CHAPTER 11

THE HUMAN EXOSOME AND DISEASE

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Abstract: Long before the RNA degrading exosome was first described in the yeast *Saccharomyces cerevisiae*, the use of autoantibodies found in the sera of certain autoimmune patients allowed the identification of a complex of polypeptides which later appeared to be the human exosome. Today, the most extensively documented association of the exosome with disease is still its targeting by the immune system of such patients. The highest frequency of autoantibodies to components of the exosome complex is found in polymyositis-scleroderma overlap patients and therefore the exosome is termed PM/Scl autoantigen in the autoimmune field. More recently, one of the core components of the exosome was identified as a protein associated with chronic myelogenous leukemia. In this chapter we will describe the identification of the PM/Scl autoantigen from a historical perspective, discuss our current knowledge on the occurrence of autoantibodies to exosome components in autoimmune diseases and end with the data that connect the exosome with cancer.

INTRODUCTION

As described in the other chapters of this book, the exosome is implicated in the processing/maturation and degradation of many different species of RNA. The exosome complex is evolutionary conserved and can therefore be found in virtually all eukaryotic and archaeal forms of life, although the composition of the complex might differ to some extent from one species to another. In eubacteria a similar complex in terms of structure and function can be discerned, which is called the degradosome. The presence of exosome or exosome-like complexes in these different species suggests that it fulfills an essential role in RNA metabolism. In agreement with the assumption that eukaryotic cells can probably not survive without a functional exosome, studies in yeast and humans have shown that an intact exosome core is required for normal cell growth.[1-3]

RNA Exosome, edited by Torben Heick Jensen.

The discovery of the human exosome complex was facilitated by its association with autoimmunity. A response of the immune system to self-components is observed in a plethora of diseases, in particular connective tissue diseases. The autoimmune response can be directed to a variety of biomolecules, including proteins, nucleic acids and lipids. In most cases, it is neither known how immunological tolerance to self-components is broken, nor whether the immune response plays a pathophysiological role or is merely an epiphenomenon. Autoimmunity is generally detected by the appearance of autoantibodies in the serum of patients. Autoantibodies in a distinct group of autoimmune patients target components of the exosome complex and these autoantibodies have been instrumental in the identification of a complex of proteins that was later found to represent the human exosome complex.

IDENTIFICATION OF THE PM/Scl COMPLEX

As already mentioned above, the first indication of the existence of a human exosome complex dates back from 1977 when Wolfe and colleagues showed that autoantibodies in the sera of patients suffering from certain muscle disorders were capable of precipitating an antigen from a calf thymus extract as determined by an immunodiffusion assay.[4] This antigen was initially called "PM-1", as 17 out of the 28 sera (61%) that were scored as 'positive' (in terms of successfully precipitating the antigen) were derived from patients that were diagnosed with polymyositis.

Polymyositis, literally meaning "many muscle inflammations", is a progressive, chronic disorder that belongs to the group of connective tissue diseases (CTD), along with other diseases including dermatomyositis (DM) and systemic sclerosis (SSc, also referred to as scleroderma, Scl). Symptoms of PM include weakening and/or loss of mass of the muscles, which is particularly evident in the legs, shoulders and pelvis of the patient, thereby severely hampering the patient's abilities in everyday's activities such as climbing stairs, standing up or even walking. While some of these PM-symptoms are shared with DM and SSc, the latter two disorders have more prominent visual effects on the skin, such as the appearance of rash, hardening of the skin and disposition of calcium under the skin (Fig. 1). When patients have symptoms of both SSc and PM or DM, the disorder is referred to as polymyositis/scleroderma-overlap syndrome (PM/Scl). A total of 8 patients suffering from this overlap syndrome were also included in Wolfe's study and all but one of these appeared to contain anti-PM-1 antibodies, indicating that these autoantibodies are more common in this group of patients. More importantly however, the disease-specificity of the autoantibodies indicated that they might be exploited as a biomarker for clinical diagnosis, which was not yet available for these patients at that time. In view of the strongest association of the autoantibodies with PM/Scl patients, the antigen was renamed "PM/Scl-antigen" in 1984. In the same period other research groups confirmed the high prevalence of these autoantibodies in patients suffering from the PM/Scl-overlap syndrome.[5,6]

An obvious next step was the identification and characterization of the molecules that make up this particular antigen. By immunoprecipitation experiments it was demonstrated that the PM/Scl-antigen consists of at least 11 polypeptides, with relative molecular masses ranging from 20,000 to 110,000.[7] Subsequently, it took about 5 years before two of the major autoantigenic proteins targeted by the sera of PM/Scl-patients were identified and their cDNAs were cloned.[8-10] The identified proteins were named

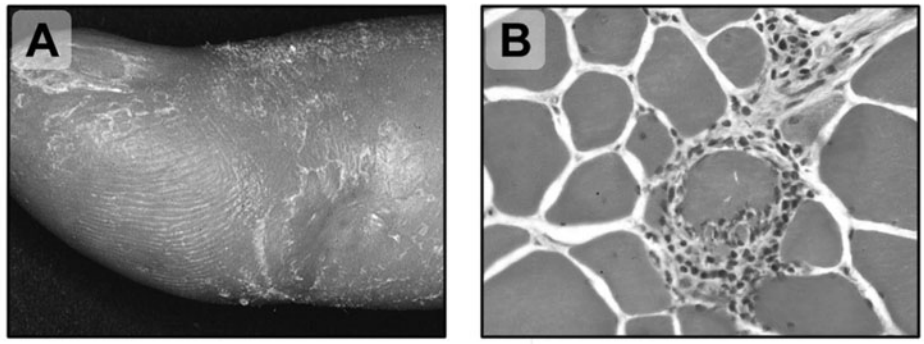

Figure 1. Clinical features of scleroderma and polymyositis. A) Sclerodactyly, a typical symptom often seen in patients suffering from scleroderma, which is characterized by thickening and tightening of the skin. B) Muscle fibers of a polymyositis patient, showing the infiltration of inflammatory cells (a.o. lymphocytes) in the endomysium.

according to their relative molecular mass as determined by SDS-PAGE: a M_r 75,000 protein designated PM/Scl-75 and a M_r 100,000 protein designated PM/Scl-100. It should be noted that the PM/Scl-75 protein migrates aberrantly in SDS-PAGE gels, most likely due to the clustering of charged amino acids in its C-terminal region.[11] In addition, four isoforms of the PM/Scl-75 protein have been described. The original cDNA reported by Alderuccio and coworkers[8] was probably incomplete in the region corresponding to the N-terminus, which was substantiated by the lack of association with the exosome complex in two-hybrid experiments.[12] By screening human EST databases sequences encoding 84 additional amino acids in the N-terminal region were found. Importantly, the polypeptide corresponding to the longer isoform did show two-hybrid interactions with components of the exosome core complex.[11] Another variation in the PM/Scl-75 sequence is resulting from an alternative splicing event, which leads to the incorporation of a 17 amino acids encoding optional exon in the C-terminal region of the protein.

When the yeast exosome complex was identified[3] and Allmang and colleagues found that two of its protein components, Rrp45 and Rrp6, were homologous to the human PM/Scl-75 and PM/Scl-100 proteins, respectively,[2] the suggestion was raised that the PM/Scl complex might in fact represent the human counterpart of the yeast exosome. This was confirmed by the cloning of cDNAs encoding the other components of the human exosome, which was based upon either the homology with their yeast counterparts or the copurification with the human exosome during affinity-purifications.[13,14]

AUTOANTIBODIES TO THE EXOSOME / PM/Scl-ANTIGEN

Originally, the detection of autoantibodies in patient sera was mainly performed by immunodiffusion (or the related technique counterimmunoelectrophoresis) and by indirect immunofluorescence (IF). With the latter technique a typical nucleolar staining pattern was indicative for the presence of anti-PM/Scl autoantibodies and therefore anti-PM/Scl autoantibodies were categorized as anti-nucleolar antibodies (ANoA), together with anti-Th/To (antibodies to protein components of the RNase MRP and RNase P particles)

Table 1. Anti-PM/Scl reactivity in PM, DM, Scl, PM/Scl-overlap and nondifferentiated idiopathic inflammatory myopathy (IIM) patients monitored by ID, IF and/or IP

	IIM		PM		DM		Scl		PM/Scl		
Study	# pos./n	%	# pos./n	%	# pos./n	%	# pos./n	%	# pos./n	%	Method
Wolfe (1977)			9/14	64	1/6	17			7/8	88	ID
Treadwell (1984)			2/22	9			2/32	6	9/77	12	ID
Reichlin (1984)	9/114	8									ID
Reimer (1988)							8/646	1			IF/IP
Reichlin (1988)			8/168	5							ID
Oddis (1992)	5/106	5					6/359	2	10/41	24	ID/IF
Hausmanowa (1997)			0/19	0	0/21	0			19/25	76	ID
O'Hanlon (2006)	65/603	11	13/227	6	19/177	11			32/101	32	ID
Selva-O'Callaghan (2006)	10/88	11	1/27	4	8/59	14					IP
Total	89/911	10	33/477	8	28/263	11	16/1037	2	77/252	31	

and anti-U3 (antibodies to proteins of the U3 snoRNP particle, in particular to fibrillarin). Although this method is not very specific for the detection of anti-PM/Scl autoantibodies, ANoA and ANA (anti-nuclear antibodies) are at least indicative for many CTD, especially since they are usually absent from healthy controls.[15,16] For this reason, this technique was often used as an initial screening method, which was followed by either immunodiffusion (ID), immunoprecipitation (IP), immunoblotting (IB) or enzyme-linked immunosorbent assays (ELISA).

A combination of the data from many studies addressing anti-PM/Scl reactivity in patient sera by either ID, IF and/or IP showed that this reactivity can be found in 31% of PM/Scl patients (Table 1). The frequency of anti-PM/Scl reactivity in patients diagnosed with PM, DM and SSc was 8%, 11% and 2%, respectively.

The cloning of cDNAs encoding individual protein subunits of the exosome and the production of the corresponding recombinant proteins in various expression systems created new possibilities to characterize the anti-PM/Scl autoimmune response and to screen patient sera for the occurrence of anti-PM/Scl autoantibodies. This facilitated the detection of autoantibodies to individual protein components of the exosome, e.g., by ELISA. Since IP assays can be quite laborious, especially when large cohorts of patients have to be analyzed and due to the poor recognition of the main antigens (PM/Scl-75 in particular) by the patient sera on IB, ELISA has become the method of choice to detect

Table 2. Anti-exosome(-associated) protein reactivity in PM, DM, Scl and PM/Scl-overlap patients monitored by ELISA[a]

	Antigen						
Diagnosis	PM/Scl –75	PM/Scl –100	MPP6	C1D	Mtr4	Ski2	hSki8
PM	0–3	2–8	0	5	2	5	0
DM	2–3	2–6	2	0	0	2	2
Scl	10	2–13	0	0	0	0	0
PM/Scl	27–28	23–55	0	23	7	0	3
References	20–22	20–22,24	0	20	0	20	20

[a] Reactivity values are given in percentages.

anti-exosome autoantibodies. During the last decade also a number of exosome-associated proteins, that are not considered to be part of the exosome core complex, but (transiently) associate with a subset of the exosome, have been identified and their cDNAs have been cloned and expressed.[14,17-20] A number of these proteins have been used as well to investigate whether they are also targeted by autoantibodies in (anti-PM/Scl-positive) patient sera (Table 2). A schematic overview of the targeting of exosome core components and of exosome-associated/auxiliary proteins by autoantibodies (as determined by ELISA) in PM/Scl-overlap sera is given in Figure 2.

The majority of anti-PM/Scl-positive patients appeared to have autoantibodies directed against PM/Scl-100 and/or PM/Scl-75 (Table 3). The other exosome core components are less frequently targeted, with the exception of Rrp4, which is recognized by 64% of the anti-PM/Scl-positive patients.[21,22] A combination of ELISA data for PM/Scl-100 and PM/Scl-75 leads to similar sensitivity scores as the conventional ID, IF, IB and IP assays (approximately 31% of PM/Scl patients are positive). After mapping a major autoepitope of PM/Scl-100,[23] a synthetic peptide corresponding to this epitope, designated "PM1-alpha", was produced to set up an ELISA, which allowed the detection of autoantibodies in 55% of the PM/Scl-overlap patients.[24] In addition, the C1D protein, a RNA-binding protein which binds to PM/Scl-100 and participates in exosome-mediated pre-rRNA processing, was found to be a major autoantigen in PM/Scl patients.[19,20]

Table 3. Anti-exosome core protein reactivity in anti-PM/Scl-positive PM, DM, Scl and PM/Scl-overlap patients monitored by ELISA[a]

	Antigen							
Diagnosis	PM/Scl –75	PM/Scl –100	hRrp4	hRrp40	hRrp41	hRrp42	hRrp46	hCsl4
PM	nd	nd	nd	nd	nd	nd	nd	nd
DM	nd	nd	nd	nd	nd	nd	nd	nd
Scl	64	100	64	0	0	36	0	0
PM/Scl	90	100	60	10	0	20	0	0

[a] Reactivity values are given in percentages.

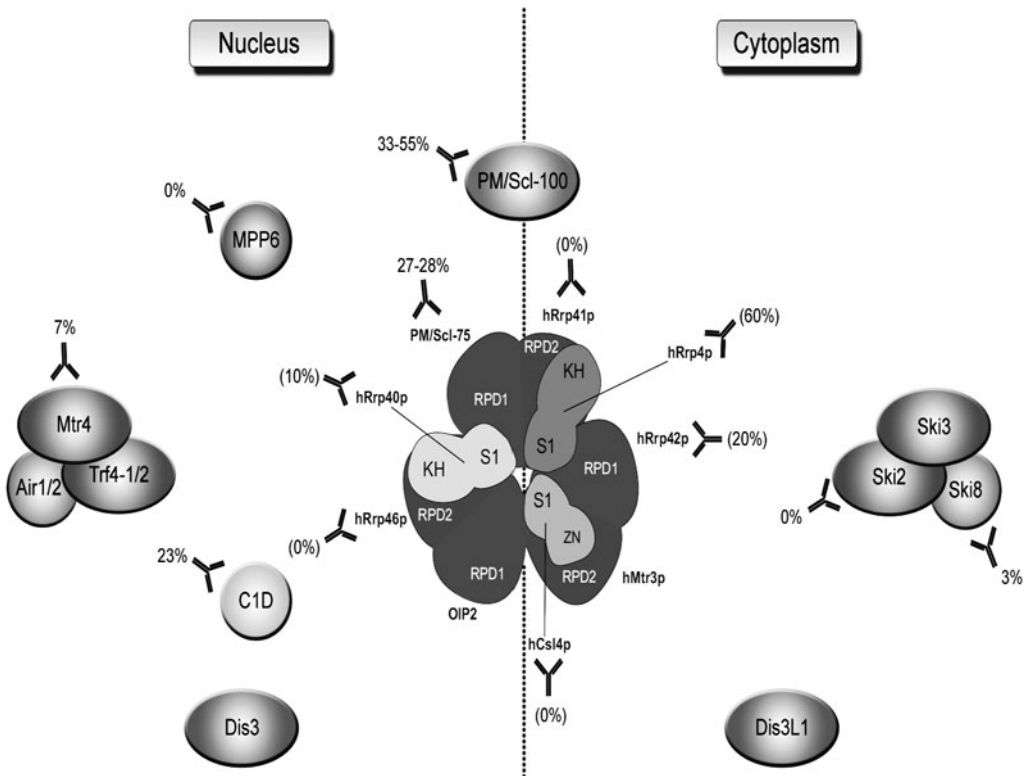

Figure 2. Targeting of exosome and exosome-associated proteins by autoantibodies from PM/Scl-overlap patients. Schematic structure of the human exosome. The nine core subunits assemble into a ring-like structure (central). RPD1 and RPD2 refer to two groups of RNase PH domains; KH, S1 and ZN to RNA-binding domains. Exosome-associated/auxiliary proteins and protein complexes that have been reported to interact with the exosome core are shown at the periphery. Proteins for which the presence of autoantibodies in PM/Scl sera has been investigated are indicated by the schematic antibody structures. Numbers (in percentages) represent the frequency of autoantibodies in patient sera. Numbers between brackets represent the frequencies for sera that were preselected based upon the reactivity with either PM/Scl-75 or PM/Scl-100.

Because many of the autoantibody studies described above have not yet been replicated with other cohorts of patients, the frequencies by which these antibody specificities occur should be interpreted with care. It is known that ethnic differences and genetic variation may affect the incidence of autoantibody production. The frequency of patients with anti-PM/Scl reactivity, for example, appears to be quite variable, as this reactivity was not found in a large cohort of 275 Japanese patients.[25] This may at least in part be due to the fact that the presence of anti-PM/Scl antibodies seems to be associated with certain MHC alleles, HLA-DRB1*0301, HLA-DQA1*0501 and HLA-DQB1*02,[26,27] whereas HLA-DRB1*15/*16 and HLA-DQA1*0101 might prevent the production of these autoantibodies.[28]

INITIATION OF THE ANTI-EXOSOME / PM/Scl AUTOIMMUNE RESPONSE

It is still an open question what triggers the anti-PM/Scl response in the initial stages of these autoimmune diseases. Several studies have proposed a role for unusual protein modifications, in particular those occurring during apoptosis and necrosis, which might play a role in breaking immunological tolerance to these proteins.[29,30] One of the core subunits of the exosome, PM/Scl-75, which is also one of the most frequently targeted proteins by anti-PM/Scl-positive sera, has been demonstrated to be cleaved by caspases in apoptotic cells.[31] Moreover, PM/Scl-100 appeared to be cleaved by granzyme B in vitro.[32] It is not known yet whether these changes lead to the formation of neo-epitopes. An alternative mechanism might be molecular mimicry, in which structural similarities between epitopes of foreign and self-proteins result in the cross-activation of autoreactive T or B cells by pathogen-derived peptides.

The targeting of multiple exosome subunits and exosome associated proteins is most likely the result of a phenomenon called intermolecular epitope spreading. When an immune response towards a particular protein is elicited, it can extend to another molecule that resides in the same complex. Data supporting this hypothesis regarding the human exosome is still scarce, although two bodies of evidence point in that direction. Firstly, immunization of rabbits with a synthetic peptide corresponding to a major epitope of PM/Scl-100 led to the generation of antibodies that targeted other components of the exosome complex as well.[33] Secondly, the serum of a patient with high anti-PM/Scl reactivity was shown to stain a single, 100 kDa polypeptide in IB. Three months later, the serum was found to stain an additional, 29 kDa polypeptide in IB, which was suggested to correspond to an aberrant form of PM/Scl-75, but might be one of the other core components as well.[34]

THE HUMAN EXOSOME AND CANCER

As already mentioned in the introduction, the viability of cells is severely impaired when the integrity of the exosome complex is disturbed. This strongly suggests that at least one of the functions of the exosome is essential to keep the cell in a proliferating state. On the other hand, when the activity of the exosome would be rate limiting in the maturation or turnover of RNAs that are crucial for cell proliferation, an overactive exosome may lead to a higher rate of proliferation. Although rather speculative, this is supported by observations of Yang and coworkers who examined patients who received a donor

lymphocyte infusion (DLI) as a treatment for chronic myelogenous leukemia (CML).[35] CML is characterized by the uncontrolled production of myeloid cells, resulting in a strong accumulation of these cells in the blood and is associated with a typical chromosomal translocation resulting in the so-called Philadelphia chromosome. This translocation leads to the production of an aberrant, overactive tyrosine kinase fusion protein, Bcr-Abl, which is thought to affect several substrate proteins involved in cell division, DNA repair and genomic instability. Of all the known cases of adult leukemia in the Western countries, about 15 to 20% have been classified as CML.[36] Before the introduction of Imatinib, a drug that specifically counteracts the activity of the Bcr-Abl fusion protein, patients suffering from CML often received a bone marrow transplantation (BMT). The patients who relapsed after this transplantation were further treated with DLI, resulting in a durable remission of the disease in 70 to 80% of the cases.[37] The success of this treatment is a result of a phenomenon called the graft-versus-tumor (GVT) effect, in which the infused lymphocytes attack any remaining cancerous cells in the bone marrow.

When Yang and colleagues used the serum of DLI-responding patients for an antibody-based screening of a CML cDNA expression library, new putative tumor-related antigens were identified.[38] Among these was a M_r 28,000 protein, which was designated CML28. Sequence analysis revealed that this protein is identical to hRrp46, a constituent of the exosome core complex (Fig. 2), although the 5' coding region of the CML28 cDNA is 33 amino acids longer than that of hRrp46. To confirm that hRrp46 is immunogenic in these patients, serum samples derived from a CML patient before and after BMT and DLI were analyzed with IB and ELISA using recombinant hRrp46. While anti-hRrp46 reactivity couldn't be detected in healthy controls and in the CML patient prior to DLI, this reactivity significantly increased 2 to 6 months post-DLI, after which the reactivity gradually declined to undetectable levels 2 years post-DLI. Moreover, the temporal pattern of anti-hRrp46 reactivity correlated well with the onset of cytogenetic remission, which is indicated by the disappearance of the Philadelphia chromosome. Since the patient didn't appear to have any symptoms indicating the development of an autoimmune disease, the anti-hRrp46 reactivity seemed to be associated with tumor rejection instead. The immune response towards hRrp46 was shown to be a common feature of other types of cancer, such as lung and prostate cancer and melanoma, in which the antibodies could be detected in 10% to 33% of the cases. The anti-hRrp46 response might at least in part be due to the overexpression of hRrp46 in these different cancers, as it was demonstrated by northern blot hybridization and IB that highly proliferating cell lines express high levels of hRrp46 when compared to normal tissues or even stable-phase CML. These findings might also prove useful for the development of an antigen-specific immunotherapy and progress in the development of such strategies has already been made.[39-41] The anti-hRrp46 immune response and elevated hRrp46 expression levels in cancer raise the question whether these phenomena are specific for hRrp46 or also occur for other exosome components. Further experiments will be required to clarify this issue.

Interestingly, the antimetabolite 5-fluorouracil, which is frequently used to treat solid tumors in a variety of cancers, was shown to inhibit the function of the exosome. Originally this drug was selected for its hypothetical ability to inhibit cell proliferation, presumably by causing thymidine starvation and thus negatively affecting DNA synthesis. However, Fang and coworkers reported that also rRNA processing was impaired by this drug.[42] The accumulated pre-rRNA precursors were similar to the precursors accumulating in exosome mutant yeast strains. Possibly related to these observations, it was demonstrated that the absence of Rrp6 (the yeast counterpart of PM/Scl-100) enhanced the 5-fluorouracil-induced

defects.[43] Taken together, these results suggest that 5-fluorouracil exerts its effect on rRNA processing at least in part by inhibiting the exosome.

CONCLUSION

In view of the central role of the human exosome in the processing and degradation of many RNAs, it is intriguing to investigate to what extent a disturbance of its function can interfere with normal cellular physiology and is associated with diseases. The two best documented examples, autoimmunity and cancer, have been discussed in this chapter, although there are still many questions that need to be addressed. It is, for example, still unclear what causes the targeting of PM/Scl-75 and PM/Scl-100 by the immune system in patients suffering from the PM/Scl-overlap syndrome and why this response is particularly associated with this autoimmune disease. In view of the physical barrier of the plasma membrane, autoantibodies are not likely to interfere with the functions of the exosome in RNA metabolism, but once exosome components are released from (dying) cells the resulting immune complexes may contribute to the progression of autoimmunity and tissue damage. In case of cancer dysfunctioning of the exosome may play a direct role, but it is clear that more work has to be done to investigate the effects of increased or decreased exosome activity on cell proliferation. It is currently not known whether exosome core subunits other than hRrp46 are overexpressed in solid tumors as well. Alternatively, a pool of non-exosome associated hRrp46 may exist and might have a completely other role independent of the exosome. In this respect, also the importance of the N-terminal extension, which may be specific for a particular isoform of hRrp46, needs to be studied.

The exosome is known to play an important role in the degradation of a special class of mRNAs, containing cis-acting adenylate-uridylate-rich sequence elements (AREs) in their 3′UTR. Transcripts containing such elements are often involved in important biological processes and their levels are therefore often tightly regulated. In theory, a perturbation of exosome function is likely to affect these levels and, as a consequence, might cause a wide variety of pathological conditions.[44]

In this chapter we focused on human diseases, but one should realize that the exosome is expressed in many other species and thus might also here be associated with disease. In plants, for instance, it was found that the effects of deletion of a particular exosome component mimicked a disorder that induced cell death in the tip of the leafs when plants are inoculated with *Blumeria graminis*, a fungus that causes mildew on grasses and that is frequently used to infect plants in a laboratory setting. Intriguingly, the deleted exosome component was Rrp46.[45]

We conclude that although targeting of exosome components in autoimmunity is known for many years, our knowledge on the involvement of the exosome, of its individual components or of its auxiliary factors in other diseases is still in its infancy and much work needs to be done to obtain more insight in this topic.

ACKNOWLEDGEMENTS

We thank Reinout Raijmakers as well as present and former members of our laboratory for stimulating discussions and contribution of ideas and information to this chapter.

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