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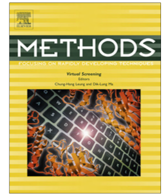

PTEN secretion in exosomes

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ABSTRACT

PTEN was discovered as a membrane-associated tumor suppressor protein nearly two decades ago, but the concept that it can be secreted and taken up by recipient cells is revolutionary. Since then, various laboratories have reported that PTEN is indeed secreted and available for uptake by other cells in at least two different guises. First, PTEN may be packaged and exported within extracellular vesicles (EV) called exosomes. Second, PTEN may also be secreted as a naked protein in a longer isoform called PTEN-long. While the conditions favouring the secretion of PTEN-long remain unknown, PTEN secretion in exosomes is enhanced by the Ndfip1/Nedd4 ubiquitination system. In this report, we describe conditions for packaging PTEN in exosomes and their potential use for mediating non-cell-autonomous functions in recipient cells. We suggest that this mode of PTEN transfer may potentially provide beneficial PTEN for tumor suppression, however it may also propagate deleterious versions of mutated PTEN causing tumorigenesis.

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1. Introduction

Traditionally, cell–cell communication can occur via gap junctions, membrane nanotubes, cell–cell adhesion and release and uptake of growth factors, neurotransmitters or hormones [1,2]. More recently, another cell–cell communication system has been identified involving the release and uptake of extracellular vesicles (EV) called exosomes [3] (Fig. 1). Exosomes were first discovered 30 years ago as vesicles for the secretion of unwanted proteins from reticulocytes [4]. Since then, exosomes have been purified from a variety of body fluids including blood, urine, saliva, amniotic fluid, milk, tears and cerebrospinal fluid [3]. Exosomes (40–100 nm) should not be confused with other vesicles that directly bud off from the plasma membrane known as ectosomes (50–1000 nm) [5]. A third category of extracellular vesicles are known as apoptotic blebs (50–5000 nm) that are released by dying cells. To avoid confusion, the main distinguishing characteristic of exosomes is their density and morphology which allows them to be isolated by differential centrifugation [6].

Another distinguishing feature is the mechanism of biogenesis (Fig. 1). Exosomes are released from multivesicular bodies (MVBs) following invagination of the MVB membrane into the lumen, engulfing components of the cytoplasm that include proteins, lipids and nucleic acids (DNA, mRNA, microRNA). These components are loaded into pre-exosomal structures called intraluminal vesicles before fusion of the MVB with the cell membrane and disgorgement of the exosomes. Under the electron microscope, exosomes assume a cup shape after fixation and have a typical size of 40–100 nm with buoyant density of 1.10–1.19 g/cm^3 [7]. Many cell types in the body secrete exosomes, therefore it is no surprise that the cargo is reflective of the cell type of origin or body fluid from which it is isolated. Indeed, as catalogued by the online resource Exocarta (www.exocarta.org) and Vesiclepedia, exosomes can reflect the pathophysiology of the cell/animal/person [8]. But perhaps the most exciting attribute of exosomes, giving rise to an entire new field of research, is their use as vehicles for short and long-range cell–cell communication in normal and disease settings. For example, cancer cells secrete exosomes containing oncogenic proteins that can initiate tumorigenesis in recipient cells [9]. Tumour cells also secrete exosomes capable of altering their host environment for metastasis by releasing proteases for cell invasion [10], or angiogenic factors (VEGF and FGF) for neovascularization [11]. Cancer cells can also receive exosomes from their stromal environment, triggering metastasis [12].

An interesting question concerns what proteins get loaded into which exosomes, and how is this process regulated? The answer to this question is largely unknown, except that exosomes from different tissues and different physiological states appear to have distinguishing cargo. So far, two biogenic pathways for generating exosomes have been identified, depending on whether they require proteins of the ESCRT complex (endosomal sorting

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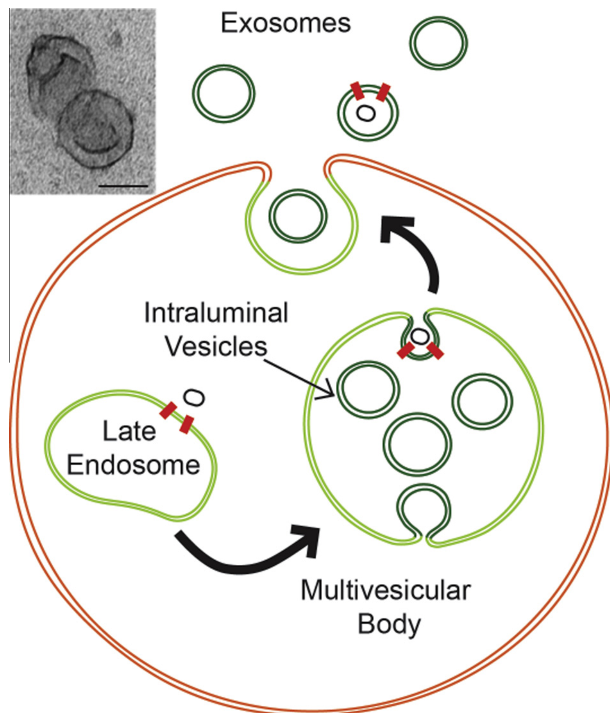

Fig. 1. Schematic diagram of exosome biogenesis from late endosome stage through to multivesicular body before fusion with cell membrane and secretion into the extracellular environment. Inset shows TEM picture of cup-shape exosome after fixation. Scale bar: 50 nm.

complex responsible for transport) [3]. (1) The ESCRT-dependent pathway, where ESCRT proteins recognise and sequester ubiquitinated proteins in the endosomal membrane for selective loading of cargo; (2) the ESCRT-independent pathway which does not require ESCRT proteins but requires the sphingomyelin ceramide and membrane partitioning protein tetraspanin [13].

Given the importance of PTEN in amassing cell defense systems for combating tumorigenesis, it was a reasonable hypothesis that PTEN may also be secreted for uptake by recipient cells with potentially non-cell-autonomous effects. Indeed, PTEN secretion in exosomes has been demonstrated by four independent groups [14–17].

In this report, we provide details of procedures for producing exosomes containing PTEN from a number of cell lines. We showed that Ndfip1 is required for exporting PTEN in exosomes, suggesting a role for Ndfip1/Nedd4 ubiquitination in this process.

2. Methods and results

2.1. Choice of cells

Traditionally, exosomes can be harvested from cultured cells following their secretion into the culture media. When choosing cell lines for generating PTEN in exosomes, it is important to ascertain if PTEN and Ndfip1 are endogenously expressed. This is best ascertained by prior Western blot analysis using commercially available antibodies. If the cell line has endogenous PTEN and Ndfip1 (e.g. mouse embryonic fibroblasts, MEFs) it is likely that PTEN would be secreted into the media without further modification [14]. In support of this, Gabriel and co-workers have showed that a prostate cancer cell line DU145 is capable of secreting PTEN in exosomes without manipulation [15]. On the other hand, cells containing only endogenous PTEN but no Ndfip1 (e.g. *Ndfip1*$^{-/-}$ MEFs) showed only negligible quantities of PTEN in exosomes, confirming the importance of Ndfip1 for driving PTEN secretion [14]. Moreover, cells without PTEN and Ndfip1 (e.g., human glioblastoma cells U87), or only expressing one of the two proteins (e.g. human glioblastoma cells LN18 has no Ndfip1) need to be transfected with the complementary plasmids (PTEN and/or Ndfip1) in order for PTEN to be exported. The choice of a cell line is also tempered by transfection difficulty; in this eventuality the alternative route of using lentiviruses may prove feasible [14]. In the present report, we describe protocols for producing exosomes containing PTEN by (1) using transfection methods to introduce exogenous PTEN and Ndfip1 into HEK293T cells; and (2) extracting endogenous PTEN from wild-type MEFs expressing both PTEN and Ndfip1. As controls to demonstrate the importance of Ndfip1, we also isolated exosomes lacking PTEN from *Ndfip1*$^{-/-}$ MEFs.

2.2. Cell culture considerations

MEFs were obtained from embryonic mice (E13) lacking *Ndfip1* (*Ndfip1*$^{-/-}$ MEFs) following homologous recombination [18]. Wild-type MEFs (*Ndfip1*$^{+/+}$ MEFs) were sourced from embryonic littermates. HEK293T and MEFs were cultured in 10 cm dishes in 10 ml of culture medium [10% fetal calf serum (FCS), 50 U of penicillin, 50 μg of streptomycin, 4 mM L-glutamate in Dulbecco's modified Eagle's medium]. Cells were grown until 90% confluence in 10 cm culture dishes containing 15 ml of culture medium. Exosome-free media was used following removal of endogenous exosomes in serum by overnight centrifugation for 18 h at 100,000*g* [19]. Depending on the experimental design, cells may also be grown in serum-free media to avoid inclusion of exosomes inherent in serum. However, if the cells survive poorly without serum, another approach would be to use serum-free media supplemented with 1% bovine serum albumin. Typically, cells were grown for 48 h before the media were harvested for exosome isolation. In cells where exogenous PTEN and Ndfip1 have been introduced, a 48 h period was observed prior to obtaining the media.

2.3. Cell transfection using plasmids encoding for PTEN and Ndfip1

Cells were grown to 90% confluence before transfection was performed. A number of transfection reagents may be used (e.g. Lipofectamine, Life Technologies). We found Effectene (Qiagen) to be the most effective, when used according to the manufacturer's instructions. To allow biochemical detection of exogenous proteins, we typically introduced the tagged forms Ndfip1-FLAG (C-terminal tagged) and PTEN-FLAG (C-terminal tagged) [14,20].

2.4. Cell death assay and trypan blue exclusion analysis

Cells undergoing apoptosis during culture can release apoptotic bodies that will contaminate exosome harvest and analysis. Hence, it is vital to assess the health of cultured cells prior to harvesting the media. An acceptable range of cell death would be <10% of the cultured population. Cell death can be assessed using the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen). In brief, after removing the supernatant for exosome isolation, the remaining cells were washed with phosphate-buffered saline (PBS) and incubated with 4 μM ethidium homodimer-1, and 2 μM calcein AM in PBS for 30 min at room temperature. Cells were mounted on coverslips and sealed with clear fingernail polish. Labeled cells indicating apoptosis can be identified using an inverted fluorescence microscope. As a positive control for dead cells, treatment with 70% methanol for 30 min at room temperature can be performed. An additional method for identifying non-viable cells is trypan blue exclusion. For this, cells were trypsinized from the culture dish and stained with 0.04% trypan blue solution. Dead and live cells were scored using a hemocytometer. Supernatant should not be used

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