametes, and the brain [13,43–45]. CTAs have been sorted into subgroups according to their distinct expression patterns: testis-restricted, testis-selective and testis/brain-restricted CTAs. All of the CTA subgroups are expressed in testes and different cancers, but testis-restricted CTAs are also expressed in the placenta, testis-selective CTAs are expressed in some other normal tissues and testis/brain-restricted CTAs are expressed in the brain [10]. Some testis-restricted CTAs are also expressed in embryonic and mesenchymal stem cells, embryonic germ and somatic cells [46–48]. With multiple gene superfamilies, not all members of the gene subfamilies in the CTA group follow the

classical CTA criteria, as some are ubiquitously expressed, e.g., members of the MAGE-D family, BORIS or STAG1.

The strictly regulated expression profiles of CTAs mainly result from epigenetic regulation in the form of DNA methylation [49–51]. Thus, individual CTA expression profiles differ due to different promoters and epigenetic modification states. Additionally, transcription factors that demethylate and activate promoters, histone modifications, cytokines and ncRNAs may also have important roles in regulating CTA expression [42,52–55].

Under pathological conditions, CTAs exhibit high expression in melanoma, hepatocellular, lung, and ovarian cancer, moderate expression in breast and prostate cancer, and low expression in colon, renal, and pancreatic cancer [56,57]. Not all CTAs are expressed in any particular cancer tissue. Typically, only up to two CTAs, if any, are simultaneously present in different tumour tissues, as reported by a systematic analysis [58]. BAGE, for example, has different expression frequencies in various tumours: 30% in infiltrating bladder carcinomas, 22% in melanomas, 10% in mammary carcinomas, 8% in head and neck squamous cell carcinomas, and 6% in non-small cell lung cancer [3]. For example, some CTAs, including MAGE and NY-ESO-1, have been shown to be more frequently expressed at a higher level in cancers of advanced grades and later stages with metastasis [59–61]. Research on the prognostic value of detected CTAs in cancer shows that it may depend on the specific characteristics of the disease, e.g., the type of CTAs expressed, the type of tumour and the grade of cancerogenic development. Reports have described CTAs as adverse prognostic factors [62–64], having no prognostic value [61,65] or even as indicators of a favourable prognosis [66,67].

1.1.5 Potential role of CTAs in anticancer therapy

In 2009, a ranking of 75 cancer-specific antigens with the highest potential for cancer therapeutics was formed, including several CTAs. MAGE-A3 and NY-ESO1 were the highest-ranking CTAs, reaching the top 10 [68]. Numerous terminated and ongoing clinical trials that are listed in the clinicaltrials.gov database include CTAs in anticancer treatments [69]. According to a review article studying such trials, the three CTAs that received the greatest attention are MAGE-A3, NY-ESO-1 and IL13Rα [70]. Several types of two general immunotherapeutic approaches are being tested: tumour vaccines and T-cell therapies. Tumour vaccines, including single and mixed peptide/protein, viral vector and cell-based vaccines, have produced promising initial results but failed to produce significant efficiency in subsequent phases. For example, single peptide vaccines based on MAGE-A3 or NY-ESO-1 induced specific immune responses, but the subsequent phases of trials enrolling placebo groups showed very little significant therapeutic effect [70]. A multiple peptide vaccine with IL13Rα or a vaccine combining NY-ESO-1 with synthetic oligodeoxynucleotides induced significant immune responses and was well tolerated by the patients [71,72]. Also mRNA based cancer vaccine against NY-ESO-1, MAGE-A3 and transmembrane phosphatase

with tensin homology (TPTE) CTAs showed similar results [73]. The aforementioned peptide vaccines and a lentiviral-based vector vaccine CMB305, which induces NY-ESO-1-specific cellular immune responses, remain the most promising approaches and await further trials [74]. T-cell therapies, including TCR-T-cell and chimeric antigen receptor T-cell therapies, have produced mixed results. Although some of the trials documented significant efficiency and mild side effects [75–77], others reported severe adverse effects, including incidences of death [78,79]. Despite the setbacks, dozens of trials remain to be completed and new approaches await testing; therefore, CTAs are still being extensively studied for anticancer therapeutics [70]. Furthermore, CTAs still maintain their high potential for tumour diagnostic and prognostic procedures.

1.2 The MAGE superfamily

1.2.1 The genes of the MAGE superfamily

The MAGE genes compose one of the largest superfamilies in the CTA group. More than 40 members in the human MAGE superfamily have been identified, whereas some are pseudogenes [20]. While a large part of the human CTA group is considered evolutionarily new, many members of the MAGE family have homologues that are present in all eukaryotes [80]. MAGE genes are divided into two large groups, type I and II (Table 1), based on the tissue expression profiles, chromosomal location and sequence homology [80].

Type I MAGE genes are considered classical CTAs, indicating that these genes are expressed primarily in the testis and aberrantly in tumour tissues and encode antigens targeted by cytotoxic T-lymphocytes [20,81]. The type I subgroup consists of subfamilies called MAGE-A, -B and -C. This subtype is evolutionarily newer than type II MAGEs and has expanded and developed rapidly during placental mammalian advancement [82]. The MAGE-C subfamily is only detected in primate species, including humans. The genes of type I MAGEs are restricted to clusters on the X chromosome. With specified expression profiles, type I genes are further categorized into three subgroups: Ia genes are expressed only in testis, Ib genes are expressed in testis and placenta, and Ic genes are expressed mainly in testis and additionally in a broader range of organs, including bladder, brain, spleen, small intestine, skeletal muscle, heart, and oesophagus [81]. During embryonic development, the expression of type I MAGEs is not restricted only to male germlines, as they are also expressed in premeiotic germ cells and the foetal ovary [83,84].

Type II MAGEs, which include subfamilies MAGE-D, MAGE-E, MAGE-F, MAGE-G, MAGE-H, MAGE-L and Necdin, are ubiquitously expressed in adult and embryonic tissues and are usually not associated with cancers [20,85,86]. Members of this subtype are more phylogenetically conserved, whereas some homologues are even found in Saccharomyces cerevisiae [80,87]. Some of the subtype II gene members have been clustered to the X chromosome, while others

are encoded on a few autosomes [20]. The general expression level of MAGE type II genes is higher than that of type I MAGEs. Type II MAGEs have additionally been divided into two subgroups based on the expression profile: type IIa genes are uniformly and highly expressed in most tissues, and type IIb MAGEs have an enriched expression profile in the brain [81].

Table 1. The classification of the members of the human MAGE superfamily based on their expression profile. The data of the MAGEs with known expression profiles were reported by Florke et al. [88].

	Type I MAGEs			**Type II MAGEs**	
Subfamilies	A, B and C			D, E, F, G, H, L and Necdin	
Gene expression	Mostly in testis and cancers			Ubiquitous	
Subtypes	Ia	Ib	Ic	IIa	IIb
Expression profile	Testis-restricted	Testis, placenta, ovary	Not restricted	Ubiquitous	Brain-enriched
Genes with known expression profiles	A1, B1, B3, B4, B5, B6, B10, B16, B18, C2	A8, A10, A11, B2	A2, A3, A4, A5, A6, A9, A12, B17, C1, C3	D1, D2, F1,G1, H1, Necdin	D3, D4, E1, E2, L2

1.2.2 Murine *Mage* genes are analogues of MAGE

The MAGE genes have also been extensively researched in mice and display striking similarities with human genes. The number of *Mage* genes in mice is similar to the number of human MAGE genes and have also been divided into type I and type II and the corresponding subgroups using the same criteria [89]. In contrast, the mouse genes are grouped differently into a slightly higher number of subfamilies, e.g., *Mage-b3* or *Mage-g2*, and chromosomal clusters [88]. Due to rapid phylogenetic development, the type I genes exhibit greater differences between their human and mouse counterparts, with gene sequence similarities ranging from approximately 40–60% [89]. Most evidently, no orthologues of the MAGE-C subfamily have been detected in mice and the *Mage-a-like* subfamily has not been identified in humans [81]. Type II MAGEs, on the other hand, may share homology of even up to 90% between human MAGE-D and murine *Mage-d* subfamily members [80]. The genomic structure, including chromosomal localization and the exon number of the genes, is comparable between the two species, with the differences that the type II *Mage* genes and even one of the type I genes are localized to a greater number of autosomes [80]. Interestingly, the protein N- and C-terminal sequences flanking the functional domain are more similar

between murine and human orthologues than paralogues of the genes in the same species [20]. In conclusion, murine *Mage* genes represent suitable analogues for use in cell culture or in vivo studies of MAGE function.

1.2.3 The MAGE homology domain

As mentioned in a previous section, MAGE superfamily proteins have a defining domain that they all share – the MHD. The MHD consists of 165–171 amino acid residues and is, on average, conserved by approximately 46% of all human MAGEs [85], but the identity of the MHD within MAGE subfamilies is even higher. For example, MAGE-D and MAGE-A subfamily members have 75 and 70% conserved MHD residues, respectively [90]. Typically, every protein has a single MHD; however, a few exceptions have duplicated or truncated versions of the domain [20,85]. The N- and C-terminal regions of the MAGE proteins flanking the MHD are poorly conserved, whereas the domain is typically positioned closer to the C-terminus [85]. The MHD comprises two tandem winged-helix (WH) motifs, termed WH-A and WH-B [90]. Analyses of the crystal structures of MAGEs and their interacting partners confirmed that MHD is at the centre of the functional region of MAGE proteins that displays binding capabilities, and a dileucine motif in WH-B has an essential role in the interactions of MAGE proteins [90,91]. As predicted for CTAs to be IDPs, the MHD was shown to form compact folded structures and disordered regions with a broad charge state distribution [92]. Thus, MAGE proteins have a versatile and flexible MHD domain that may enable them to bind to an interacting partner in different ways and allow them to interact with a relatively large number of partners.

1.2.4 MAGEs interact with E3 RING ubiquitin ligases

Although the exact biological functions of many MAGE proteins remain elusive, among putative interacting partners, E3 Really Interesting New Gene (RING) ubiquitin ligases have been claimed to interact with MAGE proteins most frequently. More than 50 E3 RING ligase interactions with MAGEs, an assembly called MAGE-RING ligase, have been reported [93]. MAGEs of both subtypes typically interact with one RING ligase, but MAGEs with higher homology might interact the same RING ligase [90]. The analysis of MAGE-RING ligases revealed that while MAGEs interact with their cognate RING ligase through the MHD, the region of the RING protein to which MAGEs bind varies in different assemblies [90]. MAGEs have been shown to regulate their cognate RING proteins within the assemblies by enhancing ligase activity, specifying novel substrates for ubiquitination, and altering the subcellular location [93]. For example, MAGE-A2, MAGE-A3, MAGE-A6, and MAGE-C2 directly bind and regulate TRIM28 (also known as KAP1), an E3 RING ubiquitin ligase with important roles in transcriptional regulation, cellular differentiation, and DNA damage repair [90,94]. These

MAGEs stimulate both TRIM28 autoubiquitination and ubiquitination of its substrates, the p53 tumour suppressor and ZNF382, resulting in proteasome-dependent degradation of the substrates [95,96]. In another example, MAGE-L2, a type II MAGE protein, forms a MAGE-RING ligase complex with TRIM27 and increases the ubiquitination of a subunit from the retromer protein complex [97]. Ubiquitination allows the retromer to recruit and activate the Wiskott-Aldrich syndrome protein and scar homologue (WASH) complex, enabling WASH-mediated endosomal protein recycling.

1.2.5 The MAGE-A subfamily

MAGE-A is a type I MAGE subfamily that includes 12 genes named MAGE-A1 to MAGE-A12 [98]. This subfamily of genes is clustered to q28 of the X chromosome and encodes proteins with 43 to 96% sequence identity, while MAGE-A3 and A6 display the highest similarity [99,100]. MAGE-A1 is the only member of the MAGE-A subfamily that belongs to the MAGE Ia subgroup, while MAGE-A8, MAGE-A10 and MAGE-A11 are part of the Ib subgroup, and MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, and MAGE-A9 are type Ic genes (Table 1) [88]. MAGE-A7 is considered a pseudogene [101].

MAGE-A proteins have been shown to increase proliferation, support anchorage, and increase the ability of cells to resist stressors such as genotoxins, starvation, and metabolic stress, which are not only necessary to maintain continuous spermatogenesis but are also very beneficial for cancer cells [81,99]. MAGE-A genes are expressed in various human cancers, including lung cancer, breast cancer, oral squamous cell carcinoma, oesophageal carcinoma, and urothelial and haematopoietic malignancies [59,102–107]. Aberrant MAGE-A expression is considered a hallmark of cancer, as different MAGE-A proteins have been shown to regulate key cancer-related pathways by targeting proteins such as SKIP, the p53 tumour suppressor, Mdm2, PML-IV, E2F1 and AMPK [94,108–112]. In several reports evaluating the prognostic value of MAGE-A proteins in different cancers, the antigens are considered indicators of a higher tumour grade, poorer patient outcome and decreased survival rates [113–116].

1.2.6 MAGE-A4

The MAGE-A4 gene encodes a 317 amino acid protein, with the MHD positioned close to the C-terminus (Figure 1) [117]. Although it is considered a type Ic MAGE, the strongest evidence suggests that it is expressed in the testis, placenta and foetal ovary, with the highest expression in primary spermatocytes, spermatogonia and embryonic gonocytes [83,84,118]. Its expression pattern in other tissues, not including various cancers, requires additional confirmation. In endogenously expressing cells, MAGE-A4 is localized either in the cytoplasm and nucleus or both, while exogenously expressed MAGE-A4 has only been detected in the cytoplasm [84,119–121].

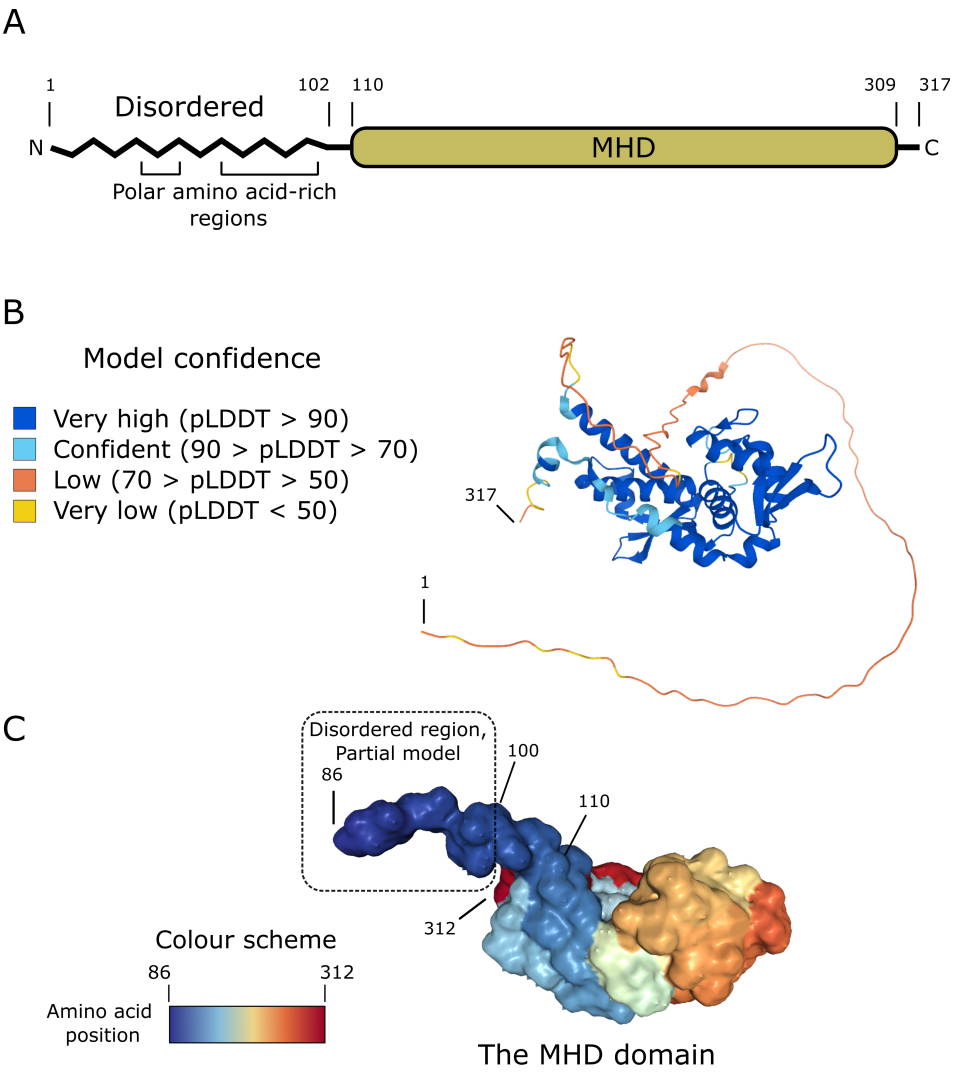

Figure 1. The MHD and the structure of MAGE-A4. A. The regions of the amino acid sequence of MAGE-A4 [117]. **B.** The 3D structure of MAGE-A4 predicted by the AlphaFold algorithm. The image was adapted from the AlphaFold Protein Structure Database [130]. AlphaFold produces a per-residue confidence score (pLDDT) ranging from 0 to 100. **C.** The crystal structure of the MHD domain of MAGE-A4. The image was adapted from the Protein Data Bank of Research Collaboratory for Structural Bioinformatics [131,132]. The amino acid sequence at positions 86–99 is a partial model of the disordered region of the MAGE-A4 protein. The rest of the disordered region was unable to be crystallized.

Similar to fellow members of the MAGE-A subfamily, MAGE-A4 is expressed in a range of cancer types and is often an indicator of a poor prognosis [66,122–127]. According to a study, if MAGE-A4 is expressed only in the cytoplasm or nucleus of tumour cells, the patient has a poorer prognosis. Interestingly, MAGE-A4 was not associated with any prognostic outcome if concurrently expressed in both subcellular regions. Additionally, coexpression with p53 indicates a better patient prognosis than patients with p53 nonexpressing tumours [120].

MAGE-A4 has been shown to promote cell growth and inhibit apoptosis by inhibiting G1 growth arrest and reducing the response of p53 targets [128]. However, the exact proteins with which MAGE-A4 may interact in cellular pathways are relatively poorly understood. At least 20 putative interacting partners have been proposed, but most need additional validation to confirm the interaction, as only two-hybrid arrays were used to detect them [117]. Since MAGE-A4 has been confirmed as an IDP [91], none of those proposed partners could be automatically excluded. Nonetheless, a few interactions have been validated.

Consistent with the finding that MAGEs interact with E3 RING ubiquitin ligases and promote resistance to environmental stressors, MAGE-A4 has been shown to contribute to translesion synthesis pathway activation, DNA damage tolerance and genome maintenance in cancer cells. More precisely, MAGE-A4 forms a MAGE-RING ligase complex with RAD18 and stabilizes it, subsequently promoting the ubiquitination of proliferating cell nuclear antigen (PCNA), the DNA polymerase processivity factor, and enabling it to activate translesion synthesis polymerases [121]. These polymerases overcome DNA replication fork stalls and continue DNA replication in the presence of DNA lesions, including some polymerases that function in an error-prone manner [129].

On the other hand, in conjunction with the immunogenic effects of MAGE-A-derived peptides, MAGE-A4 has been suggested to function as a tumour suppressor protein rather than an oncoprotein [133]. For example, a recombinant 107 amino acid C-terminal fragment of MAGE-A4 was found to induce apoptosis in vitro through p53-dependent and p53-independent pathways [134]. The C-terminal fragment is a cleaved form of MAGE-A4 that has been shown to be the product of the activated proteasome [135]. Furthermore, this fragment of MAGE-A4 inhibits p21-mediated cell cycle arrest by blocking the binding of the transcription factor Miz-1 to the p21 promoter. This property was not observed for MAGE-A4 [134]. Additionally, by also binding to the known oncoprotein gankyrin, cleaved MAGE-A4 inhibits its carcinogenic effect [119].

1.3 Extracellular vesicles

In 1946, during a blood coagulation study, researchers noted that cell-free sedimentation of blood plasma still maintains coagulation ability [136]. Approximately 20 years later, the sediment was shown to mainly contain phospholipid-rich material and was considered a residue of platelets. Hence, the material was termed “platelet dust” [137]. Nevertheless, starting in the 1980s, the scientific

community began to grasp the true nature of these microparticles, leading to the rapid increase in related research for approximately a decade [138–141]. Currently, the particles are called extracellular vesicles (EVs), which are phospholipid bilayer-limited particles released by cells. The production of EVs as a feature is evolutionally conserved in all living cells, including prokaryotic and eukaryotic cells. Virtually every cell of multicellular and unicellular organisms releases EVs to their surrounding extracellular space [142,143]. Therefore, in the human body, EVs is present in all body fluids, e.g., blood, urine, saliva, tears, synovial fluid, and cerebrospinal fluid. One of the most important biological roles of EVs is to facilitate intercellular communication by delivering bioactive cargo, such as nucleic acids, proteins and lipids, from one cell to another [144]. With such diverse roles in cellular maintenance and homeostasis, EVs are essentially involved in most physiological processes and pathological conditions, including cancer [144–149].

1.3.1 Subtypes of extracellular vesicles

Traditionally, extracellular vesicles are grouped into three main subtypes (Figure 2) that are mainly distinguished by their biogenesis and size but also by release pathways, content, and function [144,150]. The subtype comprising the largest vesicles is called apoptotic bodies (AB), which are mostly characterized to have a diameter of 1–5 μm and result from apoptosis, a form of programmed cell death [151]. Microvesicles (MV), also known as ectosomes or shedding vesicles, are derived from direct budding and fission of the plasma membrane and are smaller particles ranging from 100–1000 nm [152]. The smallest of the three subtypes are exosomes with a diameter of 50–150 nm that are produced by the endosomal pathway and released to the extracellular space through exocytosis [153]. Alternatively, EVs of the three subtypes are classified into other types based on tissue of origin or other specifications, e.g., prostasomes – exosomes or microvesicles originating from the prostate [140]. Due to high heterogeneity of EVs and overlapping characteristics of different subtypes, the isolation of particular subtypes from others is very challenging. In EV-related studies, the EVs being investigated should be validated and used to describe a particular subtype very cautiously, and the vesicles are generally recommended to be designated with the umbrella term “extracellular vesicles” instead [154].

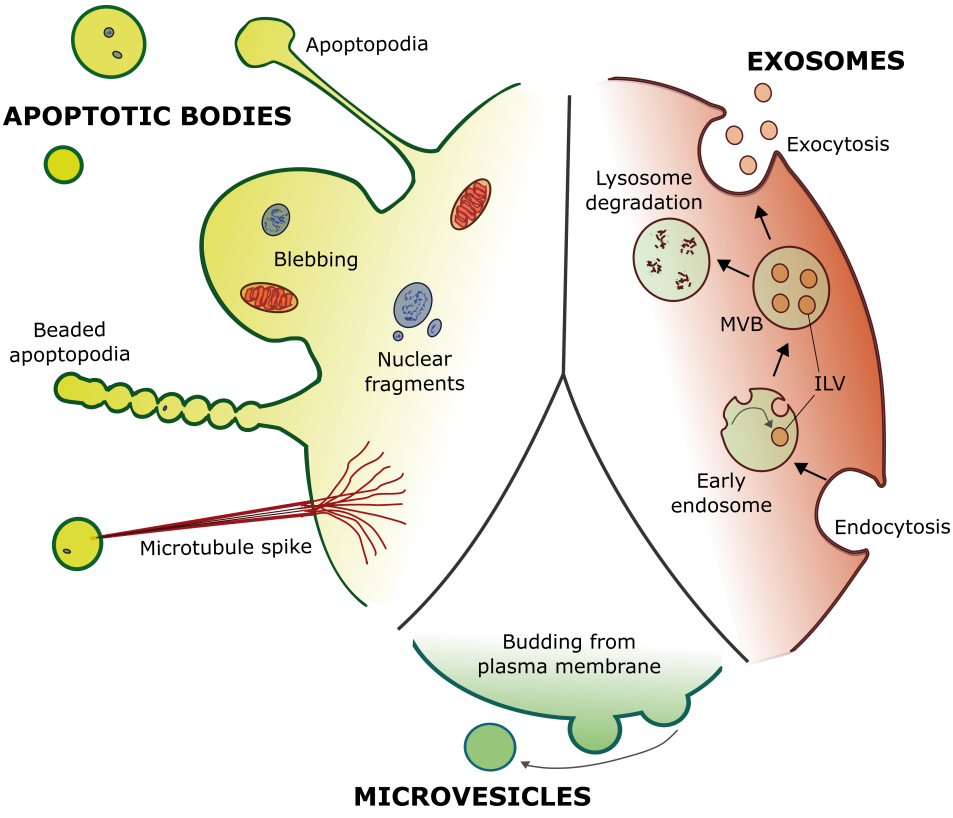

Figure 2. The formation of the three traditional EV subtypes through distinct cellular pathways. ILV – intraluminal vesicle; MVB – multivesicular body.

1.3.1.1 Apoptotic bodies

The majority of cells undergoing apoptosis are fragmented by blebbing into ABs, which are then eliminated by phagocytes [155]. Initially, apoptotic cells undergo an apoptotic volume reduction and then start to be disassembled into ABs [156,157]. Disassembly may occur by division of cells into several larger ABs or many smaller ABs through unique structures that appear during apoptosis: apoptopodia, beaded apoptopodia and microtubule spikes [155,158–160]. The formation of ABs is a complex process with several steps that include apoptotic membrane blebbing, protrusion formation, and fragmentation [159,161]. The blebbing of the plasma membrane is regulated by caspases, which induce the delamination of the membrane at specific locations [162]. Delamination occurs because of the retraction of the actin-myosin II cortex, followed by membrane blister formation and expansion due to the increased hydrostatic pressure produced by actomyosin-mediated cellular contraction [163]. The size of the blebs increases until they form ABs, separating them from the cell body [163,164]. Once ABs form, they contain virtually any components from the originating cell, including nucleic acids, proteins, chromatin or even entire organelles [165].

The defining function of ABs is considered the clearance of apoptotic cells [166]. ABs withhold so-called “find-me” and “eat-me” signals that first attract phagocytes to the scene of apoptosis and, second, initiate the engulfment process of the ABs [167–169]. Phosphatidylserine (PS), the apoptosis-related cell cycle signalling compound in the membrane, is a key recognition element for phagocytes to identify apoptotic targets and start efferocytosis – the ingestion of cellular corpses [170]. Despite the richness of AB contents, PS is considered the best marker for the identification of ABs in EV studies, although it is not unique to the subtype [155]. Specific identification could be achieved by staining the ABs with Annexin V, which indicates the presence of PS [158].

In addition to signalling phagocytes for cellular clearance, ABs, as carriers of a variety of bioactive cargo, may regulate many different processes at the cellular level when they are taken up by recipient cells [155]. For example, ABs may contain proteins implicated in many immune pathways, including Annexin A6, Heat shock protein beta-6, and LDL receptor-related proteins, and therefore are involved in immune regulation [171,172]. ABs might introduce autoantigens to professional phagocytes [173]. On the other hand, ABs might support the initiation of carcinogenesis if ABs transferred oncogenes from cancer cells to healthy recipient cells and the genes were integrated into the genome [174]. ABs have also been shown to distribute infectious agents of viruses [175,176].

1.3.1.2 Microvesicles

The detailed processes of MV formation are still relatively poorly understood. The budding of the plasma membrane is initiated by membrane and cytoskeletal remodelling. The key factors are the rearrangement of asymmetrically distributed amino phospholipids, including PS, and the contraction of the actin-myosin machinery [177,178]. ADP-ribosylation factor 6 (ARF6) initiates a cascade that activates phospholipase D (PLD), followed by recruitment of extracellular signal-regulated kinase (ERK) to the plasma membrane. ERK activates myosin light chain kinase (MLCK), which subsequently activates myosin light chain and triggers the release of MVs [177]. Additionally, Rho-associated kinase, an apoptosis-related pathway factor, inhibits myosin light chain phosphatase (MYLP) activity and subsequently enhances myosin activation [179]. The remodelling of the membrane and cytoskeleton might also result from an increase in the calcium concentration in the cytoplasm [178]. The higher level of calcium ions activates the calcium-dependent enzymes scramblase and floppase, which relocate PS to the outer leaflet of the membrane [180]. Additionally, translocase activity is inhibited, which prevents the translocation of PS and phosphatidylethanolamine back to the inner side of the membrane [178]. Calpine, a calcium-dependent enzyme, cleaves actin-capping proteins, which induces cytoskeletal disorganization and MV release [178].

The released MVs may contain a wide variety of molecules. Commonly, cytosolic and plasma membrane-associated proteins, including tetraspanins, cyto-

skeletal proteins, heat shock proteins, integrins and proteins containing post-translational modifications, have been detected in MVs [152,181]. Due to the mode of formation, MVs may be enriched with PS and phosphatidylethanolamine and contain other MV biogenesis-related compounds [178,182]. In contrast to ABs, MVs have not been assigned any major functions. Instead, as mediators of intercellular communication and cellular maintenance, MVs are involved in countless evident and putative processes [144,149,183].

1.3.1.3 Exosomes

The biogenesis pathway of exosomes is more complex than that of other EVs. First, vesicles are formed by inward budding into early endosomes, where these vesicles are called intraluminal vesicles (ILVs). The endosome, which acquires many ILVs during the process, is called a multivesicular body (MVB) [184]. The biological function of endosomes, including MVBs, is to facilitate numerous endocytic and trafficking functions, including protein sorting, recycling, transport, storage, and release [185]. Two outcomes for MVBs have been defined: one is to be assigned to the lysosomal pathway and be degraded with all its contents, and the other is to fuse with the plasma membrane and release its contents to the extracellular space [186]. The factors that determine the fate of the MVB contents to be lysed or released are still not fully understood. Evidence has suggested that the cholesterol composition is involved, cholesterol-depleted MVB are designated for degradation [187,188]. If an MVB is destined for release, also known as exocytosis, the excreted ILVs are thereafter called exosomes.

The formation of ILVs involves four protein complexes called the endosomal sorting complex required for transport (ESCRT), consisting of approximately 20 proteins [189]. The ESCRT machinery acts in a stepwise manner, where ESCRT-0 and ESCRT-I subunits cluster ubiquitylated transmembrane cargoes on micro-domains of the limiting membrane of MVBs and recruit, via ESCRT-II or an ESCRT accessory protein ALIX, the ESCRT-III subcomplexes that perform budding and fission of this microdomain [189–191]. The formation of ILVs also occurs in an ESCRT-independent manner, where ceramide and tetraspanins have been shown to have important roles [192,193]. Ceramide promotes the generation of microdomains on the membrane of MVBs that induce spontaneous curvature, or it activates G_i-protein-coupled sphingosine 1-phosphate receptor that appears essential for cargo sorting into exosomal ILVs [192,194]. Tetraspanins such as CD63, CD81, CD82 and CD9 have been shown to be involved in regulating the endosomal sorting process and cargo sorting into exosomes [193,195,196]. The balance of the pathways, and therefore the compositional repertoire of the exo-somes, depends on the cell type, its physiological and pathological state and the stage of differentiation and cellular maturation [197]. A mature MBV must be transported to the plasma membrane and primed for fusion to secrete exosomes. A series of RAB GTPases, including RAB7, RAB27A and RAB27B, are essential for these processes [198–200]. The SNARE proteins and synaptotagmin family

members have been indicated to contribute to the final step of exosome biogenesis – the fusion of the MVB with the plasma membrane and the release of the exosomes to the extracellular space [197,201].

Similar to MVs, the repertoire of processes in which exosomes may be involved is extremely diverse and wide-ranging [144,149,183]. In addition to proteins, nucleic acids and lipids are present in exosomes that are more specific for a particular type of cell with a particular physiological state, some contents may be detected in most exosomes [202]. These are proteins related to exosome biogenesis pathways, including ESCRT complex subunits and accessory proteins such as ALIX, TSG101, HSC70, and HSP90β [203,204]. These proteins are often used as exosome markers in EV studies. Tetraspanins, mostly CD63, CD81 and CD9, are also often used as exosome markers, but these membrane proteins have been identified in other EV subtypes as well [205,206]. The sequestration of cytosolic proteins into exosomes might be explained by cosorting with chaperones related to ESCRT, e.g., heat shock proteins HSC70 and HSP90β [204,207]. The lipid microdomains and lipid rafts that are important for ILV formation might explain the exosomal accumulation of membrane cargos with an affinity for them, such as glycosylphosphatidylinositol (GPI)-anchored proteins [208]. The mechanisms by which nucleic acids are sorted to exosomes remain relatively elusive, but indications of possible regulation have been reported, as some RNAs enriched in exosomes seem to possess shared motifs in their sequence [209,210].

1.3.1.4 Other types of extracellular vesicles

Evidence of special EV types that do not fit into the three traditional groups has been reported. Smaller microvesicle-like EVs derived from apoptotic cells, which have distinct functions from MVs derived from healthy cells, should be considered a different type [211]. Cancer cells, which are nonapoptotic, release large EVs called oncosomes, with diameters ranging from 1–10 μm [212]. Migrasomes are EVs released by migrating cells after the rupture of retraction fibres. The migrasomes can be as large as 3 μm and may include smaller vesicles in their lumen [213,214]. A type of EV that is even smaller than exosomes, called exomeres, has been discovered. These EVs have been shown to have distinct cargo profiles from exosomes, thus commencing to separate functions [215]. Endogenous EVs that share structural properties with retroviruses are called retrovirus-like particles (RLPs) [216]. RLPs are presumed to be the products of genes encoding functional retroviral proteins found in the endogenous retrovirus sequences embedded in the human genome [217,218]. The endogenous RLPs are expressed only due to human genome manipulation by specific cellular stressors, and since the particles lack any viral genetic material needed for infectivity, RLPs do not propagate infectious viruses [219,220].

1.3.2 Virus-like particles

The RLPs mentioned above are a specific type of particle assigned the generic term "virus-like particles" (VLPs), which is a highly valuable tool in biotechnology [221]. By definition, VLPs are supramolecular protein structures that self-assemble and are identical or highly similar to the virion structure of their corresponding native viruses [222]. VLPs typically consist of one or more viral structural proteins and are devoid of infectivity-enabling genetic material [223]. Similar to the diversity of the viruses, VLPs may have different shapes and sizes, e.g., particles of different icosahedral virions vary in diameter between 18–500 nm and may be produced by prokaryotic or eukaryotic cell systems, including yeast, plant, insect and mammalian cells [221,224]. Two main types of VLPs are classified by structure: nonenveloped VLPs and enveloped VLPs. Nonenveloped VLPs do not comprise any host structural parts, while enveloped VLPs are bound by a lipid bilayer that is obtained from the host plasma membrane [221]. VLPs have a well-defined structure, stability, biocompatibility, homogeneity and low toxicity and are conveniently utilized for diverse purposes [225]. VLPs have been loaded with antigens and are highly immunogenic, which has made them well adapted for the development of vaccines against various diseases and pathogens [226]. Additionally, VLPs are considered important tools for gene therapy, immune therapy, drug delivery or even as biomaterials [227,228].

1.3.2.1 Enveloped RLPs are similar to extracellular vesicles

While many enveloped VLPs require several viral proteins to form the complex particle, some VLPs assemble using only one structural protein, including RLPs [221,223,229]. The production of RLPs only requires retroviral group-specific antigen (Gag) to form a viral capsid in the cytosol that spontaneously buds through the plasma membrane and acquires the lipid bilayer of the host as the envelope [230–232]. In addition to the Gag protein and the capsid it forms, RLPs have several properties strikingly similar to smaller EVs. RLPs have a size ranging from approximately 80–100 nm, which is similar to the size range of exosomes [177,233]. RLPs might share cargo sorting and biogenesis pathways with exosomes and MVs, at least to some extent [234–236]. For example, ESCRT complexes are utilized by the retroviral Gag protein to induce budding at the plasma membrane [237]. Due to the highly homogenous particles combined with a robust production scheme and shared properties with EVs, RLPs might be considered an excellent model system for EV research.

1.3.3 Extracellular vesicles facilitate cancer development

EVs reflect the component profile of the parental cells they derived, indicating that EVs released by cancer cells may include components indicative of a cancerous state or bioactive cargo responsible for pro-oncogenic effects [202]. Therefore, the EVs released from tumour tissues promote processes required for cancer

proliferation and dissemination. Many studies have revealed the specific aspects of mechanisms by which EVs contribute to cancer progression, which have been merged into three main areas: modulation of the host immune response favouring immune evasion, reshaping the tumour microenvironment to support tumour growth and progression, and aiding cancer in metastatic dissemination [238]. Cancer-derived EVs regulate the function of lymphoid and myeloid cells to escape from the immune system [239]. For example, EVs induce apoptosis, proliferation and suppress the activation of T-lymphocytes [240–242]; function as decoys by binding to immunoglobulins [243,244]; contribute to the emergence of dysfunctional dendritic cells [245]; or foster immunosuppressive functions of monocytes [246]. In the cancer microenvironment, EVs support cancerous growth by driving fibroblast differentiation into activated fibroblasts or myofibroblasts [247]; promoting angiogenesis by transporting numerous proangiogenic biomolecules, such as vascular endothelial growth factor, matrix metalloproteinases, and microRNAs [248]; or inducing inflammation and fibrosis by damaging and reshaping the extracellular matrix [249]. EVs support cancer metastasis by inducing intravasation, contributing to organotropism and creating metastatic niches [250–252]. The general description of oncogenic processes named here is not conclusive. Nevertheless, not all EVs derived from cancer tissues exert a negative effect on patients. For example, EVs have also been shown to initiate the anticancer immune response by presenting cancer-specific antigens [253]. Regardless of the pro- or anti-oncogenic effect, cancer EVs undoubtedly carry cargos that are reflective of the parental cancerous tissue.

1.3.4 Extracellular vesicles as tools against cancer

The tumour-specific cargo of cancer EVs is not only relevant in terms of biological function but also has high value and potential for cancer theranostics. Tumour tissue biopsy has been the most common method for determining the cancer diagnosis and assessing progression, but it is a highly invasive approach that poses risks for patients with cancer [254]. Moreover, not all tumour tissues are reachable for this procedure, and the scission of cancerous tissue increases the risk of metastasis [255]. These drawbacks may be negated by a liquid biopsy in which samples of body fluids are acquired without disturbing the diseased tissue [256]. The main biological components in liquid biopsy include circulating tumour cells, circulating tumour DNA, tumour-educated platelets and EVs [257]. Compared to the other components, EVs are more stable in biological environments and storage conditions; the enrichment of EVs is relatively less expensive and laborious, and EVs have shown high specificity and sensitivity in cancer biomarker detection [258]. EVs have been shown to be an excellent source for early cancer diagnosis and determining the prognosis, therapeutic efficacy and disease progression, resulting in several successfully completed clinical trials and commercialisations of EV-based technologies [259,260].

EV-related anticancer therapeutic technologies are rapidly developing. EVs are considered excellent vaccines and drug carriers and delivery vehicles because of their stability, low immunogenicity, good biocompatibility, and the ability to pass through biological barriers, and EVs do not induce abnormal differentiation and tumour transformation [261–264]. EVs from B-lymphocytes or dendritic cells have been shown to display major histocompatibility complexes (MHC) on their surface, which have been exploited with cancer biomarker peptides to induce an antitumorigenic immune response [265,266]. Additionally, EVs from natural killer cells have induced antitumour effects [267]. Cancer immunotherapy might be improved by loading EVs with additional drugs. With a water-based lumen and lipid bilayer, EVs have been loaded with hydrophilic and hydrophobic drugs while protecting them from premature decomposition and preventing nonspecific toxicity [268]. EVs have been loaded with different anticancer drugs, including chemotherapeutic drugs, RNAs, proteins and viruses [269]. EVs from different cellular sources, including mesenchymal stem cells, myeloid cells or even tumour cells, exert specific therapeutic effects, such as repairing tissue, eliminating inflammation, regulating immunity, and enhancing tumour targeting and suppression [270–272]. EVs may be combined with inorganic nanocarriers and nanobioconjugates such as liposomes and hydrogels to further increase the therapeutic effect [262]. Numerous clinical trials of EV-based anticancer therapies have been conducted, and several have reported promising results for effectiveness and safety [273,274].

2. AIMS OF THE STUDY

EVs have been shown to be carriers of bioactive cargo from cancer cells, which might facilitate tumour progression or aid in acquiring immune responses against cancer. EV samples obtained through noninvasive liquid biopsy for cancer diagnostics and prognosis pose minimal risks to patients with cancer. Additionally, engineered EVs have a high potential for use as effective and safe anticancer therapeutic technologies. CTAs have a unique expression profile of being mainly expressed in the testis and aberrantly expressed in numerous cancer types. CTAs have been shown to elicit tumour-specific immune responses. This feature suggests that they are promising candidates for cancer therapeutics and diagnostics. We discovered that MAGE-A4, a known CTA, is incorporated into vesicles released by cells in our research project on melanoma-associated antigens and VLPs. Additionally, MAGE-A4 is exposed on the surface of VLPs (Study I).

A hypothesis was proposed that MAGE-A4 is incorporated into naturally occurring EVs and exposed to their surface. The aims of the study are as follows:

1. Characterise VLPs generated by MAGE-A4-expressing cells as a simplified EV model system.

2. Verify the validity of the proposed hypothesis by characterizing naturally occurring EV populations released by MAGE-A4-expressing cells.

3. Investigate the MAGE-A4-carrying EVs to study the association between EVs and MAGE-A4.

3. MATERIALS AND METHODS

Detailed descriptions of the materials and methods used in the experiments related to this dissertation are included in the corresponding publications. Here, a brief description of the materials and methods is provided as an overview.

Moloney murine leukaemia virus (MLV) VLPs and native EVs were isolated from cell culture media of mouse COP5-EBNA fibroblasts (Studies I, II and III) and human U2OS osteosarcoma cells (Study II). COP5-EBNA cells do not express MAGE-A4 endogenously and were transfected by electrophoresis with vectors enabling the coexpression of MLV Gag and MAGE-A4 for VLP generation or only MAGE-A4 for the isolation of natural EVs. The U2OS cells express MAGE-A4 endogenously and do not require any additional manipulation. All EVs or VLPs were isolated at 72 h posttransfection or, in the case of U2OS cells, at 72 h after reaching the appropriate confluence.

For VLP generation (Studies I and III), cellular debris were removed from the cell culture medium by centrifugation at 1000 × *g*, and the supernatant was filtered through 0.45 µm syringe filters. VLPs were isolated from the supernatant by ultracentrifugation at 100 000 × *g* through a 20% sucrose cushion. The pelleted material was resuspended and either analysed directly or ultracentrifuged further through a sucrose density gradient and fractionated into ten fractions with equal volumes, followed by analyses.

EVs were isolated by differential ultracentrifugation from the cell culture medium (Studies II and III). First, cellular debris was removed by centrifugation at 300 × *g*. The supernatant was used for the next centrifugation step at 2000 × *g* to pellet the largest EVs (2K EVs), followed by centrifugation of the remaining supernatant at 16 000 × *g* to pellet the medium-sized EVs (16K EVs). Finally, the supernatant from the previous step was subsequently ultracentrifuged at 120 000 × *g* to pellet the smallest EVs (120K EVs). All EV pellets were resuspended in PBS followed by purification using another centrifugation step at 17 000 × *g* for 2K and 16K EVs or at 120 000 × *g* for 120K EVs through PBS and resuspended in fresh PBS again.

The MAGE-A4-carrying EVs (MAGE-A4-EVs) isolated from MAGE-A4-expressing COP5-EBNA cells were subjected to different physicochemical treatments (Study III). Aliquots of EVs (30 µg each) were incubated at +4 °C or −80 °C for up to 21 days. After every seven days, an aliquot from both treatments was removed for analyses. The freeze–thaw cycle treatment involved subjecting aliquots up to three cycles of 1 h of freezing at −20 °C and 20 min of thawing at room temperature. After each cycle, an aliquot was removed for analyses. The chemical treatment involved suspending the EV aliquots in the following PBS-based solutions: 1 M NaCl, 0.33 M $MgCl_2$, 10 mM EDTA, 10 mM EGTA, 3.5 mM NaOH at pH 11.5, 0.02% Triton X-100 and pure PBS as the control. The samples were incubated for one hour at room temperature on the bench and washed through ultracentrifugation.

The recombinant N-His-tagged MAGE-A4 and MAGE-A4-EGFP proteins were expressed in *Escherichia coli* cells and purified using nickel Sepharose beads (Study III). The proteins were implemented in passive incubation (in vitro binding) experiments with EVs and VLPs, which included mixing 20 µg of the vesicles with 10 µg of the protein solutions and incubating them on the bench for one hour. The suspensions were washed by ultracentrifugation at 120 000 × *g* through PBS or fractionated by size-exclusion chromatography (SEC).

4. RESULTS AND DISCUSSION

4.1 MAGE-A4 is incorporated into VLPs and binds to the outer surface (Studies I and III)

4.1.1 MLV VLPs are homogenous and incorporate MAGE-A4 (Study I)

VLPs are very homogenous, undergo flexible remodelling and can be produced in relatively high quantities [275]. RLPs have striking similarities with naturally occurring EVs, including the structure and formation of the particles. The induction of RLPs is easily initiated by expressing the retroviral Gag protein in mammalian cell lines, and robust isolation protocols are well established [276, 277]. Retroviral-based VLPs are an appealing model system for EV-related research because of these features. Furthermore, VLPs are valued vectors for immunotherapies, and are primarily implemented as vaccines against different diseases.

Our first goal was to produce VLPs from mouse COP5-EBNA fibroblasts that were transiently transfected to express the MLV capsid protein Gag and MAGE-A4 to determine whether the CTA was incorporated into the particles (Figure 3). Cells transfected only with the Gag-expressing vector were used to generate the control sample of VLPs. After isolation from the cell culture medium, the VLP samples were analysed using western blotting and found to be enriched with the Gag protein, indicating the successful formation and release of MLV VLPs. By fractionating the VLP samples further into ten subsequent fractions using density gradient centrifugation, the pelleted VLP samples were purified even further from cellular contaminants that might have cosedimented with the particles. We detected the Gag protein in fractions 2 and higher in both cases, while the peak level was detected in fractions 3–6 (Figure 1C, Study I). MAGE-A4 was also successfully detected, displaying an intense signal with a peak in fractions 5–7, strongly suggesting that MAGE-A4 was indeed incorporated into the VLPs. These results supported the hypothesis that MAGE-A4 was packed into vesicles released from cells, although at this point in artificially induced particles.

The DLS analysis showed that the size of the VLPs in the control sample and the MAGE-A4 VLPs was in the expected size range for VLPs, with Z-average diameters of 143.9 nm and 119.5 nm, respectively (Figure 1D, Study I). Additionally, DLS describes the overall distribution of the particle sizes, which is presented as the polydispersity index (PDI). The PDI for the control and MAGE-A4 samples was 0.14 and 0.18, respectively (Figure 1D, Study I). As a PDI value < 0.05 describes monodisperse particles and a value $> 0{,}7$ indicates highly polydispersed particles [278], both VLPs have a very narrow size distribution and are very homogenous in size. VLPs with these PDIs surpass the nanoparticle dispersity requirement for therapeutic methods [279]. The outcome of the TEM analysis of pooled fractions 4–7 strongly correlates with the DLS results

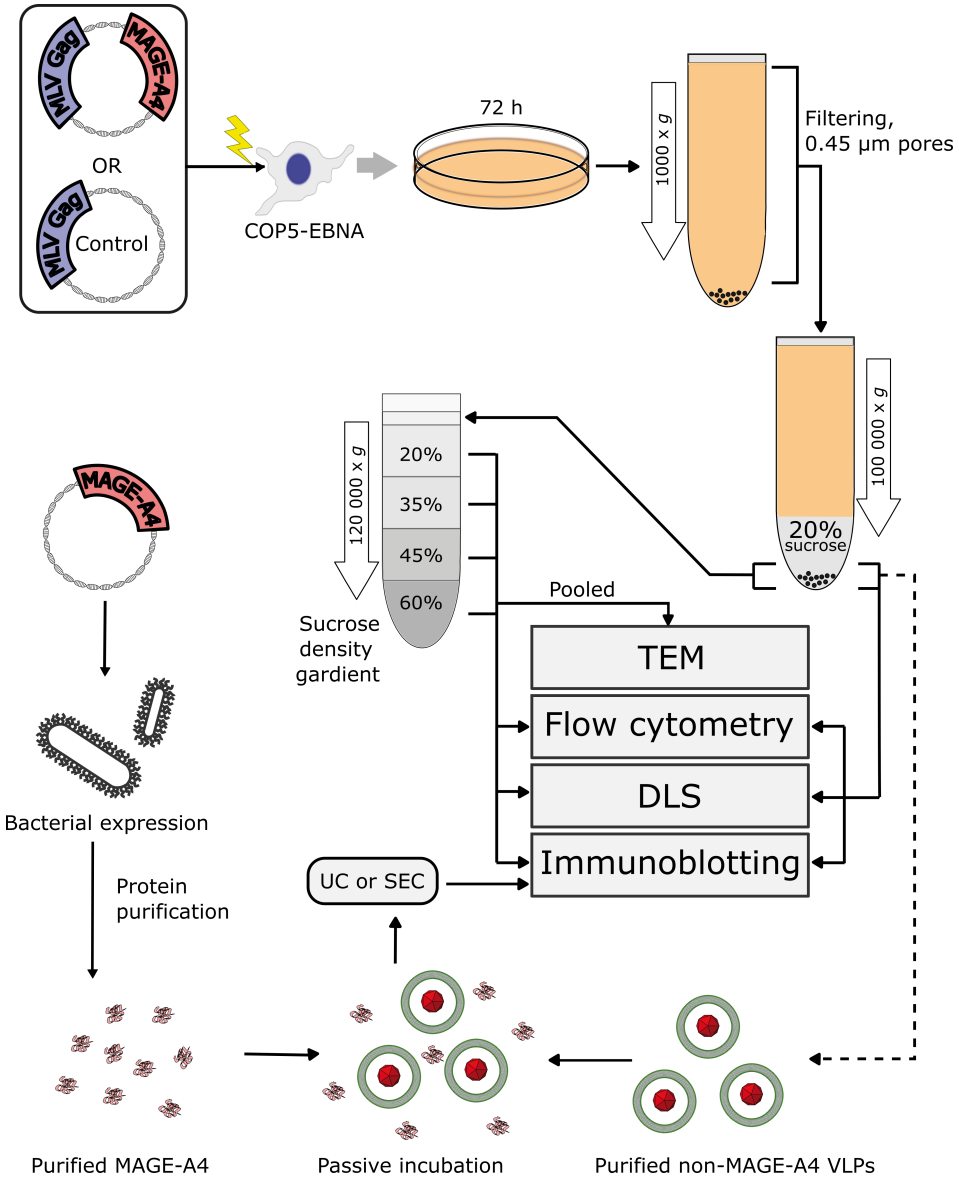

Figure 3. The scheme of VLP generation and analysis. The MLV VLPs were isolated from the filtered cell culture medium of transfected COP5-EBNA cells by ultracentrifugation through a sucrose cushion. Part of the initially pelleted VLP sample was directly used for analyses, while the other part was subsequently fractionated on a sucrose gradient column using ultracentrifugation. DLS – dynamic light scattering; TEM – transmission electron microscopy; SEC – size-exclusion chromatography; UC – ultracentrifugation.

(Figure 1E, Study I). Particles with spherical structures and a regular shape and size had a 100–140 nm diameter. Additionally, vesicular particles of 40–80 nm in diameter were also observed. The detection of Gag in immunoblots of a wide range of fractions was explained by the presence of VLPs with damaged or immature membranes observed in the TEM images. The structural description and the presence of nonintact VLPs match a description in an earlier study of MLV VLPs [280]. Our results support the hypothesis that VLPs represent suitable candidate lipidic nanocarriers with a robust generation protocol and readily display certain features acceptable for therapeutics, including high homogeneity and antigen loading.

4.1.2 MAGE-A4 is exposed to the outer surface of MLV VLPs (Study I)

VLPs themselves are already highly immunogenic adjuvants and might enhance the immune response against the antigens they carry, but presenting the antigen on the surface of the particles would induce a greater increase in humoral and cellular immune responses [281]. We analysed the isolated VLPs for exposure of MAGE-A4 on the outer surface using flow cytometry. The size of VLPs is too small for a conventional flow cytometer to detect. Therefore, we bound the VLPs to aldehyde sulfate-coated latex beads with a diameter of 4 μm before analysis. In this assay, the VLPs are not lysed, and only the antigen exposed on the surface is detected using antigen-specific antibodies. Rather surprisingly, MAGE-A4, a soluble cytosolic protein, was detected on the surface of the VLPs (Figure 2, Study I). An explanation for this finding is that the antigen was present on the outer surface of the cells. Immunofluorescence microscopy confirmed that MAGE-A4-expressing fibroblasts contained MAGE-A4 only in the cytoplasm, and no membrane localization was detected (Figure 3, Study I), although signs of a higher concentration of MAGE-A4 close to the membrane were observed in some cells. Consistent with these findings, the flow cytometry analysis of MAGE-A4-expressing cells confirmed that no MAGE-A4 was exposed to the surface of the cells, as no specific signal was detected (Figure 4A, Study I). Typically, for VLP-based cancer vaccine approaches, an epitope can be exposed to the surface of VLPs using two methods: chemical cross-linking or genetic insertion into the coding sequence of the viral structural protein [282]. Here, additional efforts to Gag and antigen coexpression are not needed. This spontaneous surface exposure avoids two challenges of VLP-based vaccine production, as no additional cost increase or protein folding complications occur that may be observed in cross-linking or genetic fusion approaches, respectively [283,284].

4.1.3 MAGE-A4 binds MLV VLPs in vitro (Study III)

We were interested in determining whether MAGE-A4 possesses properties that allow it to bind to the outer membrane of VLPs, excluding the cellular mechanisms necessary for VLP formation, to evaluate the possible pathways by which MAGE-A4 binds the outer surface of VLPs. The MLV VLPs were isolated from COP5-EBNA cells that expressed MLV Gag but not MAGE-A4. Instead, MAGE-A4 protein was purified from a bacterial production system in parallel. Both purified counterparts were mixed in a simple PBS buffer and incubated for an hour on the bench. After the incubation, the mixture was purified, and the VLPs were collected through ultracentrifugation or SEC. SEC fractionation allowed us to separate the intact VLPs from loose soluble protein in different fractions. Both the VLPs obtained from ultracentrifugation and SEC fractions positive for Gag also displayed a positive signal for MAGE-A4 (Figure 4B and 4D, Study III). Although most MAGE-A4 remained soluble, as was detected in the SEC fractions corresponding to the unbound protein, the results indicate that MAGE-A4 does have properties that enable it to bind the membrane of the VLPs. We have not determined whether MAGE-A4 binds to the phospholipid sheath or perhaps some membrane protein. The results also imply that the cellular pathways are not entirely essential for MAGE-A4 incorporation into VLPs. However, considering the abundance of the antigen in the isolated VLPs from MAGE-A4-expressing cells, the cellular pathways cannot be excluded, and these pathways are probably very important in sorting MAGE-A4 to the VLPs.

4.2 MAGE-A4 is incorporated into naturally occurring EVs (Study II)

4.2.1 MAGE-A4 is present in EVs of different sizes

One of the characteristic features of CTAs is their ability to elicit an immune response against cancer by promoting specific T-cell cytotoxicity or antibody production. As carriers of disease-specific antigens, EVs are also known to be involved in inducing immune responses [285]. Therefore, EVs derived from cancer cells that carry tumour-rejecting CTAs might be one of the pathways that support the development of specific anticancer immune responses [253]. We transfected the COP5-EBNA cells with the MAGE-A4 expression plasmid in the absence of any viral component to determine whether MAGE-A4 is incorporated into naturally occurring EVs (Figure 4). EV isolation was achieved using the so-called gold standard for EV separation – differential ultracentrifugation. Differential ultracentrifugation is a highly suitable method for isolating larger sample volumes typical for cell culture media while producing reasonably pure EV samples [286,287]. Three EV populations with different expected sizes were isolated with subsequent centrifugation of the cell culture medium at increasing speeds.

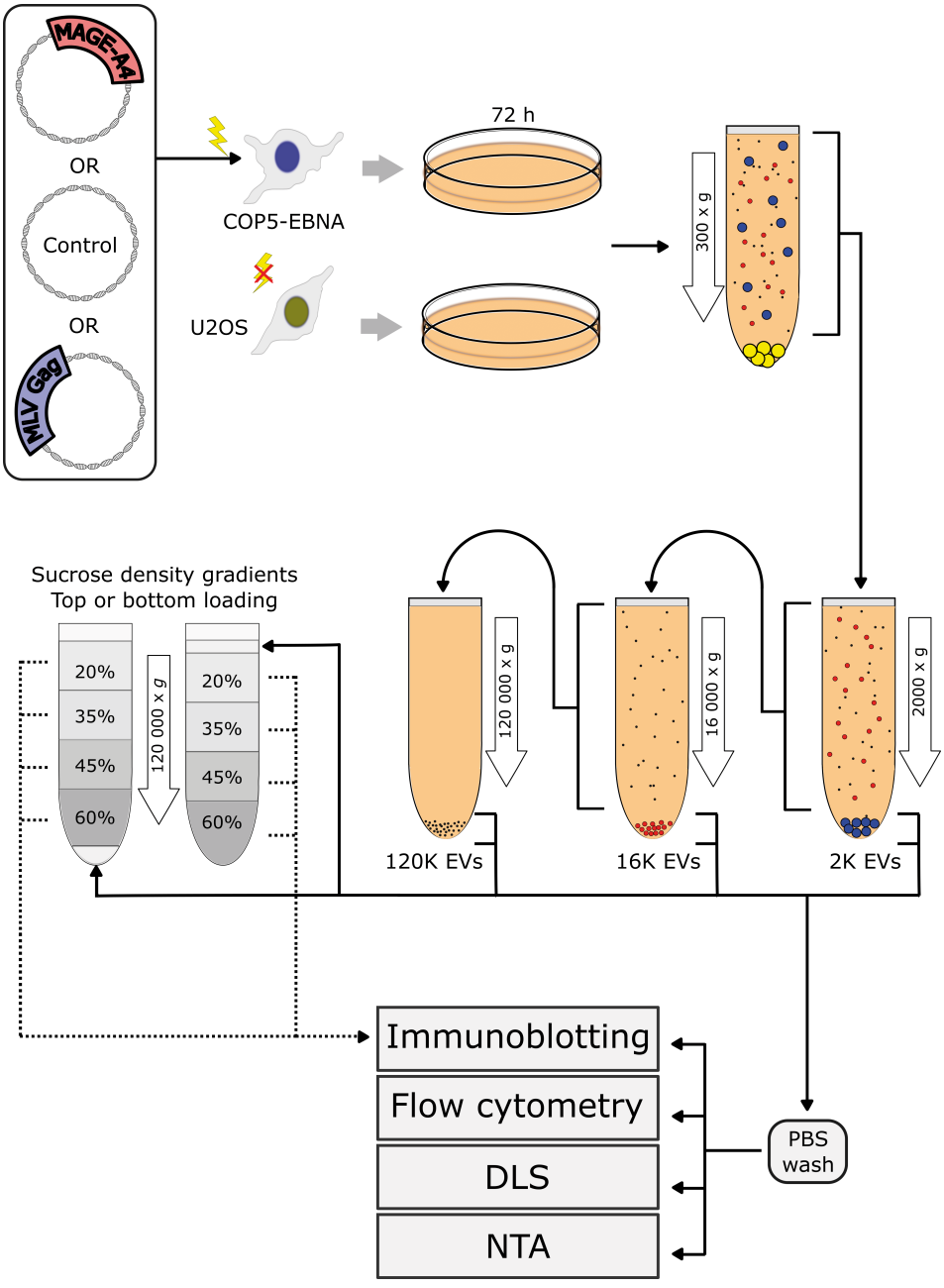

Figure 4. Schematic of EV isolation and analyses. COP5-EBNA cells were electroporated with a MAGE-A4 expression vector or an empty vector as the control. U2OS cells express MAGE-A4 endogenously and were not transfected. EVs were isolated from the culture medium of the cells using differential centrifugation. Part of the 2K and 120K EV samples was subfractionated by ultracentrifugation through a sucrose density gradient and analysed using immunoblotting. COP5-EBNA cells were transfected with MLV Gag or MAGE-A4 expression vectors for the vesicle yield assessment. The vesicles were isolated using differential ultracentrifugation and analysed using nanoparticle tracking analysis (NTA). DLS – dynamic light scattering

Western blot analysis of the EV subtypes ensured that 120K EVs were enriched with exosomes, as it is the only EV sample that displayed an intense signal for the exosomal marker TSG101 (Figure 1C, Study II). More importantly, MAGE-A4 was detected with relatively intense signals in all three EV samples, indicating that MAGE-A4 was incorporated into EVs of different subtypes.

We subfractionated the 2K and 120K EV samples by ultracentrifugation through sucrose density gradients with top and bottom loading of the samples on the column to ensure that the collected MAGE-A4-positive material had a vesicular composition and was not an aggregate of proteins. As EVs have a distinct buoyant density from protein aggregates, they will be separated into different fractions. The MAGE-A4-specific signal was detected throughout all eight fractions in both cases for the 2K EV sample, which indicates a wide variety of vesicles with different sizes and densities (Figure 1D, Study II). Regardless of the direction of loading, the MAGE-A4-specific signal was detected in fractions 2–5, indicating that the 120K EV sample contains MAGE-A4-positive vesicles of a more defined size range. The results are highly supported by the DLS measurement of the EV samples. The 2K EVs with a PDI of 0.93 are a very polydisperse population of particles, indicating the presence of many EVs with larger and smaller diameters (Figure 1E, Study II). The 16K and especially 120K EVs were more homogenous in size, as their PDIs were 0.4 and 0.27, respectively. As expected, the average sizes of the particles gradually decreased while the centrifugal force increased during isolation, and 2K, 16K and 120K EVs had average diameters of 1395 nm, 479 nm and 179 nm, respectively (Figure 1E, Study II). The DLS results suggest that the 2K EV sample was mainly enriched with ABs, the 16K EV sample was mostly enriched with MVs, and the 120K was enriched with exosomes and perhaps smaller MVs. The NTA analysis of 120K EV samples also indicated that the detected particles were mostly in the size range of larger exosomes and smaller MVs (Figure 1F, Study II). However, considering the overlapping characteristics of the EV subtypes, none of the isolated populations were explicitly considered to be in the EV subtype.

During the experiments with the EVs, we noted that transfection of cells with the MAGE-A4 expression vector increased the yield of EVs compared to cells transfected with an empty vector. The total particle amount and the protein concentration were measured to verify and assess the ability of MAGE-A4 to induce the formation and release of EVs. The MLV VLPs isolated using differential centrifugation were used as the positive control for comparison. Indeed, compared to cells transfected with the empty plasmid, MAGE-A4 and MLV Gag similarly induced the formation of a greater number of particles in all three vesicle size populations, as measured using NTA (Figure 6D, Study II). Gag induced the production of significantly more particles than MAGE-A4 only in 120K samples, as expected, because VLPs should mainly be enriched in this fraction. The EV cargo is considered the first regulator of EV formation and release [197]. Similar to the effect of viral Gag on inducing the production of RLPs in relatively high quantities, the expression of the myristoylated protein CHMP6, a known EV cargo, has also been shown to enhance the release of EVs [288]. If MAGE-A4 is

incorporated into EVs as cargo, it might increase EV secretion, especially if MAGE-A4 is overexpressed. The induction of EVs by MAGE-A4 is an indication that MAGE-A4 may be involved in the regulation of EV formation. As the cargo of the vesicles, MAGE-A4 might at least initiate the process.

In an attempt to acquire evidence that actual tumour cells release MAGE-A4-carrying EVs and to verify that the results are not an artefact of ectopically induced overexpression of the antigen, we isolated EVs from a cell line known to endogenously express MAGE-A4 – the osteosarcoma cell line U2OS [289]. Consistent with COP5-derived EVs, all three U2OS-derived EV populations were found to carry MAGE-A4, as detected using immunoblotting (Figure 5B, Study II). This result further validates our findings and suggests that cancer cells in vivo also release these vesicles. In a previous study, the endogenously expressed CTA SPANXB1 was sorted into EVs by naïve MDA-MB-231 and SUM-149 breast cancer cells, similar to our experiment [290]. A few reports of native EVs that carry CTAs, including other MAGEs, such as MAGE-A1, MAGE-A3 and MAGE-B4, originating from nonmanipulated cells or tissue have been published [290–297]. Most of the EVs in those studies were isolated from patients with specific cancer types, suggesting that MAGE-A4-EVs may also be isolated from the body fluids of patients with cancer. Our findings imply the potential of MAGE-A4-carrying EVs to be applied in cancer diagnostic analyses.

4.2.2 EVs expose MAGE-A4 on their surface

The unanticipated display of MAGE-A4 on the surface of our previously studied MLV VLPs encouraged us to investigate the phenomenon further by studying more naturally occurring EVs from that perspective. Similar to our VLP study, we bound EVs derived from MAGE-A4-expressing cells to aldehyde sulfate-coated latex beads and detected them using specific antibodies. The flow cytometry results indicate that EVs from all three size fractions expose MAGE-A4 on their surface (Figure 2A, Study II). The 120K EVs exhibited the highest signal intensity, while 16K EVs had the lowest signal intensity. Based on the results, MAGE-A4 was exposed on the surface of different EV types. The persistence of this intriguing phenomenon raises more questions before providing any answers. What mechanisms underlie the localization of the soluble cytosolic protein to the outer surface of released EVs, and does this process occur in vivo, including normally and aberrantly expressing tissues?

With the limited knowledge of MAGE-A4, any specific function cannot be inferred for this phenomenon other than direct antigen presentation. Tumour-derived EVs are known to introduce cancer antigens to professional antigen-presenting cells such as dendritic cells that initiate immune responses [298]. Research conducted by Sedlik *et al.* compared vaccines involving vesicles with an antigen packed inside the lumen and vesicles that expose it to the surface. The authors concluded that both might exert a remarkable effect on the induction of the immune response against cancer. However, vesicles exposing the antigen were shown to

exert a more significant effect on promoting the CD4+ T-cell response and the generation of IgG antibodies, resulting in a more effective therapeutic outcome [299]. Therefore, tumour-derived EVs presenting antigenic proteins might be more effective in eliciting immune responses in a biological context as well. Dendritic cells loaded with tumour-derived EVs presenting MAGE-A1 induced tumour-specialized cytotoxic lymphocytes [297], which also suggests a possible immune triggering pathway for MAGE-A4-EVs.

A few other CTA-carrying EVs have been suggested to be associated with tumour progression and drug resistance, although the antigens were not shown to be presented on the EV surface [292,300]. EVs with NY-ESO-1 exposed on their surface were described as a prognostic marker for inferior survival of patients with cancer [296]. MAGE-A3/6-carrying EVs were associated with apoptosis induction in T-cells and metastatic progression [295]. The findings suggest possible scenarios for the cancer-promoting properties of MAGE-A4-EVs, considering the adverse prognostic outcomes of MAGE-A4 in specific cancers. One of our research studies showed that patients with cancer displaying MAGE-A4- and MAGE-A10-specific antibody responses had the strongest response during stage II of cancer development [301]. However, the antibody response decreased as the cancer progressed further, indicating MAGE-related immune evasion. EVs are known for their involvement in cancer immune evasion. Therefore, MAGE-A4-EVs might also have a role in this process, e.g., acting as decoys for antibodies. Further studies are needed to elucidate whether MAGE-A4-EVs elicit antitumour immune responses or perhaps support cancer progression. Nevertheless, the spontaneous presentation of MAGE-A4 on EVs is a feature that is potentially useful cancer immunotherapeutic methods.

4.3 MAGE-A4 binds tightly to the surface of EVs (Study III)

4.3.1 MAGE-A4-EVs can be stably stored but are fragile during consecutive freeze–thaw cycles

The association between MAGE-A4 and the outer surface of EVs is a fascinating finding but is also mysterious. We studied the stability of MAGE-A4-EVs to characterize and evaluate the strength of the association and further evaluate the bond between the two components. We monitored the MAGE-A4 surface exposure and EV concentrations of the MAGE-A4-EVs by performing a 21-day trial in which they were stored at +4 °C and –80 °C and subjected them to freeze–thaw cycles.

The MAGE-A4-EVs showed persistent stability throughout the 21-day measurement period. No significant changes were detected in the analyses of the MAGE-A4 concentration, surface exposure, or particle concentration (Figure 1A, 1B, 1C and 1D, Study III). On the one hand, signs of homogenization of the samples were observed in the fluorescence intensity profiles from flow cytometry (Figure 1C, Study III). On the other hand, a slight decrease in the level of surface-

exposed MAGE-A4 was measured every week that was not significant by the end of the 21st day (Figure 1B, Study III).

In the event of several consecutive cycles of freezing and thawing, MAGE-A4-EVs are damaged and degraded. With each concessive cycle, the amount of surface-exposed and total MAGE-A4 decreased steadily, exhibiting a significant decrease by the third cycle compared to the fresh sample (Figure 2B and 2C, Study III). Although the particle concentration was not significantly changed, a slight increase in the number of particles was detected after the first cycle (Figure 2D, Study III). The increase may indicate the dismantling of some EV aggregates due to freezing that freed EVs that were now detectable as separate particles. Immature and faulty EVs might have been shattered by freezing as well. The disintegration of aggregates and removal of faulty EVs may explain the higher homogeneity of the vesicles, as reflected by the fluorescence intensity profiles from flow cytometry measurement (Figure 2A, Study III). After the first freeze – thaw cycle, the number of particles decreased steadily. Interestingly, a significant increase in the diameter of the particles was observed after the first two cycles compared to the fresh sample (Figure 2E, Study III). The swelling might result from multilamellarization, a phenomenon vesicles have been described to be prone to following freezing [302]. The reduced size observed after the third cycle might be explained by continuous disruption of the vesicles due to the detrimental effect of freezing and melting.

Not all EVs respond equally to similar conditions, as the origin, isolation methods and cargo of EVs have been shown to affect the integrity of EVs [303]. Current stability and freeze–thaw cycle experiments reveal the high stability of MAGE-A4-EVs and the robust storage conditions necessary to preserve MAGE-A4-EVs. Based on the freeze–thaw cycle experiment, the surface-exposed MAGE-A4 detectability correlates with general EV integrity. The results confirm that the association between MAGE-A4 and the surface of EVs is an imposing phenomenon and not a faint appearance. The findings might also be helpful for developing handling and preservation procedures for diagnostics or EV-based therapy development related to MAGE-A4-EVs.

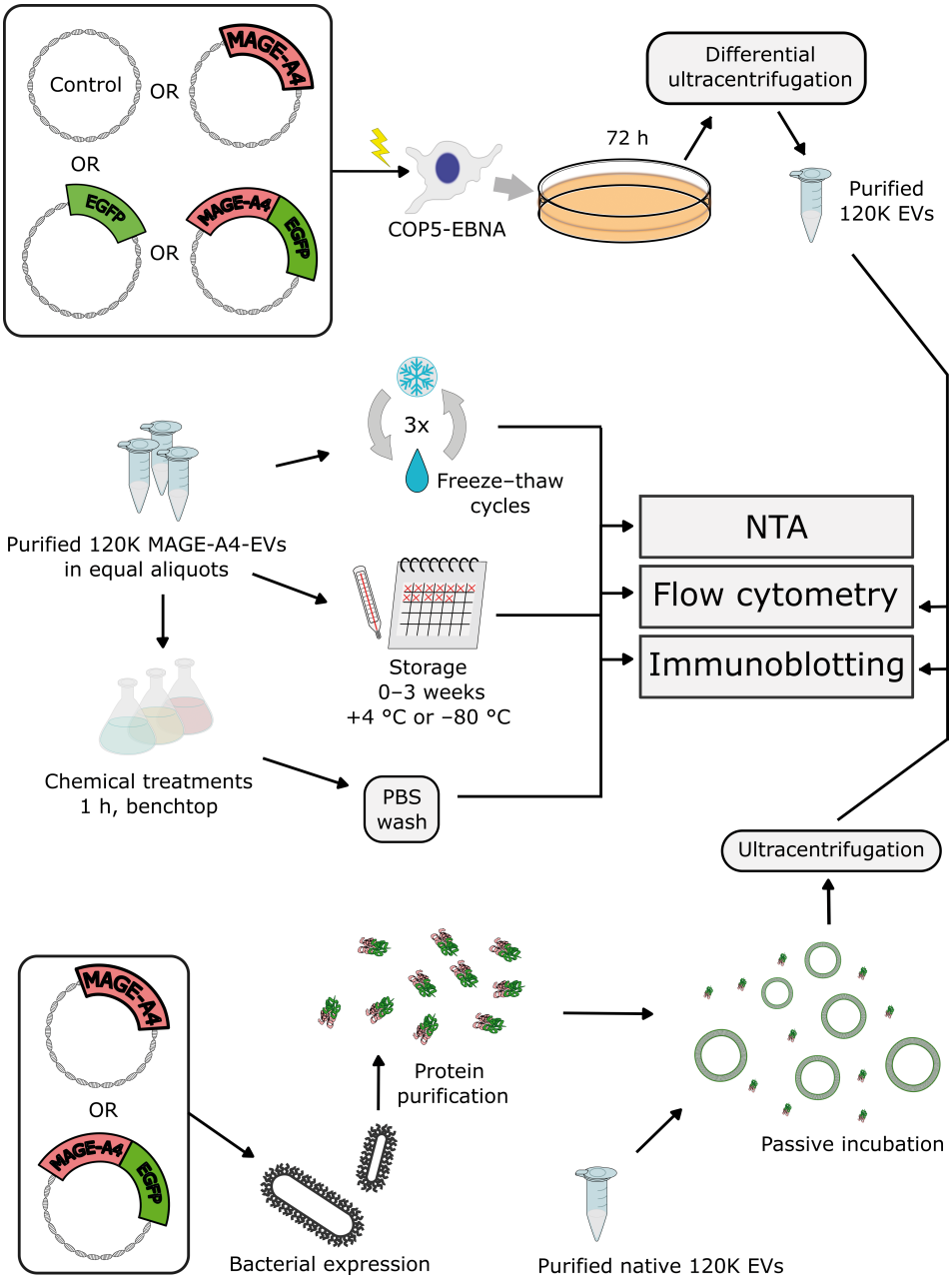

Figure 5. Schematic of the analysis of the MAGE-A4 and EV association. MAGE-A4-EVs were isolated from the transfected COP5-EBNA cell culture medium by differential ultracentrifugation, but only the 120K fraction was used. The EVs were equally aliquoted, and their physicochemical stability was investigated through storage, freeze–thaw cycles and chemical treatment experiments. The MAGE-A4 gene was genetically fused with EGFP in eukaryotic and prokaryotic expression plasmids, allowing the isolation of EVs bearing the fusion protein or purification from the bacterial expression system. The purified MAGE-A4 and fusion protein were incubated with native EVs in vitro to study the ability of MAGE-A4 to bind the EV membrane.

4.3.2 The association between the EV surface and MAGE-A4 is resistant to electrostatic manipulation

Peripheral membrane proteins are known to associate with the phospholipid membrane through various means, including electrostatic forces, direct binding through hydrophobic anchors or the interface of ions, or interacting with transmembrane proteins [304]. Since MAGE-A4 is not a transmembrane protein, we hypothesized that it must be bound to the outer surface of EVs like a peripheral membrane protein. MAGE-A4 is not known to have any hydrophobic post-translational modifications typical of binding directly to the membrane. We subjected MAGE-A4-EVs to a series of solutions used for peripheral membrane protein extraction to obtain additional insights into the possible mechanism underlying the formation of the bond between MAGE-A4 and the EV surface.

The electrostatic forces can be analysed with high ionic forces by applying high concentrations of salts. NaCl and $MgCl_2$ are salts with different levels of chaotropicity, indicating that they react differently to hydrophobic interactions. Both salts with equally high ionic strengths did not decrease the detectability of exposed MAGE-A4, but rather surprisingly, it was increased according to the flow cytometry results (Figure 3B and 3D, Study III). Perhaps these salts exerted a positive effect on antibody binding affinity to MAGE-A4. Nevertheless, MAGE-A4 presentation on EVs was not removed, suggesting that electrostatic forces are not essential factors in this association. Additionally, MAGE-A4 does not seem to bind to the membrane by interacting with metal ions, as the chelators EGTA and EDTA did not have any effect (Figure 3D, Study III). The nonionic detergent Triton X-100 and pH 11.5 buffer exerted substantial effects on MAGE-A4 detectability because these solutions forcefully degraded the EVs (Figure 3E, Study III).

In summary, these chemical treatment results indicate that MAGE-A4 is not bound to the surface of EVs through electrostatic interactions, including interactions with metal ions. Apparently, EVs would rather break before releasing MAGE-A4 from their surface. Hydrophobic interactions seem to be a more probable means of association, although no specific binding mechanism has been identified. A known soluble IDP Tau has been shown to assume a more compact conformation through interaction with the membrane and partially inserts into the lipid layer [305]. Given the intrinsically disordered structure of MAGE-A4, it might also share similar properties. Moreover, the disordered N-terminal part of MAGE-A4 is known to have polar amino acid-rich regions [117]. These amino acids have been shown to be important for transmembrane domain interactions with the plasma membrane [306]. The MHD of MAGEs may also have a role in this process, as our studies (I and II) have shown that another member of the family, MAGE-A10, shares EV sorting and surface exposure properties to a slightly lesser extent. Additionally, no EV sequestration was observed if the MAGE-A4 MHD was disrupted through genetic deletion (Study II). These findings suggest a possible interaction between an integral membrane protein of EVs and the MHD. The inclusion of more MAGE proteins in EV studies might provide a better understanding of the role of the MHD.

4.3.3 MAGE-A4 may be incorporated into EVs through cellular mechanisms or its biochemical properties

EGFP, an easily detectable marker with a similar molecular mass to the MAGE-A4 protein, was chosen for assessing the resoluteness of MAGE-A4 sorting into EVs by transfecting COP5-EBNA cells with an expression plasmid for MAGE-A4-EGFP fusion protein production. EGFP alone has been shown to be very poorly incorporated into EVs [307]. Our results confirmed the absence of EGFP in the isolated EVs if the marker was expressed alone, but when expressed as a part of the fusion protein, MAGE-A4 populated the EVs together with EGFP (Figure 5A, Study III). Moreover, the EGFP cloned at the C-terminus of MAGE-A4 did not eliminate the surface presentation of MAGE-A4 on the EVs, as determined using flow cytometry with MAGE-A4-specific antibodies and the fluorescence of EGFP (Figures 5C and 5D, Study III). The results neatly ensure that all proteins expressed in the cell are not incorporated into the EVs and that MAGE-A4 may be actively sequestered to the EVs. Some specific biomechanisms might underlie MAGE-A4 selection for EV formation. MAGE-A4 has been shown to have several ubiquitylation sites in a sequence analysis [308], which are known to be an important signal for endosomal sorting and, therefore, one of the possible factors for EV sorting of MAGE-A4. However, the "bystander" effect cannot be neglected, as MAGE-A4 might also be collaterally sorted to EVs through putative interacting partners targeted by EV sorting mechanisms. Nevertheless, the components of the EV formation machinery should be more closely studied in MAGE-A4-EVs to identify putative mechanisms.

An analysis of the VLPs and the purified MAGE-A4 protein indicated that the antigen bound the surface of VLPs after passive incubation. We performed similar passive incubation experiments with EVs to further verify the ability of MAGE-A4 to bind to membranes of vesicles more generally. The EVs washed through ultracentrifugation after the incubation were analysed using western blotting and flow cytometry; both confirmed the affinity of MAGE-A4 for the outer surface of the vesicles (Figure 4B and 4C, Study III). Furthermore, the binding ability prevailed even if MAGE-A4 was part of the fusion protein with EGFP, as indicated by the results of the passive incubation experiment with the EVs and the purified EGFP fusion protein (Figure 5E, Study III). Once more, the results suggest the innate feature of MAGE-A4 binding to the membrane or the surface components of the vesicles in vitro, indicating that cellular biomechanisms are not an essential requirement. This ability might also allow MAGE-A4 to bind the membrane on the cytoplasmic side. When bound to the plasma membrane or the endosomal membrane, MAGE-A4 could easily be sequestered into all three traditional EV subtypes. According to the fluorescence microscopy images of the MAGE-A4-expressing cells or from the MAGE-A4 passive incubation experiment with VLPs, the membrane-binding feature is a relatively marginal phenomenon compared to the soluble state of the protein, if it exists at all in the cells. The pathways of MAGE-A4 incorporation into and onto EVs remain elusive. More effort into studying the cellular mechanisms underlying the formation of

different EV subtypes and natural features of MAGE-A4 is needed to identify the critically important factors mediating the incorporation of MAGE-A4 into EVs. From a biotechnological perspective, the current results might make MAGE-A4 more appealing in the development of anticancer EV-based immunotherapies, as the additional cargo loading and spontaneous surface exposure facilitate robust antigen presentation coupled with drug loading capabilities.

5. CONCLUSIONS

MAGE-A4, a cancer-testis antigen, is expressed in numerous different cancer types. MAGE-A4 has been described to exert tumour-supporting and -inhibiting effects. Our studies showed that MAGE-A4-expressing cells incorporate the antigen into EVs or retroviral VLPs if expressed along with MLV Gag. We discovered that MAGE-A4 is spontaneously presented on the outer surface of the induced VLPs and the more naturally released EVs. The association between the outer surface of EVs and MAGE-A4 was shown to be persistent, as the presentation of MAGE-A4 on the EVs was not disrupted by 21 days of long storage or chemical treatments involving the manipulation of electrostatic forces. However, the cellular mechanisms underlying the incorporation or surface exposure of MAGE-A4 on EVs remain elusive. To some extent, MAGE-A4 is bound to the outer surface of EVs in vitro. The presence of MAGE-A4 in EVs might hypothetically facilitate the progression of cancer development in the body if the protein is delivered to recipient cells or the tumour microenvironment or used for immune evasion. On the other hand, EVs presenting MAGE-A4 as the cancer rejection antigen might elicit cancer-specific immune responses. These aspects illustrate the two areas that should be studied further in vivo to understand the functions of EVs bearing MAGE-A4.

We have shown that MAGE-A4 possesses properties that might also benefit the development of anticancer EV-based innovations. If MAGE-A4-EVs are shown to be released by MAGE-A4-positive cancer tissues in vivo in future studies, MAGE-A4 could be included in cancer diagnostic panels relying on the analysis of EVs acquired using low-risk liquid biopsy approaches. After co-expression with the retroviral Gag protein, MAGE-A4 is packed into VLPs that readily display properties suitable for immunotherapy, including antigen presentation and high homogeneity coupled with a relatively high yield compared to natural EVs. MAGE-A4 has a spontaneous EV decorating capability, allowing innovative approaches for developing EVs or VLPs that potentially elicit MAGE-A4-positive tumour-specific immune responses and load EVs with additional cargo.

The starting hypothesis of the dissertation was that MAGE-A4 is incorporated into naturally occurring EVs by cells that express it. Based on the results of the three studies included in the dissertation, I drew the following conclusions:

- The cells that express MAGE-A4 either ectopically or endogenously incorporate MAGE-A4 into naturally occurring EVs of different sizes. MAGE-A4-carrying EVs present the antigen on their surface.
- If MLV Gag and MAGE-A4 are coexpressed in mouse COP5-EBNA fibroblasts, retroviral VLPs are generated that carry and present MAGE-A4 on their surface. VLPs are produced in relatively high amounts and with high homogeneity.
- The expression of MAGE-A4 increases the release of EVs from cells.

- MAGE-A4-EVs are highly stable under common EV storage conditions for at least 21 days and withstand at least two freeze–thaw cycles without losing MAGE-A4.
- The bond between MAGE-A4 and the EV surface is chemically resistant and is not disrupted by manipulating electrostatic interactions.
- MAGE-A4 binds to the outer surface of EVs and VLPs in vitro through passive incubation and decorates EVs with recombinant proteins.

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SUMMARY IN ESTONIAN

Vähi-testis antigeen MAGE-A4 inkorporeeritakse ekstratsellulaarsetesse vesiikulitesse ja on eksponeeritud selle pinnal

Vähi-testis antigeenid (ingl lüh CTA) on valgud, mida ekspresseeritakse tavapärasel juhul peamiselt munandites ehk testistes ja vähesel määral mõnes teises koes, kuid patoloogilise seisundi korral võivad need olla kõrgelt ekspresseeritud ka erinevates vähivormides. On näidatud, et CTA-d võivad esile kutsuda vähispetsiifilist immuunvastust. Nende omaduste poolest peetakse CTA-d sobivateks kandidaatideks vähivastase teraapia- ja diagnostikameetodite väljatöötamiseks.

Inimese MAGE geenide ülemperekonda kuulub enam kui 40 geeni. MAGE-A, -B ja -C alamperekonnad moodustavad nn I tüüpi MAGE-d, mida peetakse klassikalisteks CTA-deks, olles ekspresseeritud normaalsetes kudes testistes, platsentas ja ka embrüonaalsetes munasarjades, kuid sagedasti ka erinevates vähi vormides.

MAGE-A4 on tsütoplasmas või kohati ka tuumas lokaliseeruv lahustunud valk, mille esinemine on seostatud kõrgema vähi arenguastmega, kehvema patsiendi väljavaadete ja elulemusega. On kirjeldatud, et MAGE-A4 võib vähki toetada soodustades rakulist kasvu ja inhibeerides apoptoosi. Kuid lõigatud MAGE-A4 C-terminaalne osa võib avaldada vähi kasvu pärssivat mõju.

Ekstratsellulaarsed vesiikulid (EV-d) on lipiidse kaksik-kihiga ümbritsetud nano-osakesed, mida eraldavad endast kõik rakud neid ümbritsevasse rakuvälisesse ruumi. Seega EV-sid võib leida kõikidest kehavedelikest. EV-d on võimelised endas kandma bioaktiivseid molekule ühest rakust teise, seetõttu on nad olulised rakkudevahelise suhtluse vahendajad. Nõnda laia mõjusfääriga rolli tõttu võivad EV-d olla seotud peaaegu kõigi füsioloogiliste ja patoloogiliste protsessidega kehas, seal hulgas ka vähiga. EV-d jaotatakse kolme peamisesse alatüüpi: apoptootilised kehad (AB-d), mikrovesiikulid (MV-d) ja eksosoomid, mis erinevad peamiselt biogeneesi radade ja suuruse poolest. On kirjeldatud, et erinevat tüüpi EV-d osalevad mitmekülgselt nii vähi arengus kui ka vähivastases kehalises reaktsioonis. EV-sid peetakse ka potentsiaalseteks kandidaatideks uudsete vähivastaste teraapiate väljatöötamiseks.

Viirus-laadsed partiklid (VLP-d) eralduvad rakkudest viirusosakeste komponentide indutseerimise tulemusena. Membraaniga ümbritsetud VLP-d, nagu näiteks retroviiruse-laadsed partiklid, on oma olemuselt ja tekkeviisilt natiivsetele EV-dele väga sarnased, mis teevad neist head mudelid EV-de uurimiseks

Ühe meie uuringu käigus avastati, et MAGE-A4 inkorporeeritakse VLP-desse ja selle pinnale. Käesoleva doktoritöö aluseks püstitati hüpotees, et MAGE-A4 paigutatakse natiivsetesse EV-desse rakkude poolt, mis seda ekspresseerivad.

Esimene doktoritöö eesmärk oli kirjeldada MAGE-A4 kandvaid VLP-sid. Hiire COP5-EBNA rakke transfekteeriti Moloney hiire leukeemia viiruse Gag valgu ja MAGE-A4 valgu ekspressiooniplasmiididega, mis võimaldas indutseerida rakkudest VLP-sid. Rakusöötmest läbi suhkrupadja ja tihedusgradiendi eraldatud VLP-de analüüsimisel veenduti, et MAGE-A4 on VLP-desse pakitud

ja eksponeeritakse nende pinnal. Genereeritud VLP-d olid ootuspärase suurusega ja väga homogeensed. Tehnoloogilises mõistes on MAGE-A4 kui antigeeni spontaanselt eksponeerivate VLP-de tootmine lihtne meetod, mis ei vaja täiendavaid töötlusetappe. Et hinnata MAGE-A4 VLP-de pinnale kinnitumise võimalusi, inkubeeriti puhastatud MAGE-A4 valk eraldatud VLP-dega. Selgus, et teatud määral MAGE-A4 on võimeline VLP-de pinnale seonduma *in vitro* tingimustes ehk selle fenomeni tekkimiseks pole rakulised mehhanismid täiesti vajalikud, kuid antud katsete põhjal neid välistada siiski ei saa.

Teine doktoritöö eesmärk oli veenduda hüpoteesi paikapidavuses ka naturaalsete EV-de puhul. Selleks transfekteeriti COP5-EBNA rakud ainult MAGE-A4 ekspressioonivektoriga ning seekord eraldati EV-d rakusöötmest differentsiaalse ultratsentrifuugimise teel, jaotades EV-d kolme fraktsiooni, mis erinesid suuruse poolest: kõige suuremad 2K EV-d, keskmise suurusega 16K EV-d ning kõige väiksemad 120K EV-d. Analüüside põhjal veenduti, et kõik eraldatud fraktsioonid sisaldasid MAGE-A4. EV fraktsioonide osakeste suurusi analüüsides leiti, et MAGE-A4 võib olla inkorporeeritud erinevatesse EV alatüüpidesse. Uuriti ka EV-sid rakkudest U2OS, mis ekspresseerivad MAGE-A4 endogeenselt. Kooskõlas doktoritöö eelnevate tulemustega leiti, et natiivselt ekspresseeritud MAGE-A4 on pakitud kõikidesse EV fraktsioonidesse. See tähendab, et koed kehas, sh ka vähkkasvajad, võivad eritada EV-sid, mis kannavad endas MAGE-A4. Seega MAGE-A4-l on potentsiaal olla kaasatud vähidiagnostika EV-põhistes analüüsides.

Veenduti, et MAGE-A4 on eksponeeritud erinevatest alatüüpidest EV-de pinnal. Seega see fenomen pole mitte ainult VLP spetsiifiline, vaid seotud EV-de moodustumisega üldisemalt. Teada olevate andmete põhjal on raske seletada pinnale paigutumise mehhanismi või selle funktsiooni organismis. Kõige tõenäolisemalt on tegu MAGE-A4 kui vähi antigeeni esitlemisega EV pinnal, mis võib esile kutsuda vähivastast immuunvastust.

Kolmas doktoritöö eesmärk oli uurida MAGE-A4 ja EV pinna vahelist seost lähemalt, pöörates eelkõige tähelepanu selle stabiilsusele 120K EV-des. Tulemused kinnitasid, et MAGE-A4-EV on väga stabiilsed vesiikulid, mis ei kaota MAGE-A4 EV-de pinnalt kui neid hoiustada +4 °C või –80 °C vähemalt 21 päeva. Alles kolmanda järjestikuse külmetus-sulatustsükli järel ilmneb märkimisväärne MAGE-A4 hulga langemine. Need tulemused kirjeldavad MAGE-A4-EV-sid, kui väga stabiilseid vesiikuleid.

MAGE-A4 ja EV pinna vahelise seose keemilise tugevuse hindamiseks töödeldi MAGE-A4-EV-sid erinevate perifeersete membraanivalkude eraldamise lahustega. Leiti, et MAGE-A4 ei ole seotud EV-de pinnal elektrostaatiliste interaktsioonide toel, vaid tõenäolisemalt on MAGE-A4 seostunud hüdrofoobsel viisil. Nende füüsikaliste ja keemiliste töötluste tulemusena järeldati, et MAGE-A4 ja EV-de pinna vahel on tugev seos, mitte kergelt eemaldatav kleepumine.

Katses MAGE-A4-EGFP liitvalku ekspresseerivatest COP5-EBNA-dest pärit EV-dega võimaldas kirjeldada MAGE-A4 võimekust suunata EV-desse materjali,

mida tavaliselt rakkudes EV-desse ei pakita. Tulemused ilmekalt demonstreerivad, et MAGE-A4 sattumine EV-desse ei ole juhuslik ning EGFP valk MAGE-A4 C-terminuses ei takistanud ka liitvalgul EV-de membraanile seondumast.

Katsed puhastatud MAGE-A4 valgu ja MAGE-A4-EGFP liitvalguga kinnitasid, et MAGE-A4-l on biokeemilisi omadusi, mis võimaldavad sellel EV-de pinnale seostuda passiivse inkubatsiooni teel. See tähendab, et rakulised mehhanismid pole selle fenomeni esinemiseks täiesti olulised.

Kokkuvõtvalt, MAGE-A4 valk inkorporeeritakse VLP-desse ja naturaalsematesse EV-desse. Avastati, et MAGE-A4 on eksponeeritakse ka nende vesiikulite pinnal. MAGE-A4 ja EV-de pinna vahel on stabiilne side, mida ei ole võimalik lõhkuda elektrostaatilisi interaktsioone mõjutades. MAGE-A4 on võimeline viima EV-desse ja selle pinnale täiendavat materjali ning seostuma vesiikulite membraaniga passiive inkubatsiooni teel ehk rakulisi mehhanisme kasutamata. Tänu nendele omadustele on MAGE-A4-l potentsiaali olla kaasatud EV-põhistes vähidiagnostika analüüsides. Lisaks, MAGE-A4 on võimalik rakendada innovaatilistes vähivastases EV- või VLP-põhistes immunoterapeutilistes tehnoloogiates.

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