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Title

Matrix vesicle-mediated mineralization and potential application

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ABSTRACT
Hard tissues, including the bones and teeth, are a fundamental part of the body, and their formation and homeostasis are critically regulated by matrix vesicle-mediated mineralization. Matrix vesicle has been studied for 50 years since it was first observed using electron microscopy. However, research progress has been hampered by various technical barriers. Recently, there have been great advancements in our understanding of the intracellular biosynthesis of matrix vesicles. Mitochondria and lysosomes are now considered key players in matrix vesicle formation. The involvement of mitophagy, mitochondrial-derived vesicles, and mitochondria-lysosome interaction have been suggested as potential detailed mechanisms of the intracellular pathway of matrix vesicles. Their main secretion pathway may be exocytosis, in addition to the traditionally understood mechanism of budding from the outer plasma membrane. This basic knowledge of matrix vesicles should be strengthened by novel nano-level microscopic technologies, together with basic cell biologies, such as autophagy and inter-organelle interactions. In the field of tissue regeneration, extracellular vesicles such as exosomes are gaining interest as promising tools in cell-free bone and periodontal regenerative therapy. Matrix vesicles, which are recognized as a special type of extracellular vesicles, could be another potential alternative. In this review, we outline the recent significant progress in the process of matrix vesicle-mediated mineralization and the potential clinical applications of matrix vesicles for tissue regeneration.

INTRODUCTION

Mineralization is the deposition of calcium phosphate crystals into the extracellular matrix and is a universal phenomenon in the physiological and pathological hard tissue formation of vertebrates. During the initial stages of mineralization of hard tissues such as bone, cartilage, dentin, and cementum, it is essential for hard tissue-forming cells to secrete a special type of extracellular vesicle called matrix vesicles (30-300 nm in diameter) into the extracellular space (Hasegawa 2018). Matrix vesicles contain not only calcium and inorganic phosphate, but also enzymes, lipids, and proteins necessary for hard tissue formation (Bottini et al. 2018). During hard tissue maturation, the interaction between the extracellular matrix and the matrix vesicles induces crystalline nucleation of hydroxyapatite that promotes hard tissue mineralization. This series of processes is called matrix vesicle-mediated mineralization. However, for many years, there has been no clear conclusion on how matrix vesicles are formed and secreted in hard tissue-forming cells, despite this being a fundamental mechanism of hard tissue formation. In the dental field, matrix vesicles have been observed in cementum, dentin, and alveolar bone, and it has been thought that the same mechanism promotes mineralization as in other bones throughout the body. However, matrix vesicle-related gene knockout mice have shown a specific phenotype to cementum (Thumbigere-Math et al. 2018). These data suggest that each hard tissue is formed by matrix vesicle-mediated mineralization, but the detailed mechanism may be different. There have been several exciting advancements in the field of matrix vesicle-mediated mineralization in the past three years.

Along with matrix vesicles, a line of evidence has shown that another type of extracellular vesicles, exosomes, from hard tissue-related cells, including osteoblasts, their progenitor/stem cells, and osteoclasts, play essential roles in their formation and homeostasis (Yuan et al. 2018). Moreover, exosomes can be potentially therapeutic for bone and periodontal tissues (Liu et al. 2018; Gholami et al. 2021). Ongoing research suggests exosomes are considerably heterogeneous in size, biomarker, content, function, and origin (Kalluri and LeBleu 2020), and the difference between matrix vesicles and exosomes has not been addressed yet (Shapiro et al. 2015).

In this review, we focused on two topics that have recently advanced using various new technologies. We first outline the intracellular process of matrix vesicle-mediated mineralization, highlighting areas where more research is

needed. We also discuss the possible application of matrix vesicle for regeneration therapy.

FIFTY YEARS OF MATRIX VESICLE RESEARCH

Since the first observation of matrix vesicles in cartilage under electron microscopy in 1967 (Anderson 1967; Bonucci 1967), followed by observation in bone in 1973 (Anderson and Reynolds 1973), matrix vesicles present as "needle-like structures" in the extracellular matrix prior to calcification in all biological hard tissues except enamel, and these structures have been observed within and along the membrane of matrix vesicles at the site of calcification initiation. Importantly, matrix vesicles have also been observed in ectopic calcified lesions, such as calcified vessel walls in atherosclerosis (Bakhshian Nik et al. 2017). To histologically analyze hard tissues, the tissues are generally decalcified after chemical fixation. However, during this process, the minerals and crystals are lost (Bonucci and Reurink 1978). Thus, various cytochemical methods have been explored for preventing the loss (Rohde and Mayer 2007; Boonrungsiman et al. 2012; Hasegawa et al. 2017). Further, by combining techniques such as cytochemical methods and elemental analysis, analysis of matrix vesicles at the site of calcification initiation could be performed in various tissues and in cultured cells. Many reports using such techniques have amassed, and the current concept that matrix vesicle is essential for hard tissue formation, especially at the site of calcification initiation, has been established.

However, the morphological characteristics of matrix vesicles in hard tissue-forming cells are not always consistent within these studies and many questions remain unclear. Results from previous studies showing crystalline state (amorphous or crystallized hydroxyapatite) of calcium phosphate in matrix vesicles are still controversial(Anderson and Reynolds 1973; Boonrungsiman et al. 2012; Hasegawa et al. 2017). In addition, even the composition of matrix vesicles has been partly identified (Table 1), their mechanisms remain unsolved. Thus far, the details of how matrix vesicles are formed, secreted out of cells, and interacted with extracellular matrices such as collagen to promote calcification are not fully clarified. As will be discussed later, great progress has been made in this area over the past few years, and some questions are being answered. A series of studies was sparked by a seminal report by the Stevens group in 2012 (Boonrungsiman et al. 2012). By comparing different sample

preparation methods, they first showed that calcium phosphate within intracellular matrix vesicles was mainly amorphous, suggesting that conventional chemical fixation protocols for electron microscopy cause artifacts such as needle-like structures. The study also found that extracellular matrix vesicles contained crystallized calcium phosphate, suggesting that calcium phosphate is transferred from the intracellular to the extracellular space. This conformed with previous reports showing the presence of transient amorphous calcium phosphate in calcifying tissue (Mahamid et al. 2008) and cells (Nudelman et al. 2010). Moreover, using the high-pressure freezing and freeze substitution protocol, they found that electron-dense granules containing calcium and phosphate were evident in mitochondria and that mitochondria conjoined an intracellular vesicle to probably transfer amorphous calcium phosphate (Boonrungsiman et al. 2012). In a previous biogenesis model (Anderson 1995), matrix vesicles were formed by budding from the outer plasma membrane and were observed only in the extracellular space. The notion that the intracellular process may play a role in mineralization, together with evidence from other studies such as a cryo-electron microscopic study showing calcium phosphate-containing intracellular vesicles in the developing bone (Mahamid et al. 2011), suggesting that the biosynthesis and secretion pathways of matrix vesicles should be revisited.

BIOGENESIS OF MATRIX VESICLES

Intracellular pathways

With the emerging evidence of the intracellular pathways of matrix vesicle formation, more research focused on cellular organelle relationships with matrix vesicles. Lysosomes are known as cellular degradation station, which plays important roles in intracellular trafficking. Mitochondria are known as cellular metabolic storage for metabolites, including calcium. Recent studies have suggested the involvement of these two organelles in matrix vesicle formation (Pei et al. 2018; Tang et al. 2020).

Pei et al. suggested that lysosome and mitochondria are involved in matrix vesicle biogenesis through mitophagy (Pei et al. 2018). Mitophagy is an autophagy of the mitochondria. Autophagy is a cellular process that engulfs cytoplasmic materials or organelles in autophagosomes for their degradation by fusing with lysosomes, resulting in autolysosomes. Mitophagy is mediated by PTEN-induced kinase 1 (PINK1) and E3 ubiquitin ligase (Parkin). In damaged mitochondria, PINK1 is

accumulated to initiate mitophagy. This recruits Parkin to enhance the process through further ubiquitination of mitochondrial proteins, which subsequently lead to engulfment into microtubule-associated protein 1A/2B-light chain 3 (LC3)-positive autophagosomes (Ashrafi and Schwarz 2013).

Pei et al., observed the mineralization of cultured cells using transmission electron microscopy and found the presence of matrix vesicle-containing mitochondria in both double-membrane autophagosomes and single membrane autolysosomes (Figs. 1A, B). Moreover, localization of PINK1 in the matrix vesicle-containing mitochondria and the results from functional assays suggested the role of mitophagy in matrix vesicle formation (Pei et al. 2018). Since calcium-loaded mitochondria, which subsequently transferred to matrix vesicles (Brighton and Hunt 1976; Brighton and Hunt 1978), were subjected to oxidative stress (Wuthier and Lipscomb 2011), mitophagy might be induced by an accumulation of calcium in the mitochondria. Although prior studies have detected electron-dense granules in the mitochondria (Plachot et al. 1986) or in autolysosomes of cultured cells and found an association between autophagy-related genes and mineralization (Nollet et al. 2014), this is the first study to show the involvement of the mitochondria-lysosome axis in matrix vesicle biogenesis.

This observation was supported by Tang et al., who proposed the ER-mitochondria axis in matrix vesicle formation (Tang et al. 2020). Further studies are needed to ensure the exact communication pathway of mitochondria and lysosomes since mitophagy is not a solo process for the mitochondrial quality control system; the involvement of another important pathway, mitochondrial-derived vesicles, is also possible (Sugiura et al. 2014). Instead of the engulfment of the organelle in mitophagy, the mitochondrial-derived vesicles can carry a patch of mitochondrial content to the lysosome. To understand the detailed mechanism, direct observation of nano-sized matrix vesicles in live cells without possible artifacts from matrix vesicle crystallization are needed. This has been a technical dilemma because it is not possible to directly observe live cells with a nano-level resolution electron microscopy nor to observe the nano-sized matrix vesicles under an optical microscope that can directly observe live cells.

Recently, in order to overcome this dilemma and to better understand the early stages of mineralization, we successfully observed the formation and secretion of matrix vesicles in living cells by using two new microscopic techniques:

scanning electron dielectric microscopy and super-resolution fluorescence microscopy (Iwayama et al. 2019). When mineralizing osteoblasts were observed using scanning electron dielectric microscopy, many granules accumulated inside the intracellular membranous structures, forming a multivesicular body structure (Appendix Figure 1). This suggests the involvement of lysosomes in the formation of matrix vesicles. Elemental analysis showed that these granules were rich in phosphate and calcium, had the same Raman spectrum as calcium phosphate, and their size was consistent with previously reported matrix vesicles. Furthermore, tissue-nonspecific alkaline phosphatase (TNAP) knockout osteoblasts showed no granules, even when cultured in a mineralization induction medium. These results indicated that these granules were matrix vesicles. In line with a previous electron microscopy study using the high-pressure freezing and freeze substitution protocol (Boonrungsiman et al. 2012), the matrix vesicles were not crystallized in the cells but existed in an amorphous state. In addition to the scanning electron dielectric microscopy observation, we attempted to study the dynamics of calcium-containing lysosomes using super-resolution fluorescence microscopy. Super-resolution time-lapse imaging by labeling calcium with Calcein revealed a series of processes in which Calcein-containing matrix vesicles were located near mitochondria, contacted with lysosomes, and transported (Fig. 1C). Experiments with lysosome inhibitors also suggested that the matrix vesicles formed in lysosomes. This study, for the first time, visualized the process in live osteoblasts. The limitation of this study is that the Calcein in super-resolution fluorescence microscopy imaging study is not a specific marker for matrix vesicles.

These recent advancements have emphasized the mitochondria-lysosome axis as an intracellular matrix vesicle biogenesis pathway. The remaining question is how these two organelles interact. Based on these findings, we propose three models for the intracellular pathway (Fig. 2A). All pathways end up with lysosomes, which seem to play critical roles in mineralization. i) Mitophagy: mitochondria are targeted by autophagosome, followed by fusion with lysosomes. The fission and depolarization of dysfunctional mitochondria recruit Parkin and PINK1. ii) mitochondria-derived vesicles: Recent findings show that mitochondria bud vesicles carry damaged cargo to the lysosome. Instead of mitochondrial fission and mitophagy, this mechanism can remove patches of mitochondria. iii) Direct contact: The direct interaction between mitochondria and lysosomes has

recently been identified to regulate each organelle's function (Wong et al. 2019). The presence of autolysosomes (Fig. 1A, B) suggested i) or ii), while the super-resolution fluorescence microscopy and scanning electron dielectric microscopy observations that a calcium-containing vesicle adjacent to mitochondria is fused with the lysosome (Fig. 1C) suggested ii) or iii). If matrix vesicle-specific marker molecules were identified and demonstrated *in situ* localization, our understanding of matrix vesicle-mediated mineralization would be dramatically advanced.

Secretion pathway

Previous ultrastructural analysis using primary chondrocytes or epiphyseal growth plates of chicks suggested that matrix vesicles are secreted by budding from the outer plasma membrane (Akisaka et al. 1988). Proteomic analysis of isolated matrix vesicles from human osteosarcoma Saos-2 cells showed that matrix vesicles might originate from microvilli-like membranes (Thouverey et al. 2011). Nevertheless, a study using cultured bone marrow stromal cells and the tibia of embryonic chicks showed the presence of intracellular calcium phosphate in vacuoles and an increase in crystallinity during the mineralization process, suggesting that matrix vesicles are secreted via the exocytotic process (Rohde and Mayer 2007). Endocytosis helps maintain exocytosis via recycling vesicles to prevent vesicle exhaustion (Wu et al. 2014). Matrix vesicles contain molecules linked to endocytosis and exocytosis, such as GTPase Ras-associated binding proteins, SNARE proteins, annexins, and integrins. In addition, matrix vesicles were also proposed to release from the cell in the same manner as exosomes. The endosomal and autophagic pathways are expected to be involved (Shapiro et al. 2015).

These inconsistent results might be due to the differences in the activities of cells or tissues. Even though matrix vesicle formation is relatively slow, the process can be stimulated under certain conditions. Osteoblasts can actively form matrix vesicles during embryonic development *in vivo* or upon induction with BMP-2 *in vitro*. The latter can be monitored using Alizarin Red-stained calcified nodules. For example, a murine osteoblastic cell line, KUSA-A1, can form calcified nodules as early as 4 h after induction (Eguchi et al. 2013), while other cells generally need 2-3 weeks of induction.

Recent reports have favored the exocytosis model (Pei et al. 2018; Iwayama et al. 2019). In our study, the effect of exocytosis inhibitors on vesicle secretion

was examined. In the cells treated with Vacuolin-1 or Endosidin2, vesicle secretion was inhibited. Lysosomes were restricted in the intracellular space with increased Calcein signal. These results show that matrix vesicles are secreted via exocytosis (Iwayama et al. 2019). It is well accepted that lysosomes, the central degradation unit of the cell, contain secretory proteins, and the fusion of lysosomes with the plasma membrane allows the secretion of lysosomal contents in various types of cells (Buratta et al. 2020). Whether this lysosomal exocytosis-mediated matrix vesicle transport is specific to hard tissue-forming cells such as osteoblasts and cementoblasts remains to be investigated. Cumulatively, the potential secretion mechanism of matrix vesicles could be endocytosis-exocytosis or budding from the plasma membrane (Fig. 2B). More evidence is needed to elucidate the matrix vesicle secretion pathway precisely.

Extracellular crystallization

Once matrix vesicles are released from the cells and adhere firmly to the extracellular matrix, the mineral is formed inside the vesicles (Kirsch et al. 1997). Mineralization is initiated at the inner leaflet of the matrix vesicle's membrane. An adequate supply of inorganic phosphate (Pi) is required to initiate mineralization. The accumulated Pi in matrix vesicles could be generated intravascularly by the orphan phosphatase 1 enzyme and phosphate transporter 1 (PiT-1)-mediated incorporation of Pi from the extracellular space. Moreover, Pi can also be generated extracellularly by TNAP, which can hydrolyze its substrates to produce Pi (Dillon et al. 2019). Matrix vesicles have a very high TNAP activity, suggesting their ability to induce mineralization. After mineralization, the mineral nodules are anchored to the surrounding collagen fibrils (Hasegawa et al. 2017; Yi et al. 2021), possibly via several collagen-binding proteins such as annexin proteins and TNAP (Bottini et al. 2018). One of the questions here is whether these matrix vesicle-associated proteins are not degraded in lysosomes. One possibility is that these proteins are protected by glycosylation, as lysosomal membrane proteins, e.g., Lamp, are heavily glycosylated and thus protected (Saftig and Klumperman 2009). The presence of glycosylation has been reported for both TNAP and annexin (Goulet et al. 1992; Goettsch et al. 2022), which may protect them from degradation. Nevertheless, further studies are required to clarify the precise mechanisms involved.

Despite the above-mentioned mechanism for mineralization, an explanation for the unmineralized areas among these mineralized nodules and collagen fibrils remains lacking. Recently, two widely accepted theories on collagen mineralization have been suggested (Hasegawa et al. 2017): the hole zone theory and collagen fibril superhelix theory. According to the hole zone theory, in non-mineralizing conditions, the spaces between collagen fibrils are filled with small proteoglycans. When mineralization is induced, the enzymes in the tissue fluid are activated, leading to proteoglycan degradation. Calcium and phosphate then accumulate and occupy the spaces, forming calcium phosphate nuclei and mineralizing the collagen fibrils (Landis et al. 1993; Nudelman et al. 2010). According to the collagen fibril superhelix theory, the collagen fibrils undergo transformation into superhelix structures, which strengthens the anchoring force and serves as a scaffold for mineralized nodules to expand and fill the unmineralized areas (Yi et al. 2021).

Regarding matrix vesicles, primary intrafibrillar mineralization of the newly formed collagen is purportedly regulated by matrix vesicles smaller than 40 nm containing amorphous calcium phosphate, which may be incorporated into the collagen gap region (~40 nm) (Boonrungsiman et al. 2012). These small matrix vesicles were found during the mid-to-late stages of osteoblast differentiation. However, needle-like crystals were observed at the later stage of osteogenic differentiation. These membrane-free, needle-like crystals were less able to induce mineralization than small vesicles with amorphous calcium phosphate. It is suggested to be matrix vesicles, in which the membrane is disrupted by crystals (Wei et al. 2019). Therefore, this indicates that matrix vesicles with amorphous calcium phosphate play an important role in initiating primary or intrafibrillar mineralization. However, the exact process remains unclear.

APPLICATION OF MATRIX VESICLE AS BIOLOGICAL THERAPEUTICS

Regenerative therapies for periodontal and bone tissues have been developed, and cytokine-based therapies have shown some success so far. For more severe cases, cell therapy using transplantation of tissue stem cells derived from bone marrow, adipose tissue, or periodontal ligament is expected. It has been suggested that not only the transplanted cells directly differentiate into local cells, but also trophic factors such as cytokines, chemokines, and growth factors secreted by the transplanted cells may be involved in the mechanism of action (Phinney and Prockop 2007). Recently, it is becoming clear that

extracellular vesicles secreted by transplanted cells play an important role (Johnson et al. 2021). While the safety and efficacy of cell transplantation therapies have been established to date, they are accompanied by difficulties in quality control due to lot-to-lot differences. Conversely, extracellular vesicles garner greater interest as uniform and easily quantifiable therapeutic tools. Since the therapeutic application of exosomes in bone and periodontal tissue has been extensively reviewed (Liu et al. 2018; Gholami et al. 2021), we focus on the potential use of matrix vesicles in this review.

As discussed above, matrix vesicles are essential for mineralization, and there has been an idea to utilize matrix vesicles for hard tissue regeneration as biological therapeutics (Table 2). In 1997, a group demonstrated an increase in calcium and phosphate concentrations in a gelatin gel containing isolated matrix vesicles from the chondrocytes culture supernatant. However, the gel showed only limited evidence of mineralization (Boskey et al. 1997). Another study utilized matrix vesicles isolated from the supernatant of MC3T3-E1 cells mixed with collagen hydrogel (Kunitomi et al. 2019). They confirmed that isolated matrix vesicles could not promote mineral formation. However, nanofragments obtained by sonication of isolated matrix vesicles could induce rapid mineralization *in vitro* shown by Alizarin Red staining. The use of isolated matrix vesicles containing amorphous calcium phosphate might be ideal as biologics. However, their isolation and purification require a technological breakthrough since the crystalline condition and probably the protein composition are significantly different after secretion.

One novel approach is the fabrication of artificial matrix vesicles or matrix vesicle mimics. Currently, nanosheets of black phosphorus (BP), an emerging metal-free layered material, have been focused on as a specific biological trigger for osteogenesis and osseointegration (Yang et al. 2018). A recent report (Wang et al. 2019) prepared nanospheres by mixing BP with poly (lactic-co-glycolic acid). The nanospheres are biodegradable, biocompatible, and can incorporate local calcium phosphate. The efficacy of these artificial matrix vesicles were demonstrated by positive Alizarin Red staining and increased osteogenic mRNA expressions *in vitro* and enhanced bone regeneration in calvarial defect models (Yang et al. 2018; Wang et al. 2019).

Taken together, extracellular vesicles can be used in potential new treatments (Fig. 4). For exosomes, many clinical trials and basic research are currently underway. On the other hand, the detailed mechanism of matrix vesicle-

mediated mineralization is not fully elucidated. Therefore, to apply matrix vesicles in a clinical setting, further studies are encouraged.

FUTURE PERSPECTIVES ON MATRIX VESICLE STUDY

Imaging the matrix vesicle-mediated mineralization

Electron microscopes have been extensively utilized as a primary tool for most studies. In the future, novel technologies such as super-resolution fluorescence microscopy, scanning electron dielectric microscopy, and cryo-electron microscopy (Table 3) will be crucial for studying ultrastructure in the cell, including extracellular vesicles. Of note, scanning electron dielectric microscopy, originally developed (Okada and Ogura 2016) by Dr. Ogura of the National Institute of Advanced Industrial Science and Technology, Japan, enables the observation of live cells in an aqueous solution at a nano-level resolution (Appendix Figure. 1). While calcium phosphate (both in amorphous and crystalized form) can be identified as electron-dense granules in scanning electron dielectric microscopy and electron microscopy, other optical microscope-based method needs immuno-staining or probes. Moreover, cryo-electron microscopy outperforms conventional electron microscopy in its ability to directly observe the morphological evolution of mineral precursor phases at different stages of biomineralization with nanoscale spatial resolution and sub-second temporal resolution in two or three dimensions. The use of cryo-electron microscopy for mineralization research has been recently reviewed (Lei et al. 2021 Dec 17). Accordingly, these novel imaging tools could be beneficial for studying matrix vesicles, especially in terms of organelle colocalization.

Matrix vesicle-specific biomarkers

While abundant and large organelles can be recognized by their known morphology and sizes, identification of matrix vesicles could be challenging because of their nanoscale. One approach to overcome this hurdle is tagging matrix vesicles with a specific marker. Recently, Syntenin-1 has been successfully identified as a candidate biomarker for exosomes, which have a comparable size to matrix vesicles (Kugeratski et al. 2021). However, the studies on specific biomarkers for matrix vesicles are still lacking. So far, several matrix vesicle-associated molecules have been reported, for example, alkaline phosphatase, annexins, ectonucleotide pyrophosphatase, and sodium-dependent phosphate transporters (Hasegawa et al. 2017; Bottini et al. 2018).

However, none of them are specific to matrix vesicles. Moreover, it has been shown that the function of these molecules may be site-specific in hard tissues, including bone and cementum as discussed below.

Matrix vesicles have been found to localize in all hard tissues. In periodontium, matrix vesicles are found in cementum and alveolar bone. In knockout mice of the *Phospho1* gene, an enzyme that accumulates inorganic phosphate in matrix vesicles, hypoplasia of alveolar bone and cellular cementum were evident, as found in long bones. However, the formation of the acellular cementum was not affected (Zweifler et al. 2016). In contrast, mice lacking alkaline phosphatase, which degrades pyrophosphate (an inhibitor of mineralization) to produce inorganic phosphate, showed hypoplasia of acellular cementum (Foster et al. 2012). The loss of function of this gene in humans is known as hypophosphatasia. A subset of patients exhibited cementum hypoplasia and early deciduous tooth loss due to the absence of the periodontal ligament (van den Bos et al. 2005). ENPP is crucial for producing pyrophosphate. *Ank* gene encodes transport protein for pyrophosphate. Both genes are also important for matrix vesicle-mediated mineralization. In *Enpp1*- and *Ank*-knockout mice, cementum hyperplasia and ectopic cementum granule formation in the periodontal ligament were reported (Ao et al. 2017; Thumbigere-Math et al. 2018). These findings suggest that matrix vesicle-mediated mineralization may be tissue-specific and involve different molecules.

Both bone and cementum are essential components in periodontal tissue regeneration. However, definite criteria to distinguish bone from cementum have not been established (Zhao et al. 2016). Taken together, identifying matrix vesicle-specific molecules along with clarifying the differences between alveolar bone and cementum will lead to a better understanding of matrix vesicle-mediated mineralization and new approaches to bone and periodontal tissue regeneration therapy.

CONCLUSIONS

In the last three years, our understanding of intracellular formation and secretion of matrix vesicles, which are essential in the early stages of hard tissue formation, has advanced dramatically. Although there are hurdles to elucidate detailed mechanism of matrix vesicle formation (Appendix Table 1), it may be possible to promote bone formation by stimulating endogenous matrix vesicle production.

Extracellular vesicles such as exosomes have recently shown promise as biologics. Similarly, matrix vesicles, a special type of extracellular vesicles, could be another potential alternative. Once matrix vesicles can be efficiently purified, they can also be utilized as biological agents to induce hard tissue regeneration.

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FIGURE LEGENDS

Figure 1 The mitochondria-lysosome axis as an intracellular matrix vesicle biogenesis pathway

A. Transmission electron microscopy images of mineralizing cultured cells showing matrix vesicle containing mitochondria inside double-membrane autophagosome (open arrow)

B. Degrading matrix vesicle containing mitochondria (pointer) inside single membrane autolysosomes (black arrow)

C. Time-lapse super-resolution fluorescence microscopy images of mineralizing osteoblasts suggesting involvement of mitochondria and lysosome in matrix vesicle biogenesis. A calcium-containing vesicle adjacent to mitochondria is fused with the lysosome.

The red arrow indicates Lysotracker-stained lysosome, the green arrow indicates Calcein-stained vesicle, and the purple structure indicates Mitotracker-stained mitchondria.

Panels A–B is derived from Pei et al. 2018, and panels C are modified from Iwayama et al. 2019. Scale bars, 50 nm in A; 200 nm in B; 1 μm in C.

Figure 2 Schematic illustration of possible intracellular pathways of matrix vesicle biogenesis and secretion

A. Possible biogenesis pathway

i) Mitophagy: a part of fissioned mitochondria is engulfed into autophagosomes, followed by fusion with lysosomes.
ii) Mitochondria-derived vesicle: mitochondria bud vesicles, which are then fused with lysosomes
iii) Direct contact: lysosome interacts with mitochondria through direct contact before transport to extracellular space

Mt: Mitochondria, Ly: Lysosome, AP: Autophagosome, AL: Autolysosome, MDV: Mitochondria-derived vesicle.

B. Possible secretion pathways

i) Endocytosis-Exocytosis: secretion of lysosomal content (matrix vesicles) upon lysosome fusion with the plasma membrane
ii) Budding: a membrane protrusion or bleb buds off from the outer plasma membrane as a matrix vesicle

Figure 3 Possible application of extracellular vesicles in periodontal tissue regeneration

Tables

Table 1 Commonly found components in matrix vesicles (adapted from(Golub 2009; Thouverey et al. 2011; Wuthier and Lipscomb 2011; Shapiro et al. 2015; Cui et al. 2016))

Proteins	**Lipids**
Enzyme	*Cholesterol*
Alkaline phosphatase (TNAP)	
Phosphoethanolamine (PHOSPHO-1)	*Free fatty acids*
Ectonucleotide phosphodiesterase1 (ENPP1)	
Matrix metalloproteinase (MMP) II, III, IX, XIII	*Phospholipids*
	Phosphatidylcholine
Transport proteins	Phosphatidylethanolamine
Annexin V, VI, II	Sphingomyelin
Progressive ankylosis protein (ANK)	Phosphatidylserine
Sodium-dependent phosphate transporter1 (PiT-1)	
Cytoskeletal & surface proteins	
Actin-B	
Integrin β_1, β_5, α_5, α_1, α_3	

Table 2 Studies utilized matrix vesicles as biological therapeutics

Study	Source of MVs	MV isolation	Scaffold	Evaluation of Mineralization	Results of Mineralization
Boskey et al. 1997	Chondrocytes	Differential centrifugation	Gelatin gel	*In vitro*: Ion concentration Fourier Transform Infrared Spectroscopy	*In vitro*: Increased Ca and Phosphate accumulation Bone-like apatite formation
Kunitomi et al. 2019	MC3T3-E1 cells	Differential centrifugation	Collagen hydrogel	*In vitro*: ARS staining Elemental analysis	*In vitro*: No mineralization
	MC3T3-E1 cells	Differential centrifugation + ultrasonication 30 s			*In vitro*: Positive for ARS staining Hydroxyapatite-like mineral formation
Yang et al. 2018	MV mimics: Black phosphorus nanosheet	N/A	3D printing Bioglass	*In situ*: ARS staining Osteogenic gene expression Elemental analysis *In vivo*: Rat cranial defect model	*In situ*: Positive for ARS staining Formation of calcium phosphate nanoparticles Increased *OPN*, *OCN*, *TNAP*, *COL1*, *RUNX2* mRNA expression *In vivo*: Promoted bone regeneration
Wang et al. 2019	MV mimics: Black phosphorus nanosheet	N/A	PLGA nanoparticles	*In vitro*: ARS staining Osteogenic gene expression *In vivo*: Mouse cranial defect model	*In vitro*: Positive for ARS staining Increased *ALP*, *RUNX2* mRNA expression *In vivo*: Promoted bone regeneration

N/A: not applicable, ARS: Alizarin Red S

Table 3 Technical comparison of microscopic tools utilized in matrix vesicle studies

	Optical microscope	Super-resolution microscope	Scanning electron microscope	Transmission electron microscope	Cryo-electron microscope	Scanning electron dielectric microscope
Resolution	~200 nm	~10 nm	~1 nm	~0.1 nm	~0.5 nm	~10 nm
Live cells observation in aqueous media	Applicable	Applicable	Not applicable: need sample processing	Not applicable: need sample processing	Not applicable: need sample processing	Applicable
Cellular damage	Laser damage (if used)	Laser damage	Electron beam damage	Electron beam damage	Electron beam damage	No or little
Identification of matrix vesicles	Need specific biomarker staining	Need specific biomarker staining	Electron dense granules	Electron dense granules	Electron dense granules	Electron dense granules

Fig.1

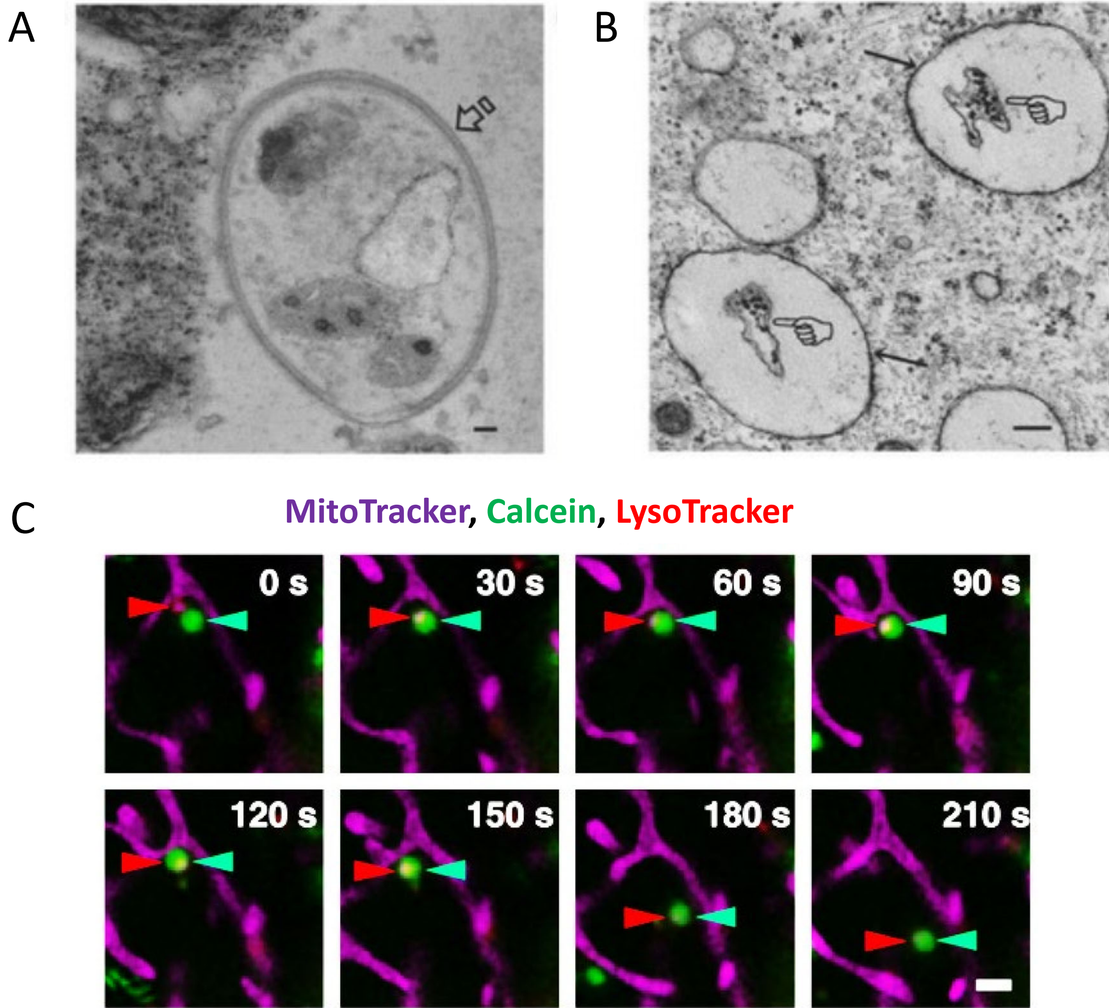
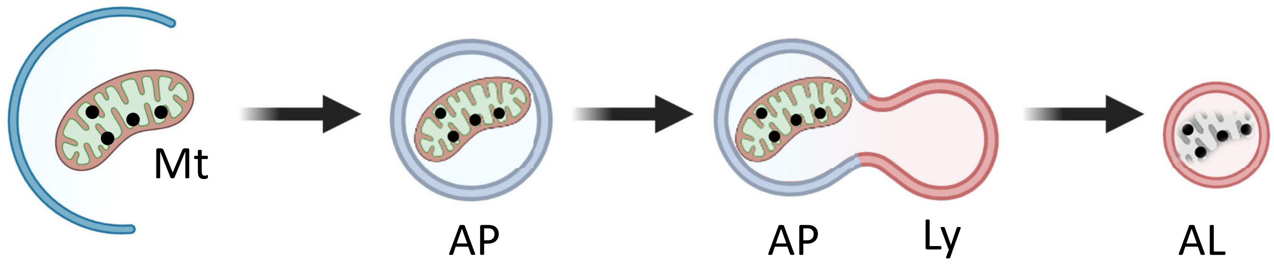


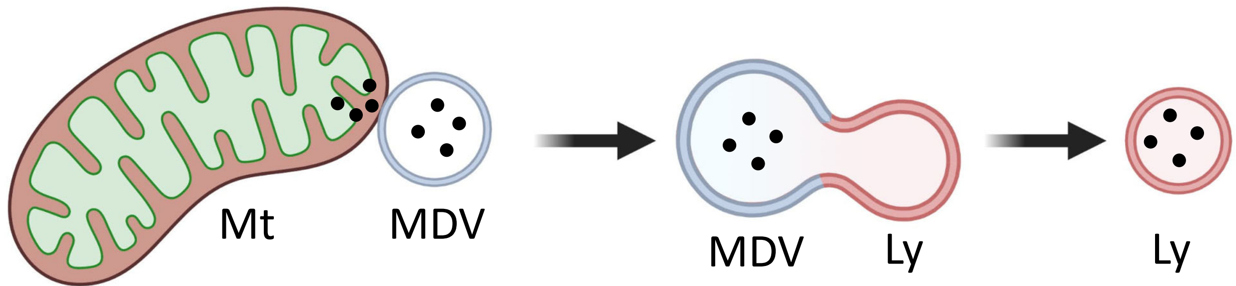
Fig.2

A. Possible intracellular pathways

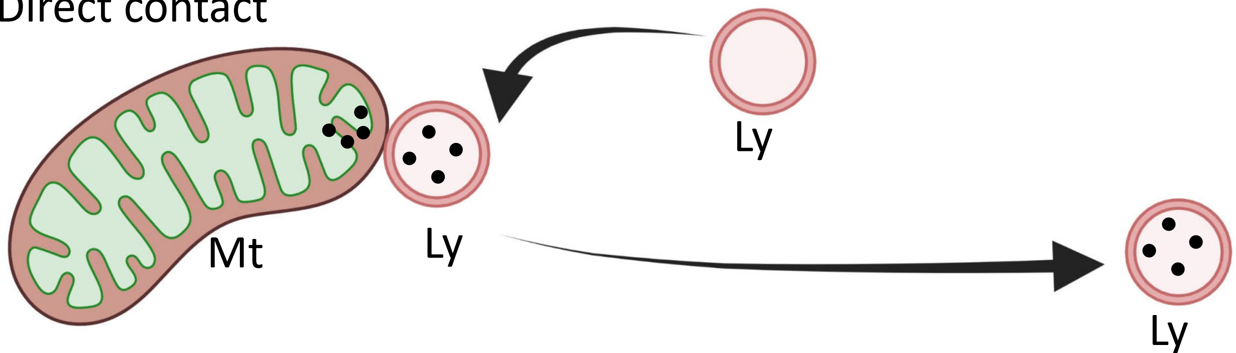
i) Mitophagy



ii) Mitochondria derived vesicle

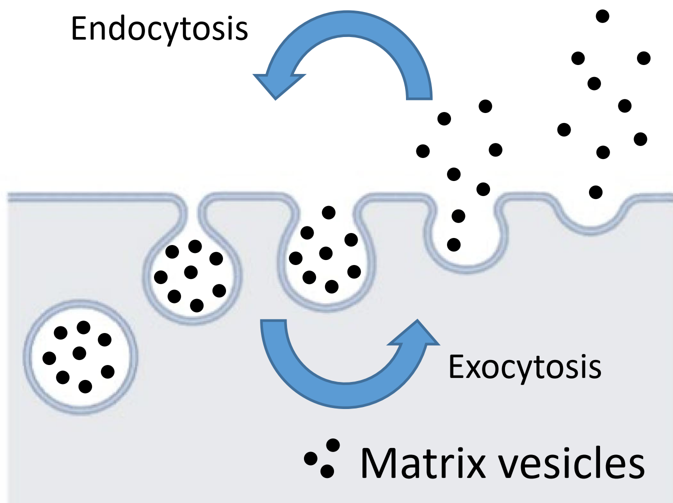


iii) Direct contact

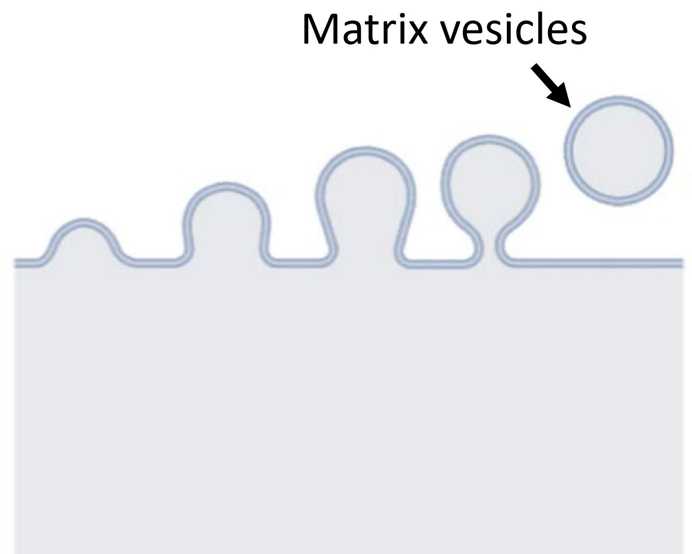


B. Possible secretion pathways

i) Exocytosis and endocytosis



ii) Budding



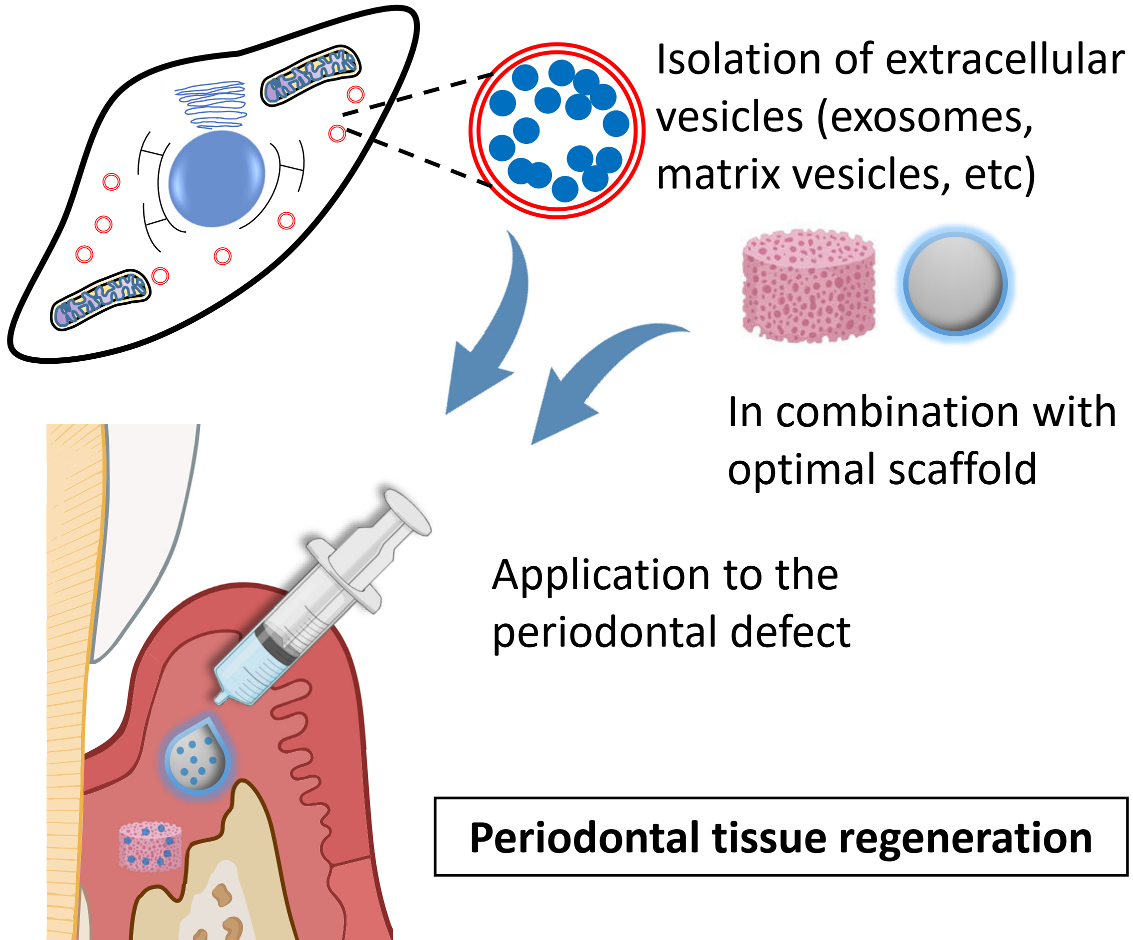


Fig.3
Isolation of extracellular vesicles (exosomes, matrix vesicles, etc)
In combination with optimal scaffold
Application to the periodontal defect
Periodontal tissue regeneration

Supplemental Appendix

Matrix vesicle-mediated mineralization and potential application

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Appendix Figure 1

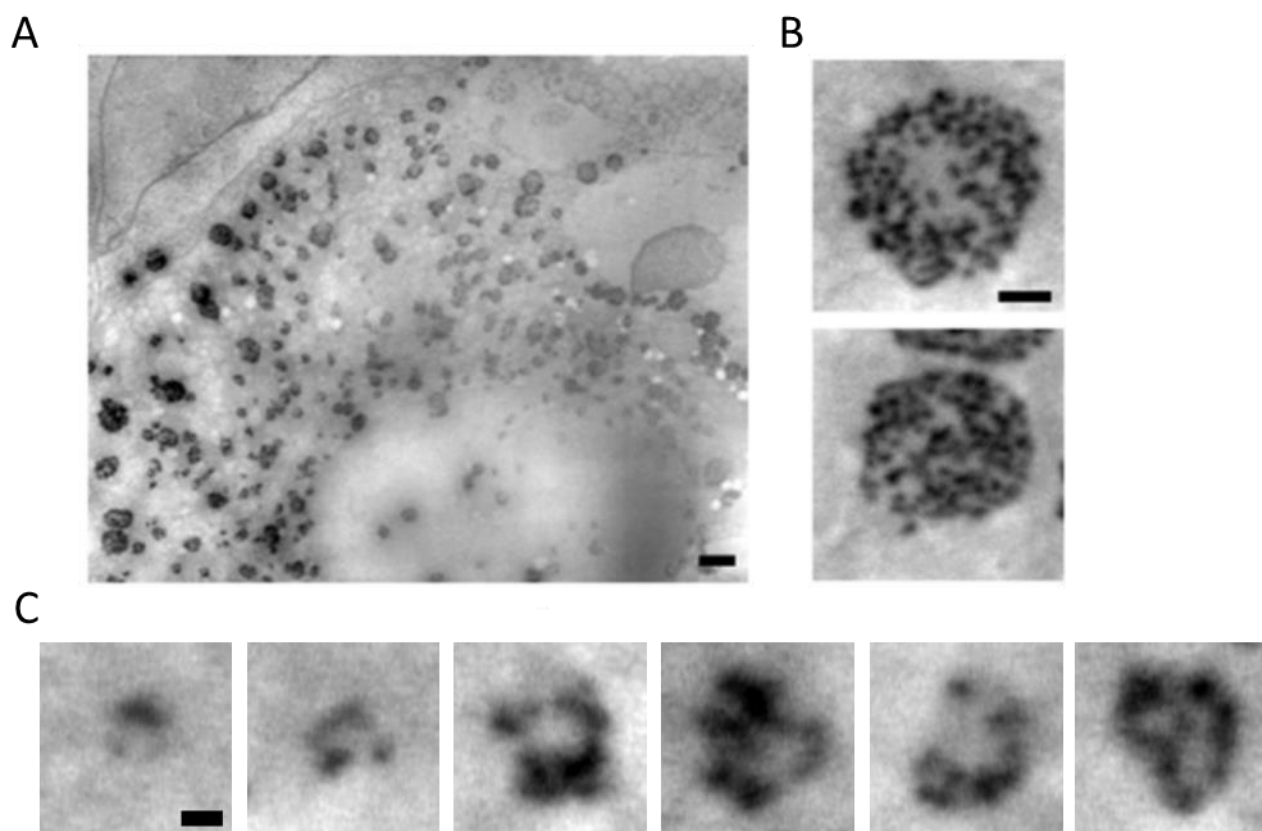

FIGURE LEGENDS

Appendix Figure 1 Various objects in an aqueous solution analyzed under scanning electron dielectric microscope

A. Scanning electron dielectric microscopy images of mineralizing osteoblasts showing the granules accumulation inside the intracellular membranous structures, forming multivesicular bodies

B. Higher magnification of multivesicular bodies

C. Various sizes of matrix vesicles in the osteoblastic cells.

Adapted from Iwayama et al. 2019. Scale bars, 1 μm in A; 500 nm in B; 200 nm in C.

Appendix Table1 Hurdles in the progression of research based on matrix vesicles

Difficulty in imaging
- Possible artifact by chemical fixation and preparation for electron microscopy
- Use of super-resolution fluorescence microscopy, scanning electron dielectric microscopy, and cryo-electron microscopy

Difficulty in isolation
- No specific biomarker
- Might have tissue specificity
- Need to develop a more specific isolation method