Vesicles of the intracellular and extracellular transport – key structures in the process of tissue differentiation towards bone and cartilage

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Abbreviations: AFM – atomic force microscopy; ALP – alkaline phosphatase; AnxA – vertebrate annexin; Abs – apoptotic bodies; BM – bone mineralization; BSP – bone sialoprotein; CHOL – cholesterol; ECs – ectosomes; ENs – endosomes; EVs – extracellular vesicles; EXs – exosomes; FCM – flow cytometry; FTIR – Fourier transform infrared spectroscopy; HA – hydroxyapatite; IVs – intracellular vesicles; MMP – matrix metalloproteinase; MicroVs – microvesicles; MVs – matrix vesicles; MVBs – multivesicular bodies; OMFs – organelle membrane fractions; PM – plasma membrane; PMVs – plasma membrane vesicles; P_i – inorganic phosphate; PP_i – inorganic pyrophosphate; PS – phosphatidylserine; PTH – parathyroid hormone; ROCK – Rho-associated coiled-coil kinase; SM – sphingomyelin; SPM – scanning probe microscopy; Src – sarcoma proto-oncogene tyrosine-protein kinase; TEM-EDX – transmission electron microscopy with energy dispersive X-ray microanalysis; TNAP – tissue non-specific alkaline phosphatase; VC – vascular calcification

ABSTRACT

Differentiation of cells of the skeletal tissue, such as osteoblasts and chondrocytes, into mineralization-competent cells is a necessary step of the physiological process of bone and cartilage mineralization. Vascular cell calcification accompanies a pathological process of atherosclerotic plaque formation, which occurs due to trans-differentiation of vascular smooth muscle cells into cells resembling bone mineralization-competent cells. The activity of tissue-nonspecific alkaline phosphatase (TNAP), an enzyme necessary for physiological mineralization, is also induced in vascular cells in response to inflammation. TNAP acquires its mineralizing function when anchored to the plasma membrane (PM) of mineralizing cells and to the surface of vesicles derived from these cells. Numerous important reports indicate that various types of vesicles play a crucial role in initiating cell differentiation. In this review, we would like to highlight various functions of different types of vesicular structures of the cellular transport machinery such as intracellular vesicles (IVs), extracellular vesicles (EVs) or matrix vesicles (MVs) at distinct stages of both physiological and pathological processes of tissue differentiation.

INTRODUCTION

Mineralization is a physiological process by which growth plate chondrocytes and osteoblasts deposit calcium phosphate crystals during endochondral and membranous ossification, respectively. This process is initiated by TNAP activity [Narisawa *et al*. 2013]. Deficiency of this enzyme in human cells results in a severe disease called hypophosphatasia causing in utero death of fetuses devoid of a mineralized skeleton [Millan & Whyte 2016]. TNAP activity is necessary for physiological bone mineralization, but also for the induction of pathological vascular calcification. Vesicular structures produced by mineralization-competent cells may resemble rafts, that is, membrane microdomains. In this article we would like to consider a possible role of vesicles of the intracellular and extracellular transport in the process of tissue differentiation and to discuss the stimulating effect of their cargo on bone mineralization and vascular calcification processes.

TYPES AND STRUCTURES OF VESICLES

INTRACELLULAR VESICLES

The ability of cells to interact with one another and with their environment relies on a large number of proteins that need to be in the right amount and in the right location. For this reason, eukaryotic cells have evolved complex sorting machineries to ensure a dynamic and strictly controlled flow of proteins between cellular compartments. This machinery exists in both plants and animals, and it involves vesicular structures called intracellular vesicles (IVs) [Otegui *et al*. 2010]. Endosomes (ENs) are vesicles between 100-500 nm in diameter, formed *via* a complex series of processes collectively known as endocytosis, and found in the cytoplasm of virtually every animal cell. The basic mechanism of endocytosis is the reverse of what occurs during exocytosis or cellular secretion. It involves the invagination (folding inward) of a plasma membrane (PM) to surround macromolecules or another matter diffusing through the extracellular fluid. The encircled foreign material is then brought into the cell and, following a pinching-off of the membrane (termed budding), is released to the cytoplasm in a sac-like vesicle.

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ENs are primarily intracellular sorting organelles which regulate trafficking of proteins and lipids among subcellular compartments of the secretory and endocytic pathway, specifically the plasma membrane (PM) Golgi, trans-Golgi network (TGN) and vacuoles/lysosomes [Marsh 2001; Bottini *et al.* 2018]. They receive cargo (proteins and lipids) from both the biosynthetic and the endocytic pathways. Most proteins are synthesized at the endoplasmic reticulum, trafficked through the Golgi, sorted at the TGN, and then sent to ENs for their final vacuolar/lysosomal delivery. ENs also receive PM proteins that are internalized by endocytosis and these proteins are either recycled back to PM or are sorted for degradation. The recycling of PM proteins occurs at early and recycling ENs; whereas their degradative sorting is achieved in intermediate/late ENs, which are also called multivesicular bodies/multivesicular endosomes (MVBs/MVENs), 500–5000 nm in size.

Three primary mechanisms of endocytosis may be distinguished [Marsh 2001]. Receptor-mediated endocytosis (RME) is the most specifically-targeted form of the endocytic process. Through RME, active cells are able to take in significant amounts of particular molecules (ligands) that bind to receptor sites extending from the cytoplasmic membrane into the extracellular fluid surrounding the cell. These receptor sites are commonly grouped together along coated pits in the membrane, which are lined on their cytoplasmic surface with a bristle-like collection of coat proteins. The coat proteins are thought to play a role in enlarging the pit and forming a vesicle [Marsh 2001]. As plasma membrane vesicles (PMVs) 50-500 nm in diameter we refer to caveolae and clathrin-coated vesicles or pits (CCVs/CCPs). Clathrin-mediated endocytosis (CME) is the major endocytic pathway in mammalian cells. It is responsible for the uptake of transmembrane receptors and transporters, for remodeling PM composition in response to environmental changes, and for regulating cell surface signaling. CME occurs *via* the assembly and maturation of CCPs that concentrate cargo as they invaginate and pinch off to form CCVs. In addition to the major coat proteins, clathrin and adaptor protein complexes, CME requires endocytic accessory proteins and phosphatidylinositol lipids. It is regulated at multiple steps (initiation, cargo selection, maturation, and fission) and monitored by an endocytic checkpoint that induces disassembly of defective pits *via* posttranslational modifications, allosteric conformational changes, isoform and splice-variant differences among components of the CME machinery, including the GTPase dynamin [Mettlen *et al.* 2018]. Vesicles produced *via* RME may internalize other molecules in addition to ligands, though the ligands are usually brought into the cell in higher concentration. One of the main factors causing bacterial diarrhea are AB_5 enterotoxins. The AB_5 enterotoxins contain a catalytic subunit A and pentameric subunit B, which binds to the cell surface within lipid rafts. The Cholera toxin family cause the constitutive activation of Gsα protein, which results in cAMP production, opening of the chloride channels and release of chloride ions into the lumen of the small intestine. In contrast, the Shiga toxin family has a cytotoxic effect on epithelial cells. It can inhibit protein synthesis leading to cell death. Although AB_5 has a toxic activity, the B_5 subunit has a significant potential as a transporter for proteins with anticancer activity and as a tool for the visualization of lipid rafts and cancer cells (Fig. 1) [Komiazyk *et al.* 2015].

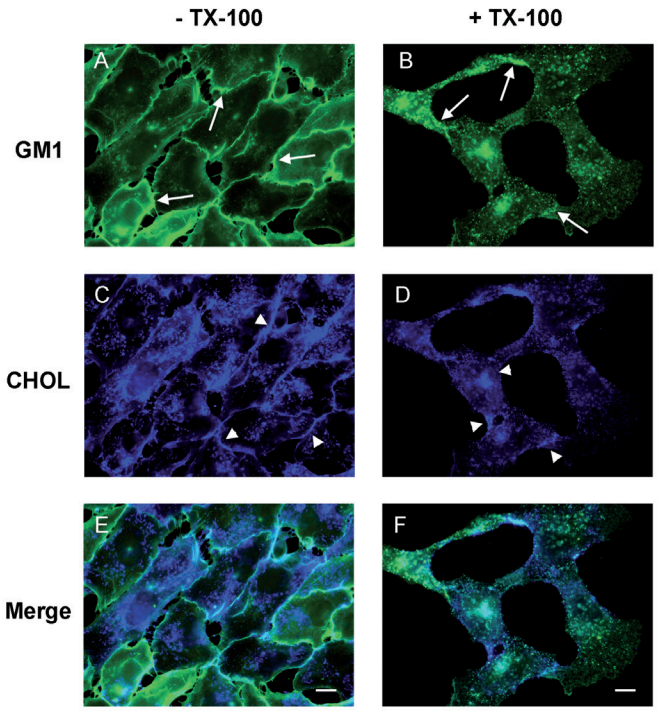

Figure 1. Binding of bacteria enterotoxin to membrane rafts. Monkey African Green kidney Vero cells (ECACC 84113001) were fixed, permeabilized (B, D, F) or non-permeabilized (A, C, E) in 0.08% TX-100 and analyzed by fluorescent microscopy with structured illumination ApoTome. Cholera Toxin B subunit FITC conjugate binds to ganglioside GM1 (GM1) (A, B, green, arrows). Cholesterol (CHOL) was visualized by staining with filipin (C, D, blue, arrowheads). Sites of GM1 and CHOL co-localization are visible in cyan on merge images (E, F), scale bar 10 µm. (MK and JD, unpublished results).

A less specific mechanism of endocytosis is pinocytosis [Marsh 2001]. By means of pinocytosis, a cell is able to ingest droplets of liquid from the extracellular fluid. All solutes found in the droplets outside of the cell may become encased in the vesicles formed *via* this process, with those present in the greatest concentration in the extracellular fluid also becoming the most concentrated in the membranous sacs. Pinocytotic vesicles tend to be smaller than vesicles produced by other endocytic processes.

The final type of endocytosis, termed phagocytosis, is probably the most well-known manner in which cells may import outside materials [Marsh 2001]. By means of phagocytosis, a single-celled organisms engulf by stretching out pseudopodia, encircling of the particles and subsequent packaging of them into vesicles. Phagocytic vesicles are usually large enough to be referred to as vacuoles or phagosomes. Phagocytosis is commonly associated with amoebas, but in multicellular organisms, phagocytic cells function in bodily defense rather than as a means to gain nourishment. For example, leukocytes in the human body often phagocytose protozoa and bacteria or dead cells and cellular components in order to help stave off infections or autophagy problems [Altman & Rathmell 2009].

When both phagocytosis (of particles) and pinocytosis (of solutes) was investigated in macrophages in the absence or presence of metabolic and cytoskeletal inhibitors, Percoll (30 nm diameter) was captured by pinocytosis, whereas polystyrene beads (100, 300, 600, 800 or 1100 nm diameter) were taken

by phagocytosis with increasing Endocytic Index (microliter/10^6 cells per h) rate. The pattern of inhibition of particles uptake suggests that there is no radical discontinuity between pinocytosis and phagocytosis, but that the contribution of phagocytosis steadily increases with increasing particle diameter [Pratten & Lloyd 1986].

EXTRACELLULAR VESICLES

Extracellular vesicles (EVs) are membrane-delimited subcellular particles, which contain multiple classes of bioactive molecules including markers of the cell from which they are derived. EVs are implicated in an increasing number of physiological and pathological contexts as mediators of local and systemic intercellular communication and detection of specific EVs may be useful in monitoring disease progression [Lynch *et al.* 2017].

EVs can be released from cells both constitutively and in response to activation or stress. Although there is a lack of consensus on the properties and nomenclature within the EV field, it is generally accepted that there are at least three different types of EVs, which have been classified according to size, biogenesis, or isolation technique, composition and function [Kanada *et al.* 2016; Lawson *et al.* 2016; Lynch *et al.* 2017; Bottini *et al.* 2018]. Exosomes (EXs) are the smallest category (30–150 nm) formed by inward budding of the endosomal membrane *via* an endosomal pathway, which involves formation of MVBs that are trafficked to the PM, where they fuse to release their contents by exocytosis. Microvesicles/microparticles (MicroVs/MicroPs) (100–1000 nm) are produced by budding or blebbing directly from the PM. Apoptotic bodies (ABs) are formed in cells undergoing programmed cell death named apoptosis, and apoptotic cell-derived vesicles have very broad size ranges (typically described as 1000–5000 nm). Relatively large ectosomes (ECs), >1 µm in diameter and not apparently associated with apoptosis, have also been described. They contain intact organelles but not nuclear components. EV cargoes are diverse, ranging from genetic material such as mRNA, genomic DNA and microRNA (miRNA) to lipids, and cytosolic or membrane proteins such as growth factors, enzymes and cytokines. Thus, they can be considered as transport vehicles, which may influence on the behavior of distant cell types within the body, as well as may transfer information between individual organisms of the same or separate species [Lawson *et al.* 2016].

It is widely accepted that EVs contain biomolecules indicative of the cell from which they derive, its state of activation, its metabolic activity, and in some cases its genotype. Since EVs are accessible in various body fluids including plasma, urine, milk, tears, sweat and semen, their cargoes may be useful as non-invasive biomarkers for diagnosis and prognosis of many diseases including cancer, cardiometabolic diseases and diabetes [Lawson *et al.* 2016]. Recent investigations serve to highlight the association of EVs and their cargoes with diverse cancer types. Because of their structure and highly specific cargoes, EVs are likely to prove to be most useful targets for biomarker screening in cancer diagnosis, prognosis and therapy, as well as in other disease settings. EVs are involved in the regulation of tumor growth, progression (pre- and post-therapy), and antitumor immunity, in the latter context showing potential application in antitumor vaccination. Lately it has been proposed that the "onco-regenerative niche" (ORN) represents a microenvironmental signaling network driven by apoptosis and involving tumor cells, non-tumor stromal and immune cells, connective tissue, soluble factors, and EVs [Lynch *et al.* 2016].

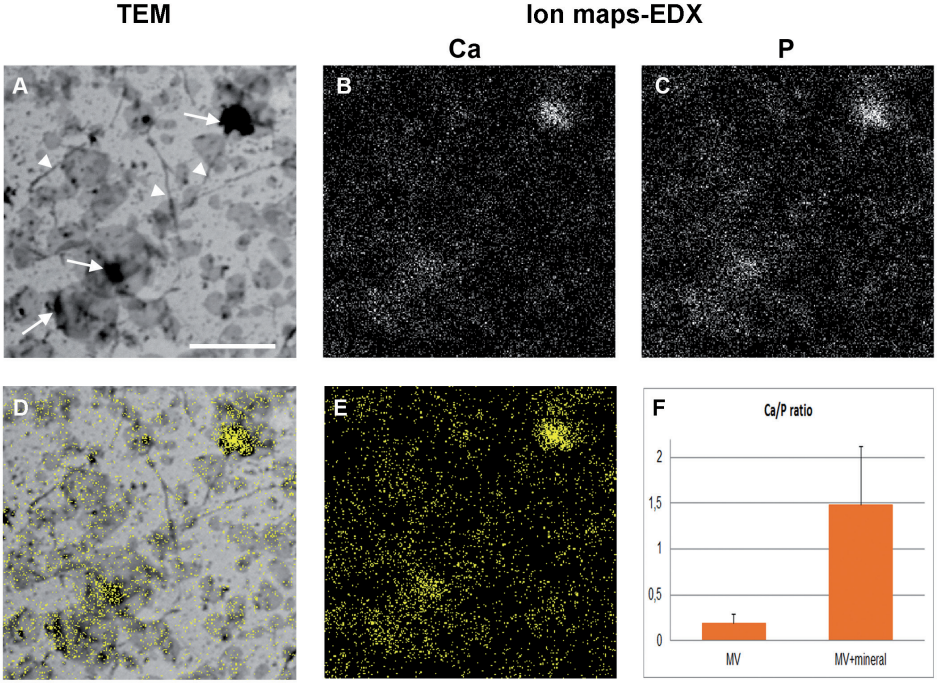

Figure 2. Localization of calcium and phosphate ions within matrix vesicles during bone mineralization. Matrix vesicles (MVs) were isolated from 17-day old chicken embryo leg bones, digested with collagenase type-I (200 U/g) at 37°C for 3h in synthetic cartilage lymph (SCL), centrifuged at 13 000 x g for 20 min at 4°C followed by 80 000 x g for 60 min at 4°C, They were subsequently incubated in SCL with 2 mM Ca^{2+} at 37°C for 24 h, negatively stained and analyzed by transmission electron microscopy with energy dispersive X-ray microanalysis (TEM-EDX). A – TEM image of MVs with minerals (arrows) and attached to collagen fibers (arrowheads), scale bar 1 µm. B, C – Ion maps for Ca (B) and P (C) measured by TEM-EDX. Sites of Ca and P co-localization are visible in yellow on merge images (D, E). F – Ca/P ratio in empty MVs (MV) or in MVs containing minerals (MV + mineral) calculated from TEM-EDX spectra; n=4 ± s.d. (ASK and LB, unpublished results).

The putative network engenders pro-repair and regenerative responses that promote tumor cell proliferation, angiogenesis, and invasiveness while at the same time suppressing antitumor immunity. EVs, whose membranes protect their cargo from degradation, are being explored for their ability to deliver targeted molecules to specific cell types for directed therapy [Kanada *et al.* 2016].

MATRIX VESICLES

Matrix vesicles (MVs) are released by budding from PM of hypertrophic chondrocytes and mature osteoblasts, that is cells responsible for endochondral and membranous ossification (Fig. 2) [Anderson 2003, 2007; Thouverey *et al.* 2009, 2011]. In addition, MVs from odontoblasts contribute to the onset of mineralization of mantle dentin during its genesis [Goldberg *et al.* 2011]. Under pathological conditions, MVs can also be released from non-skeletal tissues for example from vascular smooth muscle cells (VSMCs) (Fig. 3) [Fakhry *et al.* 2017; Roszkowska *et al.* 2018]. MV release from apical membrane microvilli is triggered by depolymerization of the actin cytoskeleton [Thouverey *et al.* 2009]. MVs are 100-500 nm in diameter, able to bind to collagen in vitro and to form apatite within 1-3 h in a medium containing 2 mM calcium and 1-2 mM phosphate (equivalent to a Ca/P molar ratio around 1.5) (Fig. 2) [Buchet *et al.* 2013; Bottini *et al.* 2018].

Three phosphatases have been implicated in the concerted regulation of inorganic pyrophosphate (PP_i): orphan phosphatase 1 (PHOSPHO1) present in the lumen of MVs, nucleotide pyrophosphatase/phosphodiesterase (NPP1) and tissue-nonspecific alkaline phosphatase (TNAP). The latter two act on the outer surface of MVs to regulate the extracellular P_i/PP_i ratio, a turning point between physiological and pathological mineralization [Garimella *et al.* 2006; Millan 2013]. Phosphate transporter 1 (PiT-1) helps incorporate P_i into MVs [Yadav *et al.* 2016], while annexins may be involved in the binding and transport of Ca^{2+} and in the biophysical process that initiates mineralization in the MV lumen [Wang *et al.* 2003]. Several members of the vertebrate annexin (AnxA) family (AnxA1, AnxA2, AnxA5, AnxA6 and AnxA7) are present in MVs [Balcerzak *et al.* 2008; Thouverey *et al.* 2011; Cmoch *et al.* 2011, Roszkowska *et al.* 2018]. They may be found in the lumen, on the inner leaflet of the bilayer in contact with phosphatidylserine (PS), or on the outer surface of MVs. AnxA5 [Kirsch 2012; Bolean *et al.* 2015], AnxA6 or an unidentified calcium channel (UCC) may function as calcium carriers [Bottini *et al.* 2018]. Annexins (AnxA2, AnxA5 and AnxA6) and TNAP also have collagen-binding capacity [Kirsch 2012], a property that may help align MVs along collagen fibers to promote propagation of mineralization onto the extracellular matrix (ECM) scaffold.

Fetuin A and osteopontin are inhibitors of apatite formation and can restrict further propagation of mineralization within the ECM [Jahnen-Dechent *et al.* 2008]. MVs are enriched in lipid markers of lipid rafts such as cholesterol (CHOL), sphingomyelin (SM) and PS as well as in protein markers of lipid rafts such as AnxA6, H^+ ATPase, G-proteins and glycosylphosphatidylinositol (GPI)-anchored TNAP (Fig. 3) [Balcerzak *et al.* 2008; Wuthier & Lipscomb 2011; Roszkowska *et al.* 2016, 2018]. MVs can be considered as a model of membrane microdomains due to their selective lipid and protein recruitments, with the additional advantage that they can be extracted without detergent [Bottini *et al.* 2018].

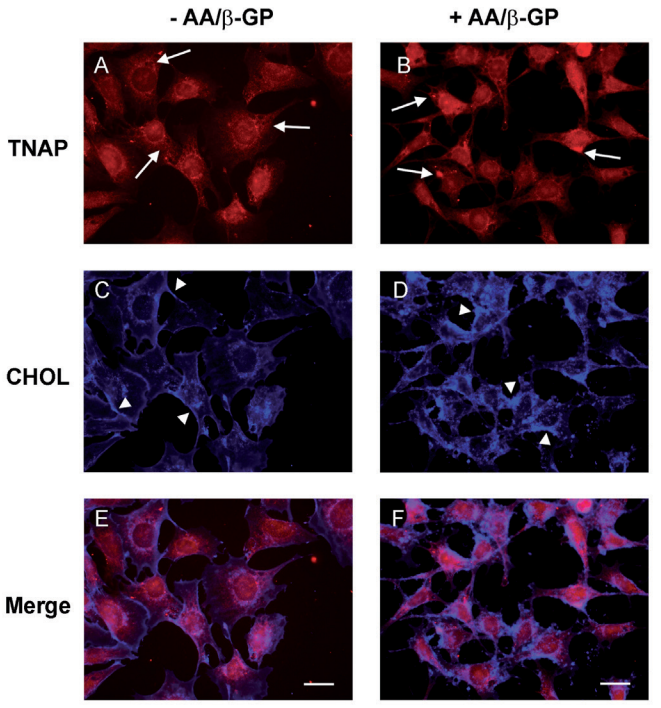

Figure 3. Localization of tissue non-specific alkaline phosphatase and cholesterol during vascular calcification. Mouse vascular smooth muscle MOVAS cells (ATCC CRL-2797) were trans-differentiated (B, D, F) or non-differentiated (A, C, E) in 50 µg/ml ascorbic acid (AA) and 10 mM β-glicerophosphate (β-GP) for 7 days, fixed and analyzed by fluorescent microscopy with structured illumination ApoTome. Tissue non-specific alkaline phosphatase (TNAP) was immunostained with anti-TNAP primary antibody conjugated with Alexa Fluor 594 secondary antibody (A, B, red, arrows). Cholesterol (CHOL) was visualized by staining with fillipin (C, D, blue, arrowheads). Sites of TNAP and CHOL co-localization are visible in magenta on merge images (E, F), scale bar 10 µm. (MK and JG, unpublished results).

BIOGENESIS AND PROPERTIES OF EXTRACELLULAR VESICLES

SEPARATION AND PURIFICATION OF EXTRACELLULAR VESICLES

The size range of different vesicles may overlap, and the markers which are displayed on the outer leaflet of the surrounding membrane may be common to both types of vesicles: intracellular (IVs) and extracellular (EVs). Thus, most isolation methods do not guarantee a pure population of vesicles. With this in mind, and based on the most common protocols involving sequential/differential ultracentrifugation steps, some researchers now refer to the speed at which each vesicle population is pelleted, rather than to vesicle type [Lawson *et al.* 2016; Bottini *et al.* 2018].

There are guidelines available for the collection of blood in particular [Lacroix *et al.* 2012] and several protocols have been published for the sequential isolation of different populations of EVs *via* ultracentrifugation. In the first step, bodily fluid or culture medium is centrifuged at low speed (1500-3000×g (times gravity)) to remove cellular material and debris. The supernatant from this step can then be further centrifuged at 10000–17000×g to pellet MicroVs, with a final centrifugation

at 100000×g to pellet EXs. In addition to EXs this population is likely to contain small MicroVs and possibly some lipoproteins. Density gradient ultracentrifugation may be further employed for a purer exosome population [Thery *et al.* 2006]. There is still no perfect method to isolate MVs [Wuthier & Lipscomb 2011]. One of the most challenging aspects of sample preparation is establishing approaches that faithfully preserve biological material *in situ* in a fully hydrated state. During extraction and purification of chondrocyte-released MVs without collagenase, usually two bands of membranous layers are visible on Percoll gradients, while four bands of membranous layers are found in sucrose gradients [Balcerzak *et al.* 2007]. The less dense membrane fraction with the highest lipid-to-protein ratio (around 3 mg/mg), the highest specific TNAP activity and the highest ability to form apatite, corresponds to MVs and the addition of collagenase increases the yield of released MVs [Cmoch *et al.* 2011; Bottini *et al.* 2018; Roszkowska *et al.* 2018].

In addition, commercially available kits utilize size exclusion chromatography and magnetic separation based on CD9 or CD63, cell surface markers which are exposed on exosomes but not thought to be expressed on MicroVs. EXs can be directly precipitated from plasma or culture supernatant using these approaches [Lawson *et al.* 2016]. However, there is no clear consensus as to the efficacy of these methods in the published literature. For these reasons, it is imperative to characterize the vesicle population of interest as fully as possible.

CHARACTERIZATION AND IMAGING OF EXTRACELLULAR VESICLES

A selection of methods have been utilized for characterization of EVs. Transmission electron microscopy (TEM) is considered the gold standard of imaging techniques, as it can give accurate information about the sizes of all classes of vesicles. However, as it is not a quantitative technique and requires specialized expertise and equipment, TEM may be of limited appeal. Energy dispersive X-ray microanalysis (EDX) spectra quantitation and ion mapping give quantitative and qualitative results of ion ratios and replacements in MVs or apatite crystals [Strzelecka-Kiliszek *et al.* 2017a]. The overall goal of using TEM-EDX was to find a simple method for imaging and quantifying the distribution of multiple ions in various minerals from different types of cells during distinct stages of bone mineralization [Bozycki *et al.* 2018] or vascular calcification [Roszkowska *et al.* 2018] processes (Fig. 2). Mineralization profiles may also be analyzed by different optical methods: Alizarin Red-S (AR-S) staining or von Kossa silver nitrate or lactate staining and light microscopy with RGB filters may be used for imaging of cells and calcium-phosphate minerals [Strzelecka-Kiliszek & Bozycki 2017b] while ultraviolet (UV) light facilitates visualization of cell lysates or apatite powders [Strzelecka-Kiliszek *et al.* 2017c, Bozycki *et al.* 2018]. The formed minerals may also be analyzed by Fourier transform infrared spectroscopy (FTIR). It was proved that the infrared spectrum of the mineral deposit formed by MVs of mineralization-competent cells was identical to the spectrum of the HA standard [Thouverey *et al.* 2009, 2011]. The mineralization process may also be imaged using different macro- and microscopy techniques. This can be achieved by clearing organs such as leg bone or AS-R staining and macrophotography. In addition, the process can be observed by staining of crucial cellular enzymes such as Src kinases or Rho small GTPases and Rho-associated coiled-coil kinase (ROCK) [Strzelecka-Kiliszek & Bozycki 2017b] or ganglioside GM1, TNAP and CHOL (Fig. 1 and 3) with fluorophores and imaging by fluorescent microscopy. Some advanced microscopy techniques like scanning probe microscopy (SPM), contact mode and dynamic mode of atomic force microscopy (AFM), scanning tunneling microscopy and scanning near-field optical microscopy have been recently proposed to study proteoliposomes, as models of biological membranes. Analysis of proteoliposomes facilitates the understanding of lipid-protein interactions with emphasis on the properties of physicochemical and biochemical reactions during the biomineralization process [Bolean *et al.* 2017, Bottini *et al.* 2018]. Proteoliposomes provide a means of reconstituting lipid vesicles that function like MVs, making these structures an advantageous and convenient experimental model to understand MV-mediated mineralization. They may be constituted of a single type of lipid or a mixture of lipids, with proteins and/or electrolytes. Comparisons of proteoliposomes and osteoblast-derived MVs or MVs deficient in TNAP, NPP1 or PHOSPHO1, using natural substrates such as ATP, adenosine 5′-diphosphate (ADP) and PP_i, confirmed the validity of proteoliposome models [Yadav *et al.* 2011]. The ultimate goal of the *in vitro* MVs biomimetic models is to replicate in vitro the key events leading to the domain-induced MV budding, and to initiation of apatite crystal formation in chondrocyte- and osteoblast-derived MVs. Once the proteoliposomes are obtained and characterized, they can be added to fixed amounts of MVs, either WT or deficient in specific enzymes, to modulate their *in vitro* mineralization properties [Bottini *et al.* 2018].

Several other non-optical methods have also been utilized but are limited [Lawson *et al.* 2016]. Thus, many researchers have opted for one of a range of optical techniques, of which flow cytometry (FCM) is the most widely reported for MV detection. FCM enables EVs phenotyping using fluorochrome conjugated antibodies to determine the parental cell type, and is also quantitative. Small particle size at the limit of detection may be an issue; however, there are a number of sophisticated protocols to eliminate background noise. Newer instruments have lower detection limits and image stream technology (IST) is able to further differentiate between different types of particles in whole blood and plasma [Headland *et al.* 2014]. Accurate detection of EXs using FCM should be carried out with caution, as their size is below the limit of detection of many instruments. However, alternative methods are available including dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS). These techniques enable quantitation of EXs and small MicroVs, but they have limited capabilities for phenotyping and may require additional time for purification of vesicles before measurement [van der Pol *et al.* 2010]. Raman spectroscopy, which is a highly sensitive quantitative technique for analysis of the biochemical composition of vesicles without labelling, has also been used to define vesicular populations [Lawson *et al.* 2016].

FUNCTIONS OF VESICLES IN THE PROCESS OF TISSUE DIFFERENTIATION

Vesicles derived from normal cells and those from diseased and malignant cells exhibit unique 'molecular signatures' that differentially modulate cellular functions in re-

Table 1. Main vesicles involved in tissue differentiation.

Type	Biogenesis	Reference
IVs	ENs	Marsh 2001; Otegui *et al*. 2010; Bottini *et al*. 2018
	MVBs	
	PMVs	
	OMFs	
EVs	ECs	Lawson *et al*. 2016; Kanada *et al*. 2016; Lynch *et al*. 2017; Bottini *et al*. 2018
	ABs	
	MicroVs	
	EXs	
MVs	Collagen-free	Wuthier & Lipscomb 2011; Roszkowska *et al*. 2018; Bottini *et al*. 2018
	Collagen-attached	Balcerzak *et al*. 2008; Thouverey *et al*. 2009, 2011; Cmoch *et al*. 2011; Buchet *et al*. 2013; Fakhry *et al*. 2017; Roszkowska *et al*. 2018; Bottini *et al*. 2018

cipient cells [Kanada *et al.* 2016]. Intracellular vesicles (IVs), produced in different cellular compartments or extracellular vesicles (EVs), released by different cell types may participate in physiological and pathological processes [Lener *et al.* 2015; Malda *et al.* 2016; Karpman *et al.* 2017, Silva *et al.* 2017]. They mediate intracellular or intercellular communication and transmit specific information from their cell of origin to their target. As a result of these properties, vesicles of defined cell types may serve as novel tools for various therapeutic approaches, including (a) anti-tumor therapy, (b) anti-pathogen vaccination, (c) immune-modulatory and regenerative therapies and (d) drug delivery.

The translation of vesicles into clinical therapies requires the categorization of vesicle-based therapeutics in compliance with existing regulatory frameworks. As the classification defines subsequent requirements for manufacturing, quality control and clinical investigation, it is of major importance to define whether vesicles are considered the active drug components or primarily serve as drug delivery vehicles. For an effective and, particularly, safe translation of vesicle-based therapies into clinical practice, a high level of cooperation between researchers, clinicians and competent authorities is essential.

The main function of MVs is the initiation of apatite formation in bone, cartilage and dentin due to their high TNAP activity [Bottini *et al.* 2018]. Pathological mineralization concerns not only skeletal but also non skeletal tissues [Bottini *et al.* 2018]. It is manifested by apatite deposition, often mediated by MVs, in soft tissues of tendons and/or ligaments (calcific tendinitis and ankylosing spondylitis), in arterial media, in atherosclerosis, in articular cartilage, and during vascular mineralization induced by chronic kidney disease or by type 2 non-insulin-dependent diabetes mellitus, in atherosclerosis, and in articular cartilage.

It was recently speculated that MVs might mediate cell signaling by bone morphogenetic proteins (BMP-2 and BMP-4) and serve as carriers of morphogenetic information to nearby chondrocytes and osteoblasts [Nahar *et al.* 2008]. The presence of microRNA (miRNAs) in MVs [Lin *et al.* 2016] suggests that MVs can function as signalosomes in cell-cell communication during cartilage and bone development *via* transfer of specific miRNAs. However, it remains to be determined whether such cell-cell communication occurs *in vivo,* because the average life span of MVs as determined *in vitro* is 1–3 h. Once apatite forms, MV membranes break, releasing their lumen content, and possibly also signaling molecules. Secondly, MVs bind to collagen produced by the cells from which they originate, which prevents long-distance displacement. Thirdly, MVs are probably surrounded by many dying cells from which they were released during late stages of differentiation. Taken together, these findings suggest that cell-cell communication mediated by MVs, if it exists, would be of a limited occurrence and of a short distance, rather autocrine or paracrine in nature [Bottini *et al.* 2018].

Some recent reports of basic and clinical scientists, members of the International Society for Extracellular Vesicles (ISEV) and of the European Cooperation in Science and Technology (COST) program of the European Union, namely European Network on Microvesicles and Exosomes in Health and Disease (ME-HaD), summarize recent developments and the current knowledge of vesicle-based therapies [Lener *et al.* 2015; Malda *et al.* 2016; Karpman *et al.* 2017, Silva *et al.* 2017; Bottini *et al.* 2018]. They highlight many aspects of safety and regulatory requirements that must be considered for pharmaceutical manufacturing and clinical application. They discuss the production and quality control processes and address the strategies to promote the therapeutic im-

plementation of vesicles in future clinical studies. Finally, what is most important, these reports consider how transport of vesicles and proteoliposomes can be easily detected and blocked. However, there is still much left to understand regarding the mechanisms of vesicles formation and release, cargo loading and their specific targeting to a particular tissue.

CONCLUDING REMARKS

There has been a growing interest in using vesicles of the intracellular and extracellular transport as carriers of biomarkers of disease, especially since they carry markers of their cell of origin and may represent the pathophysiological status of the cells. With increasing evidence for the importance of them as mediators of signal transduction, their potential as biosensors and/or delivery vehicles for signaling molecules to specific target cells, interest in their measurement and isolation has grown exponentially over the last decade. This has led to the availability of an ever-increasing range of instruments and isolation kits, but more established techniques of separation, characterization and imaging, should be accessible to individuals wishing to start exploring this exciting new field to better understand mechanisms of the process of tissue differentiation.

REFERENCES

1. Altman BJ, Rathmell JC (2009) Autophagy: not good OR bad, but good AND bad. Autophagy 5: 569-570
2. Anderson HC (2003) Matrix vesicles and calcification. Curr Reumatol Rep 5: 222-226
3. Anderson HC (2007) The role of matrix vesicles in physiological and pathological calcification. Curr Opin Orthop 18: 428-433
4. Balcerzak M, Malinowska A, Thouverey C, Sekrecka A, Dadlez M, Buchet R, Pikula S (2008) Proteome analysis of matrix vesicles isolated from femurs of chicken embryo. Proteomics 8: 192-205
5. Bolean M, Simao AM, Kiffer-Moreira T, Hoylaerts MF, Millan JL, Itri R, Ciancaglini P (2015) Proteoliposomes with the ability to transport Ca^{2+} into the vesicles and hydrolyze phosphosubstrates on their surface, Arch Biochem Biophys 584: 79-89
6. Bolean M, Simao, AM, Barioni MB, Favarin BZ, Sebinelli HG, Veschi EA, Janku TAB, Bottini M., Hoylaerts MF, Itri R, Millan JL, Ciancaglini P (2017) Biphysical aspects of biominralization. Biophys Rev 9: 747-760
7. Bottini M, Mebarek S, Anderson KL, Strzelecka-Kiliszek A, Bozycki L, Simão AMS, Bolean M, Ciancaglini P, Pikula JB, Pikula S, Magne D, Volkmann N, Hanein D, Millán JL, Buchet R (2018) Matrix vesicles from chondrocytes and osteoblasts: Their biogenesis, properties, functions and biomimetic models. Biochim Biophys Acta 1862: 532-546
8. Bozycki L, Komiazyk M, Mebarek S, Buchet R, Pikula S, Strzelecka-Kiliszek A (2018) Analysis of minerals produced by hFOB 1.19 and Saos-2 cells using transmission electron microscopy with energy dispersive X-ray microanalysis. J Vis Exp 136: e57423, doi:10.3791/57423
9. Buchet R, Pikula S, Magne D, Mebarek S (2013) Isolation and characteristics of matrix vesicles. Methods Mol Biol 1053: 115-124
10. Cmoch A, Strzelecka-Kiliszek A, Palczewska M, Groves P, Pikula S (2011) Matrix vesicles isolated from mineralization-competent Saos-2 cells are selectively enriched with annexins and S100 proteins. Biochem Biophys Res Commun 412: 683-687
11. Fakhry M, Roszkowska M, Briolay A, Bougault C, Guignandon A, Diaz-Hernandez JI, Diaz-Hernandez M, Pikula S, Buchet R, Hamade E, Badran B, Bessueille L, Magne D (2017) TNAP stimulates vascular smooth muscle cell trans-differentiation into chondrocytes through calcium deposition and BMP-2 activation: Possible implication in atherosclerotic plaque stability. Biochim Biophys Acta 1863: 643-653
12. Garimella R, Bi X, Anderson HA, Camacho NP (2006) Nature of phosphate substrate as a major determinant of mineral type formed in matrix vesicle-mediated *in vitro* mineralization: an FTIR imaging study. Bone 38: 811-817
13. Goldberg M, Kulkarni AB, Young M, BoskeyA (2011) Dentin: structure, composition and mineralization. Front Biosci 3: 711-735
14. Headland SE, Jones HR1, D'Sa AS1, Perretti M1, Norling LV (2014) Cutting-edge analysis of extracellular microparticles using ImageStream(X) imaging flow cytometry. Sci Rep 4: 5237
15. Jahnen-Dechent W, Schafer C, Ketteler M, McKee MD (2008) Mineral chaperones: a role for fetuin-A and osteopontin in the inhibition and regression of pathologic calcification. J Mol Med (Berl) 86: 379-389
16. Kanada M, Bachmann MH, Contag CH (2016) Signaling by Extracellular Vesicles Advances Cancer Hallmarks. Trends Cancer 2: 84-94
17. Karpman D, Stahl AL, Arvidsson I (2017) Extracellular vesicles in renal disease. Nat Rev Naphrol 13: 545-562
18. Kirsch T (2012) Biomineralization - an active or passive process? Connect Tissue Res 53: 438-445
19. Komiażyk M, Palczewska M, Pikula S, Groves P (2015) Bacterial type AB_5 enterotoxins-structure, function and mechanism of action. Postepy Biochem 61: 430-435
20. Lacroix R, Judicone C, Poncelet P, Robert S, Arnaud L, Sampol J, Dignat-George F (2012) Impact of pre-analytical parameters on the measurement of circulating microparticles: towards standardization of protocol. J Thromb Haemost 10: 437-446
21. Lawson C, Vicencio JM, Yellon DM, Davidson SM (2016) Microvesicles and exosomes: new players in metabolic and cardiovascular disease. J Endocrinol 228: R57–R71
22. Lener T, Gimona M, Aigner L, Borger V, Buzas E, Camussi G, Chaput N, Chatterjee D, Court FA, del Portillo HA, O'Driscoll L, Fais S, Falcon-Perez JM, Felderhoff-Mueser U, Fraile L, Gho YS, Gorgens A, Gupta RC, Hendrix A, Hermann DM, Hill AF, Hochberg F, Horn PA, Dominique de Kleijn D, Kordelas L, Kramer BW., Kramer-Albers E-M, Laner-Plamberger S, Laitinen S, Leonardi T, Lorenowicz MJ, Lim SK, Lotvall J, Maguire CA., Marcilla A, Nazarenko I, Ochiya T, Patel T, Pedersen S, Pocsfalvi G, Pluchino S, Quesenberry P, Reischl IG., Rivera FJ, Sanzenbacher R, Schallmoser K, Slaper-Cortenbach I, Strunk D, Tonn T, Vader P, van Balkom BWM, Wauben M, El Andaloussi S, Thery C, Rohde E, Giebel B (2015) Applying extracellular vesicles based therapeutics in clinical trials – an ISEV position paper. J Extracel Ves 4: 30087
23. Lin Z, Rodriguez NE, Zhao J, Ramey AN, Hyzy SL, Boyan BD, Schwartz Z (2016) Selective enrichment of microRNAs in extracellular matrix vesicles produced by growth plate chondrocytes. Bone 88: 47-55
24. Lynch C, Panagopoulou M, Gregory CD (2017) Extracellular vesicles arising from apoptotic cells in tumors: roles in cancer pathogenesis and potential clinical applications. Frontiers Immunol 8: 1174
25. Malda J, Boere J, van de Lest CH, van Weeren P, Wauben MH (2016) Extracellular vesicles – new tool for joint repair and regeneration. Nat Rev Rheumatol 12: 243-249
26. Marsh M (2001) Endocytosis. (Frontiers in Molecular Biology) 1st edition. Oxford University Press
27. Mettlen M, Chen PH, Srinivasan S, Danuser G, Schmid SL (2018) Regulation of clathrin-mediated endocytosis. Annu Rev Biochem 87: 871-896
28. Millan JL (2013) The role of phosphatases in the initiation of skeletal mineralization. Calcif Tissue Int 93: 299-306
29. Millan JL, Whyte MP (2016) Alkaline Phosphatase and Hypophosphatasia. Calcif Tissue Int 98: 398-416
30. Nahar NN, Missana LR, Garimella R, Tague SE, Anderson HA (2008) Matrix vesicles are carriers of bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF), and noncollagenous matrix proteins. J Bone Miner Metab 26: 514-519
31. Narisawa S, Yadav MC, Millan JL (2013) In vivo overexpression of tissue-nonspecific alkaline phosphatase increases skeletal mineralization

and affects the phosphorylation status of osteopontin. J Bone Miner Res 28: 1587-1598

32. Otegui M, Reyes FC (2010) Endosomes in Plants. Nature Education 3: 23

33. Pratten MK, Lloyd JB. (1986) Pinocytosis and phagocytosis: the effect of size of a particulate substrate on its mode of capture by rat peritoneal macrophages cultured *in vitro*. Biochim Biophys Acta 881: 307-313

34. Roszkowska M, Strzelecka-Kiliszek A, Magne D, Pikula S, Bessueille L (2016) Membranes and pathophysiological mineralization. Postepy Biochem 62: 511-517

35. Roszkowska M, Strzelecka-Kiliszek A, Bessueille L, Buchet R, Magne D, Pikula S (2018) Collagen promotes matrix vesicle-mediated mineralization by vascular smooth muscle cell. J Inorg Biochem 186: 1-9

36. Silva AM, Teixeira JH, Almeida MI, Gonçalves RM, Barbosa MA, Santos SG (2017) Extracellular Vesicles: Immunomodulatory messengers in the context of tissue repair/regeneration. Eur J Pharmac Sci 98: 86-95

37. Strzelecka-Kiliszek A, Bozycki L, Mebarek S, Buchet R, Pikula S (2017a) Characteristics of minerals in vesicles produced by human osteoblasts hFOB 1.19 and osteosarcoma Saos-2 cells stimulated for mineralization. J Inorg Biochem 171: 100-107

38. Strzelecka-Kiliszek A, Bozycki L (2017b) Cross-talk between Src kinases and Rho small GTPases regulates biomineralization and simplify imaging of the mineralization process. Postepy Biochem 63: 93-109

39. Strzelecka-Kiliszek A, Mebarek S, Roszkowska M, Buchet R, Magne D, Pikula S (2017c) Functions of Rho family of small GTPases and Rho-associated coiled-coil kinases in bone cells during differentiation and mineralization. Biochim Biophys Acta 1861: 1009-1023

40. Thery C, Amigorena S, Raposo G, Clayton A (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol chapter 3, unit 3.22

41. Thouverey C, Strzelecka-Kiliszek A, Balcerzak M, Buchet R, Pikula S (2009) Matrix vesicles originate from apical membrane microvilli of mineralizing osteoblast-like Saos-2 cells. J Cell Biochem 106: 127-138

Thouverey C, Malinowska A, Balcerzak M, Strzelecka-Kiliszek A, Buchet R, Dadlez M, Pikula S (2011) Proteomic characterization of biogenesis and functions of matrix vesicles released from mineralizing human osteoblast-like cells. J Proteomics 74: 1123-1134

42. Wang W, Xu J, Kirsch T. (2003) Annexin-mediated Ca^{2+} influx regulates growth plate chondrocyte maturation and apoptosis. J Biol Chem 278: 3762-3769

43. Wuthier RE, Lipscomb GF (2011) Matrix vesicles: structure, composition, formation and function in calcification. Front Biosci 16: 2812-2902

44. van der Pol E, Hoekstra AG, Sturk A, Otto C, van Leeuwen TG, Nieuwland R (2010) Optical and non-optical methods for detection and characterization of microparticles and exosomes. J Thromb Haemost 8: 2596-2607

45. Yadav MC, Simao AM, Narisawa S, Huesa C, McKee MD, Farquharson C, Millan JL (2011) Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: a unified model of the mechanisms of initiation of skeletal calcification. J Bone Miner Res 26: 286-297

46. Yadav MC, Bottini M, Cory E, Bhattacharya K, Kuss P, Narisawa S, Sah RL, Beck L, Fadeel B, Farquharson C, Millan JL (2016) Skeletal mineralization deficits and impaired biogenesis and function of chondrocyte-derived matrix vesicles in Phospho1(−/−) and Phospho1/Pit1 double-knockout mice. J Bone Miner Res 31: 1275-1286

Pęcherzyki transportu wewnątrzkomórkowego i zewnątrzkomórkowego – kluczowe struktury w procesie różnicowania tkanki kostnej i chrzęstnej

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Słowa kluczowe: różnicowanie tkanek, pęcherzyki wewnątrzkomórkowe, pęcherzyki zewnątrzkomórkowe, pęcherzyki macierzy pozakomórkowej, transport

STRESZCZENIE

Różnicowanie komórek tkanki kostnej, takich jak osteoblasty i chondrocyty, do komórek kompetentnych w procesie mineralizacji jest ważnym etapem fizjologicznego procesu mineralizacji kości i tkanki chrzęstnej. Wapnienie komórek naczyń krwionośnych towarzyszy także patologicznemu procesowi powstawania płytki miażdżycowej, która jest wynikiem różnicowania komórek mięśni gładkich ściany naczyń krwionośnych do komórek przypominających kompetentne w procesie mineralizacji komórki tkanki kostnej. Tkankowo-niespecyficzna fosfataza alkaliczna (TNAP), enzym niezbędny dla fizjologicznej mineralizacji, ulega aktywacji także w komórkach naczyń krwionośnych w odpowiedzi na stan zapalny. TNAP osiąga swoją funkcję w formie związanej z błoną plazmatyczną (PM) komórek ulegających mineralizacji oraz z błoną pęcherzyków wydzielanych przez te komórki. Liczne doniesienia wskazują, że różnego typu pęcherzyki odgrywają podstawową rolę w zapoczątkowywaniu różnicowania komórek. W niniejszym artykule przeglądowym, opisano odmienne funkcje różnych typów struktur pęcherzykowych transportu komórkowego, takich jak pęcherzyki wewnątrzkomórkowe (IV), pęcherzyki zewnątrzkomórkowe (EV) i pęcherzyki macierzy pozakomórkowej (MV), na kolejnych etapach fizjologicznego lub patologicznego różnicowania tkanek kostnej i chrzęstnej.