metabolites, (iii) identification of orthologous vesicular components, (iv) an array of tools for bioinformatic analyses including sequence search, set analysis, Gene Ontology enrichment analysis and network analysis, as well as (v) 'My EVpedia', a personalized function of EVpedia. Using 'My EVpedia', users privately store their own datasets, analysis results and publications of interest by creating their own accounts. New functions of this updated EVpedia are indicated in red texts in Figure 2.

We invite the research community to submit EV-related multiomics data and publications to EVpedia.

As of May 2014, a total of 6879 EV-related publications with 3336 principal investigators have been cataloged in EVpedia. In addition, a total of 172 080 vesicular components from 263 high-throughput datasets are listed (Table 1). All of these vesicular components could be searched by their sequences and browsed. Furthermore, in the 'Top 100+ EV markers' menu, the current vesicular components are sorted in the descending order of their identification counts, which are the numbers of datasets identifying those vesicular components or their orthologs.

5 Community participation and annotation in EVpedia

After the initial launch in January 2012, EVpedia has been globally accessed more than 65 000 times from more than 750 cities (Table 1). For community annotation of EVpedia, we built 'EVpedia Community'. About 350 members from 73 international EV research groups have joined this community, in which they can exchange EV-related information and submit their multiomics data via the 'User forum' and 'Upload' menu bars in EVpedia, respectively. In addition, non-members can easily join the 'EVpedia Community' by adding their information via clicking the 'Sign In' menu. Moreover, EVpedia has been cross-linked with the website of the 'International Society of Extracellular Vesicles' (http://www.isev.org).

6 Concluding remarks

EVpedia is a comprehensive database of EVs derived from prokaryotes and eukaryotes. Currently, a total of 6879 EV-related publications and 172 080 vesicular components (proteins, mRNAs, miRNAs, lipids and metabolites) are deposited in this public repository. For the systematic analysis of EVs, EVpedia also provides integrated systems biology research tools such as 'Experiment', 'Browse', 'Analysis', 'Top 100+ EV markers' and 'My EVpedia' menu bars. In the future, additional multiomics datasets and publications will be deposited, and we expect more researchers to join the 'EVpedia Community' and to share their research data. The community database is scheduled to be updated every 3 months. EVpedia, a community web portal for EV research, should serve as a useful resource to stimulate the emerging field of EV biology research and to help us to elucidate the fundamental roles of these complex extracellular organelles.

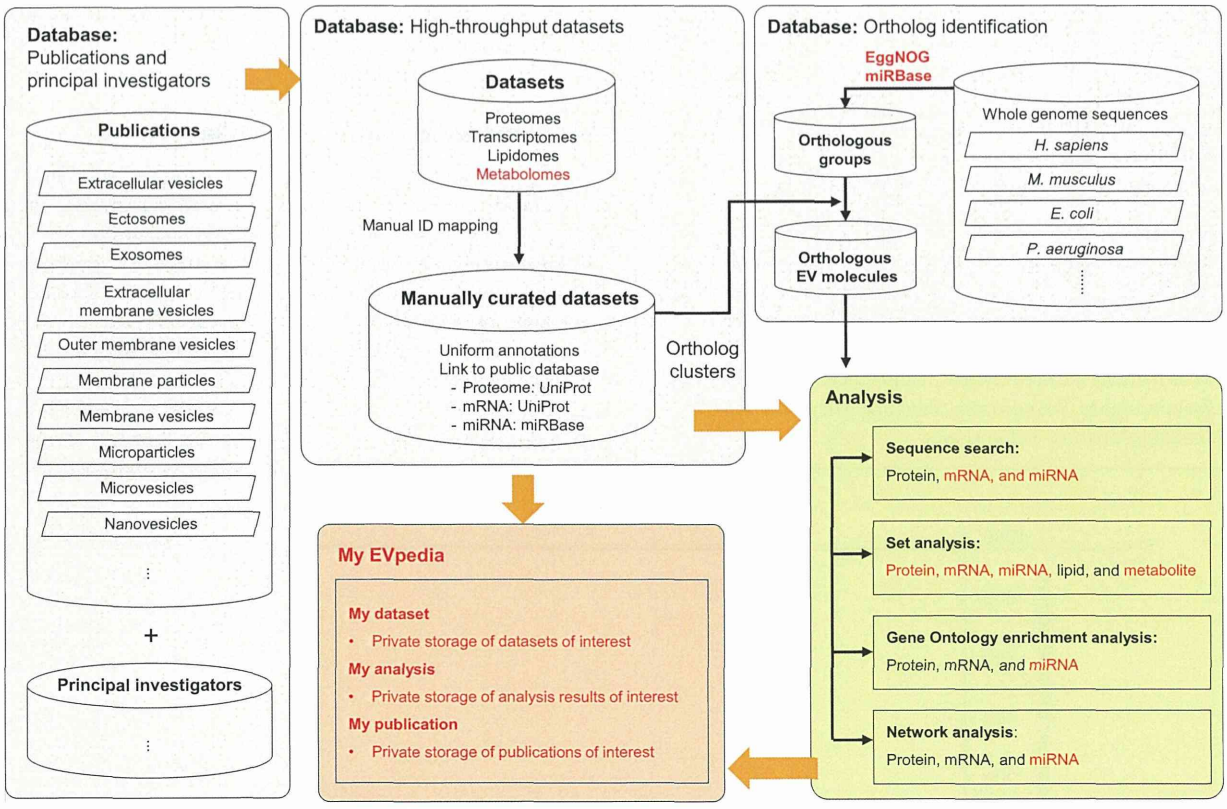

Fig. 2. Overall structure of EVpedia. EVpedia provides a comprehensive database for (i) publications and principal investigators, (ii) vesicular proteins, mRNAs, miRNAs, lipids and metabolites and (iii) identification of orthologous vesicular components. For systematic analyses of vesicular components, there is an array of tools for sequence search, set analysis, Gene Ontology enrichment analysis and network analysis. 'My EVpedia' is a personalized function of EVpedia to deposit the user's own datasets, analysis results and publications of interest. Note that red texts indicate newly included functions in the updated version of EVpedia

Table 1. EVpedia statistics

	All	Eukaryotes	Prokaryotes
Publications			
Articles	6879	6021	858
Principal investigators	3336	2886	483
Proteomes			
Studies	117	97	20
Datasets	176	148	28
Proteins	78 971	74 696	4275
Transcriptomes			
mRNA			
Studies	17	17	0
Datasets	28	28	0
mRNAs	74 430	74 430	0
miRNA			
Studies	11	11	0
Datasets	29	29	0
miRNAs	18 119	18 119	0
Lipidomes			
Studies	22	21	1
Datasets	29	28	1
Lipids	550	534	16
Metabolomes			
Studies	1	1	0
Datasets	1	1	0
Metabolites	10	10	0
Participating			
Laboratories (countries)	73 (20)		
Accesses (countries)	66 617 (73)		

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Journal of Extracellular Vesicles

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EDITORIAL

Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles

Secreted membrane-enclosed vesicles, collectively called extracellular vesicles (EVs), which include exosomes, ectosomes, microvesicles, microparticles, apoptotic bodies and other EV subsets, encompass a very rapidly growing scientific field in biology and medicine. Importantly, it is currently technically challenging to obtain a totally pure EV fraction free from non-vesicular components for functional studies, and therefore there is a need to establish guidelines for analyses of these vesicles and reporting of scientific studies on EV biology. Here, the International Society for Extracellular Vesicles (ISEV) provides researchers with a minimal set of biochemical, biophysical and functional standards that should be used to attribute any specific biological cargo or functions to EVs.

Keywords: *extracellular vesicles*; *microvesicles*; *microparticles*; *exosomes*; *ectosomes*; *extracellular RNA*

Over the past decade, there has been a rapid growth in studies of secreted membrane vesicles, collectively called extracellular vesicles (EVs). Publications in high-impact journals have proposed exciting functional roles of EVs. In particular, the knowledge that EVs can shuttle functional nucleic acids between cells (mRNA, miRNA or other RNA species) has fundamentally changed the thinking about gene regulation, as the EVs can regulate the recipient cell at a post-transcriptional level (1–3).

However, the extracellular milieu is more complex as several body fluids (especially serum/plasma) harbour extracellular RNA (exRNA) in other non-EV carriers, including protein complexes (AGO2) (4) and lipoproteins [HDL and LDL (5)]. Separation of these non-vesicular entities from EV is not fully achieved by common EV isolation protocols, including centrifugation protocols or commercial kits that claim EV or "exosome" isolation/purification. Also, the composition of recovered EVs vary vastly according to the protocols used (6–8). In particular, polymer-based methods to precipitate EVs (used by some commercial kits) do not exclusively isolate EVs, and are likely to co-isolate other molecules, including RNA–protein complexes. Consequently, there is a need to determine the distinct contribution of EVs in any experiment that describes the molecular content or the functional consequences of the isolated material.

We recognize that different experimental systems, sources of biological specimens, investigator's experience and instrumentation used contribute to the heterogeneity of published protocols and the interpretation of results. A framework for providing data and attributing functions to EVs was discussed by the Executive Committee of the International Society for Extracellular Vesicles (ISEV), a group of scientists with collective long-term expertise in the field of EV biology. Here, we propose a series of criteria, based on current best-practice, that represent the minimal characterization of EVs that should be reported by investigators. Adoption of these criteria should aid researchers in planning studies as well as reporting their results. In addition, we suggest appropriate controls that should be included in EV-related functional studies. These controls should support conclusions regarding the functions of EVs and their relationship to physiologic and pathologic mechanisms.

The term "exosomes" is the most commonly used word to designate any type of EV (Fig. 1), and this has become a "buzz term" for EV-related science. The actual meaning of this word, however, is not universally accepted [see letter by Gould and Raposo (9)]. Many publications specify that exosomes are formed in endosomal multivesicular compartments and are secreted when these compartments fuse with the plasma membrane. However, the isolated material generally studied contains a mixture of EVs. Unfortunately, the field of EV research has not matured to the point that we can propose a list of EV-specific "markers" that distinguish subsets of EVs from each other, for example, EVs produced via budding from the cell membrane or produced via endosomal compartments.

The criteria we provide here can be used by researchers to guide them in discriminating EV from non-EV components. These criteria will be updated with improvements

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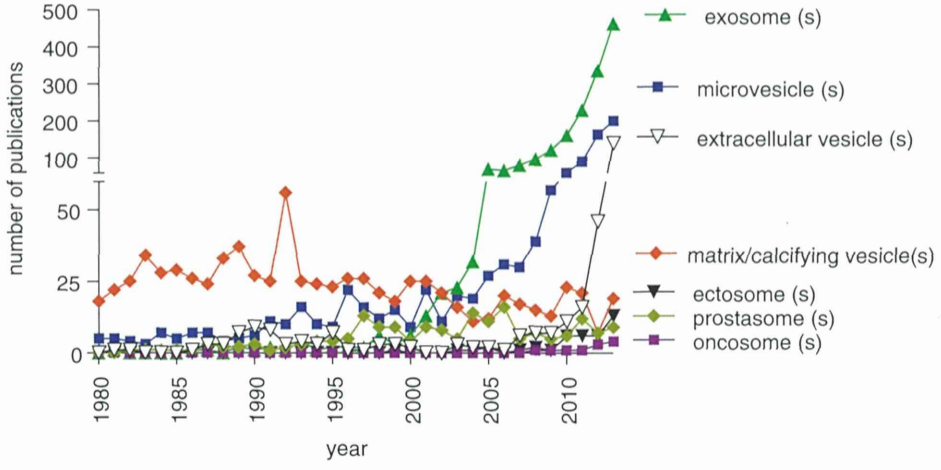

Fig. 1. Comparative evolution of the use of different terms for EVs in the literature. An advanced search was performed in PubMed at the end of December 2013 to find, for each year of publication, all articles using the given term (singular or plural) as text word: exosome(s), microvesicles, oncosome(s), ectosome(s), prostasome(s), matrix/calcifying vesicle(s). Year of final publication (and not advanced online date) of articles in English (and not other languages) was taken into account. Manual elimination of articles describing non-EV-related work was performed for exosome(s) (RNA-excision machinery) and microvesicle(s) (intracellular secretory vesicles). Use of the term microparticle(s) could not be reliably evaluated, since it is massively used to refer to non-vesicle-related particles. Notably, from 2004 onwards, the term "exosome" has become the most often used in published articles describing EVs, whereas the term "extracellular vesicles," chosen as generic term at creation of ISEV in September 2011, is steadily growing. This figure is not intended to show expansion of the EV field as compared to other fields, since numbers are not normalized to the total number of scientific medico-biological publications per year.

in the "state of the art," and we hope to eventually be able to provide specific markers and characteristics of EV subtypes. In the meantime, readers can also refer to 2 detailed Position Papers of ISEV published in 2013, listing recommendations on EV isolation (10) and EV/exRNA analysis (11).

Minimal requirements to claim the presence of EVs in isolates

One of the first criteria to define EVs is that they are isolated from extracellular fluids, that is, from conditioned cell culture medium or body fluids. Importantly, collection of the EV-containing fluid must be gentle, limiting cell disruption. Mechanical disruption of cells or tissues can result in isolation of vesicles that originate from the intracellular compartments, which obviously would reduce the purity of EVs. Therefore, the term "EVs" may not be appropriate for materials isolated in such ways.

Since there is currently no consensus on a "gold-standard" method to isolate and/or purify EVs, it cannot be claimed that there is an "optimal" method that should be uniformly used. The reader should be aware that the methods that are most efficient probably depend on (a) the specific scientific question asked and (b) on the downstream applications used. However, we urge researchers to describe in detail the methods used for EV isolation, to allow interpretation and replication by other researchers. Further, we also suggest a format of characteristics of EVs that should be analyzed and then provided in publication.

General characterization of EVs

A general overview of the protein composition of each EV preparation should be provided, at least in a first publication, including description or quantitation of components not necessarily expected to be present on or in EVs (see Table I). Although numerous proteomic analyses have highlighted proteins commonly found in exosome preparations, it is becoming clear that these do not represent "exosome-specific" markers but rather "exosome-enriched" proteins, as different subsets of secreted EVs contain many common markers. However, the relative proportions of different proteins seem to vary in the different types of EVs. Therefore, we suggest that investigators report the amount of *several* proteins (3 or more) in at least a *semi-quantitative* manner in any EV preparation, including EV isolates from body fluids or obtained from secreting cells *in vitro*. The proteins described and characterized should be proteins *expected* to be present in the EVs of interest, especially transmembrane proteins and cytosolic proteins with membrane-binding capacity (Table I, groups 1 and 2). In addition, the level of presence of proteins *not expected* to be enriched in EVs of endosomal origin should also be determined (Table I, group 3). This description will cast light on the extent of co-isolation of EVs of different intracellular origins and nature in the isolates (Table I). Furthermore, investigators can compare their protein isolates with those described in other EVs, by searches within databases [EVpedia and Vesiclepedia (12,13)].

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Table I. Different categories of proteins and their expected presence in EV isolates, including some examples (non-exclusive)

1. Transmembrane or lipid-bound extracellular proteins	2. Cytosolic proteins	3. Intracellular proteins	4. Extracellular proteins
Argues presence of a membrane in the isolate	With membrane- or receptor-binding capacity	Associated with compartments other than plasma membrane or endosomes	Binding specifically or non-specifically to membranes, co-isolating with EVs
Present or enriched in EVs/ exosomes	Present or enriched in EVs/ exosomes	Absent or under-represented in EVs/exosomes, but present in other types of EVs	Variable association with EVs
Examples:	Examples:	Examples:	Examples:
Tetraspanins (*CD9*, *CD63*, *CD81*)	Endosome or membrane-binding proteins (*TSG101*, annexins = *ANXA**, Rabs = *RAB**)	Endoplasmic reticulum (Grp94 = *HSP90B1*, calnexin = *CANX*)	Acetylcholinesterase (*ACHE*)
Integrins (*ITG***) or cell adhesion molecules (*CAM**)		Golgi (*GM130*)	Serum albumin
Growth factor receptors		Mitochondria (cytochrome C = *CYC1*)	Extracellular matrix (fibronectin = *FN1*, collagen = *COL*A**)
Heterotrimeric G proteins (*GNA***)	Signal transduction or scaffolding proteins (syntenin)	Nucleus (histones = *HIST*H**)	Soluble secreted proteins (cytokines, growth factors, matrix metalloproteinases = *MMP**)
Phosphatidylserine-binding *MFGE8*/lactadherin		Argonaute/RISC complex (*AGO**)	

At least one protein of each category 1, 2 and 3 should be quantified in the EV preparations. EV association of proteins of category 4 should be demonstrated by other means.
Italics: official gene names; *, ** denotes different possible family members.

Analytic approaches can include Western blots (WB), (high resolution) flow cytometry (FACS) or global proteomic analysis using mass spectrometry techniques to identify e.g. transmembrane proteins. We recommend that analyses should be performed in a semi-quantitative manner, for example, using intensity analysis of Western blot signals or specific mean fluorescence intensity as compared to isotype control in FACS. When EVs secreted *in vitro* by cultured cells are analyzed, their composition should ideally be *compared with that of the secreting cells*, to determine level of enrichment of the EV components. This is *not possible for biological fluid-derived EVs*, as these are produced by a vast array of cells in the tissues. In that case, we recommend that reports include the relative proportion of different EV-associated proteins.

Table I lists the different categories and examples of proteins whose presence/absence should be simultaneously analyzed. Caution should be taken when using the enzymatic activity of proteins to indirectly determine the concentration of vesicles in any sample. An example of this is acetylcholinesterase (ACHE), a GPI-anchored protein localized in the membrane of reticulocytes, which is present in multiple membrane-anchored and non-membrane-anchored secreted forms also in other cells (14). While the activity of ACHE has been used as a marker of EVs released by reticulocytes, the use of this (or any other proteins in which activity can be measured) requires confirmation of the presence of the protein by Western blotting or functional inhibition by a specific enzyme inhibitor, as well as the recognition that these do not represent specific markers of EVs or exosomes (Table I, group 4). Therefore, their use should be restricted to cases where it is not possible to use other quantitative measures as described above, and the reasons for using them should be clearly justified.

Given the variable quality of commercial and home-made antibodies used for quantitation studies, appropriate *negative controls* should also be used and their results should be presented. These controls are best provided in the first reports using these antibodies. Ideally, the signal obtained in EVs should be compared to signals obtained from the biological fluid or conditioned medium depleted of EVs (i.e. recovered after the isolation procedure) and/or from complete medium non-conditioned by cells but processed for EV purification as conditioned medium. The reader should be aware that the supernatant, for example, after a 70-minute post-ultracentrifugation, still contains significant quantities of remaining EVs (15). The Methods section of reports should also contain details of the antibodies used (source, catalogue number and dilution) and conditions of preparations of the samples (e.g. reducing/non-reducing conditions for Western blot, an important issue to analyze some tetraspanins).

Characterization of single vesicles

We recommend characterization of single vesicles within a mixture to be performed, to provide an *indication of the heterogeneity* of the EV preparation studied. As a general rule, at least 2 different technologies should be used to characterize individual EVs. For electron microscopy

(Transmission EM) or atomic force microscopy (AFM), images should show a *wide field* encompassing multiple vesicles in addition to close-up images of single vesicles. For larger vesicles such as apoptotic bodies, cytospins and/or immunofluorescent images may be presented to provide an overview of vesicles isolated, again not focusing on a single vesicle. *Size distribution* measurements of EVs, such as nanoparticle-tracking analysis, dynamic light scattering, or resistive pulse sensing provide diameters of a large number of vesicles. However, the values acquired with these techniques should be compared with TEM, AFM or other microscopy techniques, since they do not distinguish membrane vesicles from co-isolated non-membranous particles of similar size.

Studies of the functional activity of EVs: recommendations for controls

When *in vitro* functional studies are performed with isolated EVs, a *quantitative analysis* of the dose–function relationship should be presented. This dose–response curve should be supplemented by data on the volume of starting fluid and/or the number of producing cells used to isolate the range of functional EVs.

It is important to make use of systematic *negative controls* which should exhibit minimal functional effects. These may include "mock" EVs obtained from culture medium that has not been conditioned by the cells of interest (but incubated at 37°C as if used in culture) or the fluid remaining after the EV isolation (for body fluids and conditioned medium). These controls provide insights into the "background" functional activity or signal and possibly the proportion of functional "activity" present in the soluble versus EV-associated components of the isolated fluid. Clearly, there is value to negative controls being performed at concentrations of negative EVs approximating those of functional EVs. Foetal calf serum EVs and their protein and RNA cargo can influence measurements (16,17). Thus, there should be efforts to perform studies in the absence of the serum-derived EVs. In this regard, it should be noted that 70 minutes of high speed centrifugation is insufficient to remove EV RNA cargo in foetal calf serum (18).

The ISEV Executive Committee remains concerned about the future reporting of functional changes ascribed to specific single or small clusters of molecules (protein, RNA or other) associated with EVs. This will increasingly be important, as EV biomarkers, EV therapeutics and fundamental mechanisms of EV function are brought to clinical utility or claimed in patent protection drafts. *Demonstration of association* of these molecules to EVs should therefore be provided for such use. Some proteins (Table I, group 4), but also different RNA species (5), have been variably described as co-isolated with EVs, but may not necessarily be harboured in EVs. For instance, MMP9 has been described as secreted with EVs (19,20) or, conversely, as a soluble non-EV-associated molecules (21).

A direct approach to prove association of these molecules to EVs can be fractionation of the EV preparation using density gradients. Separation of EVs from other particulate material can be guaranteed only by floatation (=upward displacement). However, for some other separations, sedimentation (=downward displacement) may be more appropriate. Such separation should be followed by qPCR or other biochemical detection methods, and the functional moiety and/or biomarker cluster should be co-fractionated with the transmembrane or EV-enriched cytosolic protein used to characterize EVs (Table I). Thus, the functional activity should be resident within defined density gradient fractions specifically containing the EV proteins. Importantly, we are aware that some density gradients often used may alter or impede functional tests performed.

An alternative approach to link functional activity, or specific molecules, with isolated EVs may be based upon antibody-mediated capture or depletion of EVs from the biofluid or conditioned medium. The antibodies used should be specific to the transmembrane protein of the characterized EVs. In these studies, depleted preparations will have lost functional activity, whereas the antibody-captured EVs should retain it (if proper and non-destructive elution from the antibody-coated beads used for capture is technically possible). We realize that EVs with functional activity but without the transmembrane protein also exist, and thus would not be depleted nor captured with this approach.

Another approach would include the use of fluorescent labels of EVs incubated with target cells. Unstained EVs and non-EV dye materials and aggregates must be eliminated with appropriate technology when this method is used. As EVs elicit their function by binding to, fusing with or being uptaken into recipient cells, it could be possible to determine a functional activity in fluorescent cells (EV-associated cells) versus non-fluorescent cells.

In the absence of any of the above proposed controls, investigators may still conclude that an extracellular functional activity exists and affects recipient cells, but the specific EV nature of this function should not be claimed.

Conclusion

The EV field is rapidly expanding and becoming increasingly complex, especially as it overlaps with the even newer field of exRNA-mediated communication. A generic biological standard of EVs, or of "exosomes," would be very useful as a baseline to compare EV preparations obtained by individual laboratories, and we are aware that European and US networks of researchers are working towards establishing such standards. When available, these standards may provide comparative EV preparative data

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and also could support inter-laboratory comparisons. However, such standards are not yet available, due to the lack of universal or unequivocally specific markers of EVs, a situation linked to the fact that the content of EVs is probably highly context-dependent. Such tools will therefore only become available with increased knowledge of the core composition and, perhaps, core functions of EVs recovered from diverse sources. Nonetheless, to harmonize research practice in the field of EV research, and to ensure an acceptable level of data comparability, we herein propose that technical and experimental information is provided in significant detail in any published scientific article, and that the characterization includes *a minimal* set of proofs of the EV relationship to the observations reported. We hope that the minimal requirements presented in this editorial therefore will increase awareness of all researchers for potential confounders in their EV-related results, and thus help editors and reviewers of journals other than *J Extracell Vesicles*, less specialized in EVs, to better assess and promote advances in the exceptionally promising field of EV research.

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Declaration of interest and/or relationship

JL: Co-owner of patents from 2006 to 2012 (approved and pending) for using exosomes as therapeutics. AFH: Co-owner of patent (pending) for using exosomal miRNA as a biomarker; shareholder in D-Gen Ltd. DDV: Inventor of 2 patents on EVs as circulating biomarkers of cancer (pending). YSG: Inventor of patents for using EVs as therapeutics, diagnostics and vaccines; founder of Aeon Medix and own stock in the company; sponsored research agreements with AmorePacific. IK: Co-inventor of a technology for purification of exosomes licensed to Cell Guidance Systems Ltd that uses it for manufacturing Exo-spin™ kits. SS: Co-inventor of a patent (pending) on the use of exosomes as therapeutics in cardiovascular diseases. Sponsored research agreements with Baxter Healthcare and NeoStem. HT: Founder of MiRTeL, and own stock in the company. MHW: Collaborative research agreement with Friesland Campina on bovine milk EVs and with BD Biosciences on high resolution flow cytometry of EVs, and partnership between Technology Foundation STW, Utrecht University and Danone Nutricia research on EV-based biomarker profiling in human breast milk. KWW: Scientific research agreement with AgriSciX, Inc. Other authors declare no significant relationships or conflicts.

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DSE-FRET: A new anticancer drug screening assay for DNA binding proteins

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Nuclear factor-κB (NF-κB) is a key regulator of cancer progression and the inflammatory effects of disease. To identify inhibitors of DNA binding to NF-κB, we developed a new homogeneous method for detection of sequence-specific DNA-binding proteins. This method, which we refer to as DSE-FRET, is based on two phenomena: protein-dependent blocking of spontaneous DNA strand exchange (DSE) between partially double-stranded DNA probes, and fluorescence resonance energy transfer (FRET). If a probe labeled with a fluorophore and quencher is mixed with a non-labeled probe in the absence of a target protein, strand exchange occurs between the probes and results in fluorescence elevation. In contrast, blocking of strand exchange by a target protein results in lower fluorescence intensity. Recombinant human NF-κB (p50) suppressed the fluorescence elevation of a specific probe in a concentration-dependent manner, but had no effect on a non-specific probe. Competitors bearing a NF-κB binding site restored fluorescence, and the degree of restoration was inversely correlated with the number of nucleotide substitutions within the NF-κB binding site of the competitor. Evaluation of two NF-κB inhibitors, Evans Blue and dehydroxymethylepoxyquinomicin ([−]-DHMEQ), was carried out using p50 and p52 (another form of NF-κB), and IC_{50} values were obtained. The DSE-FRET technique also detected the differential effect of (−)-DHMEQ on p50 and p52 inhibition. These data indicate that DSE-FRET can be used for high throughput screening of anticancer drugs targeted to DNA-binding proteins.

DNA-binding proteins such as transcription factors play key roles in normal biological processes and in development of disease. Nuclear factor-κB (NF-κB) is a DNA-binding protein involved in inflammation and tumorigenesis, and thus inhibition of NF-κB signaling is a potential target for cancer therapy; however, there are few direct inhibitors of NF-κB binding to DNA. In general, DNA-binding proteins are attractive therapeutic targets,[1,2] but conventional methods for detecting protein–DNA binding, such as a gel-shift assay,[3] DNA footprinting assay,[4,5] and ELISA,[6] are laborious and time-consuming, and thus not amenable to combinatorial screening.

Fluorescent-based homogeneous methods have been exploited for detection of sequence-specific protein–DNA binding,[7–9] including use of the split probe "molecular beacon" method in a FRET-based assay.[7] In this method, a DNA fragment containing a protein binding site is split into two parts in the middle of the site. One part is labeled with a donor fluorophore and the other has an acceptor fluorophore. Fluorescence resonance energy transfer between the two fluorophores is produced as a result of protein-dependent association of the split DNA fragments. The molecular beacon is simple in principle and is cost-effective. However, probe designs are complicated for three reasons. First, protein-independent association of the two DNA molecules may occur. Second, fluorophores can disturb protein–DNA binding or fluorescence can be changed by the protein because the fluorophores are introduced into nucleotides proximal to the protein binding site. Finally, nicks in the associated DNA fragment can influence protein binding because proteins interact with the phosphate backbone, in addition to the bases. Fluorescence-based DNA footprinting can overcome these drawbacks.[8,9] This method is based on a DNA-binding protein protecting its target DNA against exonuclease III digestion. However, the method requires careful quality control of exonuclease III activity to obtain stable data, and the half-life of the protein–DNA complex must be long compared with the time required for the exonuclease III reaction.

The Holliday junction is a four-way DNA structure that is the central intermediate in homologous recombination. Branches of the structure migrate spontaneously *in vitro* as a result of strand exchange between two DNA molecules, and DNA-binding proteins including the histone octamer, p53, TRF1, and TRF2 suppress the strand exchange.[10–12] We have found that NF-κB also suppresses this strand exchange *in vitro*. Based on these findings, we developed a new homoge-