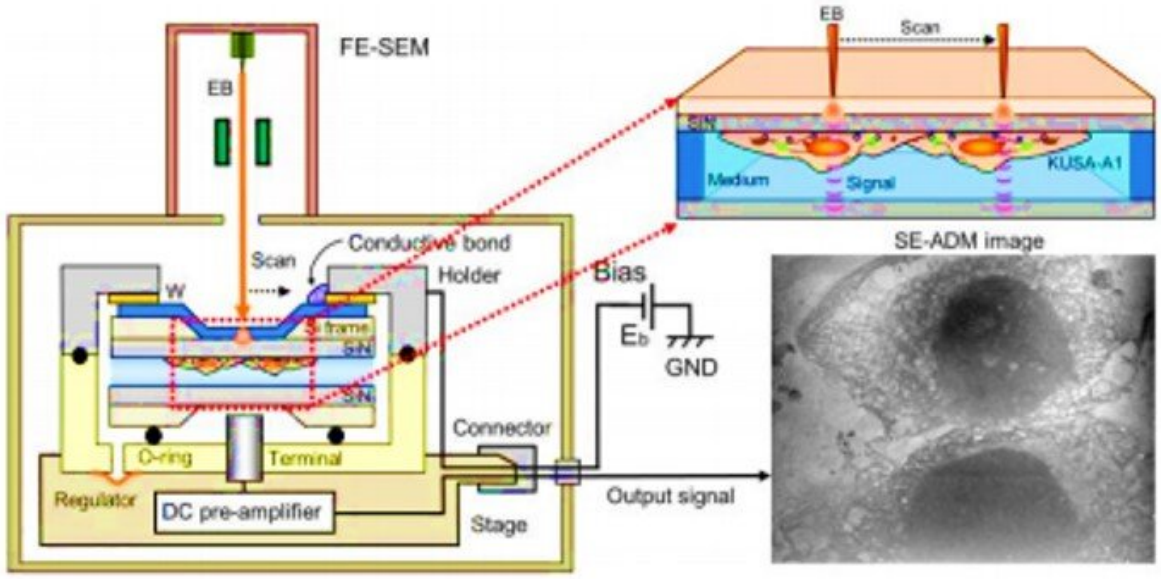
Osteoblastic lysosome plays a central role in mineralization

July 11 2019, by Thamarasee Jeewandara



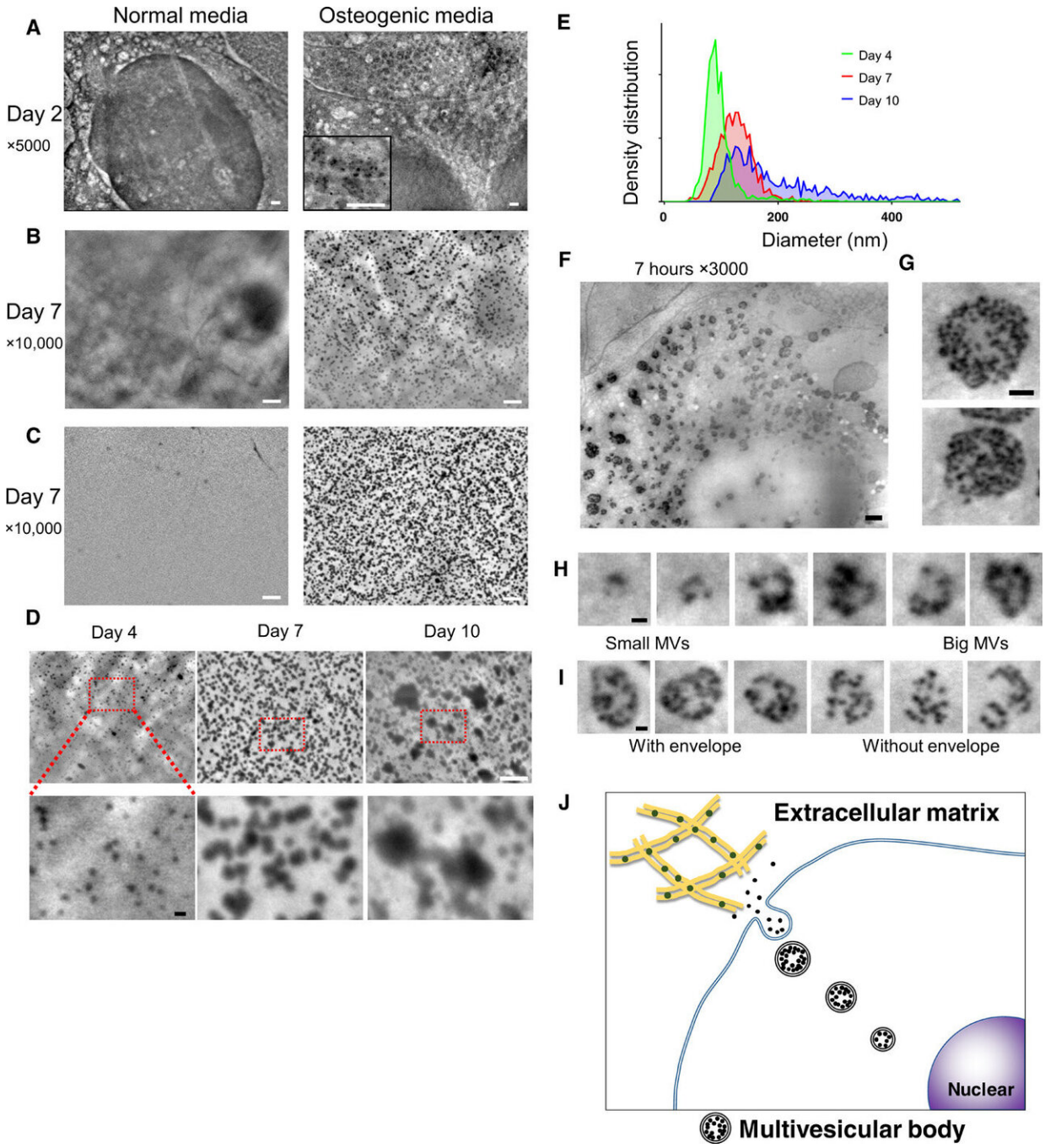
Scanning electron-assisted dielectric microscopy (SE-ADM) observation of osteoblasts. (A) High-resolution SE-ADM set up for osteoblast observation. Liquid-sample holder including osteoblasts is mounted on the pre-amplifier-attached stage, which is introduced into the specimen SEM chamber. The scanning electron beam is applied to the W-coated SiN film at a low acceleration voltage. Measurement terminal under the holder detects electrical signals through liquid specimens. Clear intracellular structures are visible (right image). Credit: Science Advances, doi: 10.1126/sciadv.aax0672

Mineralization is mediated by osteoblasts, which secrete mineral precursors through matrix vesicles (MVs) as a fundamental process in vertebrates. The vesicles are calcium and phosphate rich, containing organic materials such as acidic proteins. In a new study now published in *Science Advances*, Tomoaki Iwayama and colleagues at the departments of periodontology, biomedical research, oral science, biomaterials and oral anatomy development used scanning electron-assisted dielectric microscopy (SE-ADM) and super-resolution microscopy (SRM) to assess live osteoblasts during conditions of mineralization at nano-level resolution. They found the calcium-containing vesicles to be multi-vesicular bodies containing mineralizing nanovesicles or matrix vesicles (MVs). According to the observations, the MVs could be transported together with lysosomes and secreted by exocytosis. Iwayama et al. presented proof that the lysosomes could transport amorphous calcium phosphate in mineralizing osteoblast cells.

During the physiological process of bone mineralization, the deposition of calcium phosphate crystals occurs in the extracellular matrix as a fundamental process in all vertebrates. In 1967, biologists Clarke Anderson and Ermanno Bonucci, individually visualized mineral-related particles in the extracellular space using electron microscopy (EM). Scientists later recognized these particles as mineralizing nano-vesicles or matrix vesicles (MVs). During the past 50 years of EM studies on MVs, biologists have grappled to understand the mechanism of MV formation and secretion, which remains largely unknown.

Clarifying the mineralizing process of live cells with EM is challenging since sample preparation for EM requires steps on both chemical fixation and alcoholic dehydration. The steps can induce artefacts and even dissolve or remove unstable mineral precursors leaving an organic scaffold known as a "crystal ghost". While scientists had successfully used the process of EM using fixed and dehydrated tissue to view the structure of mineralized collagen fibrils in bone, to study mineral

precursors, they must employ cryo-EM processes to avoid dehydration and facilitate costly, extremely fast cooling with small specimens.



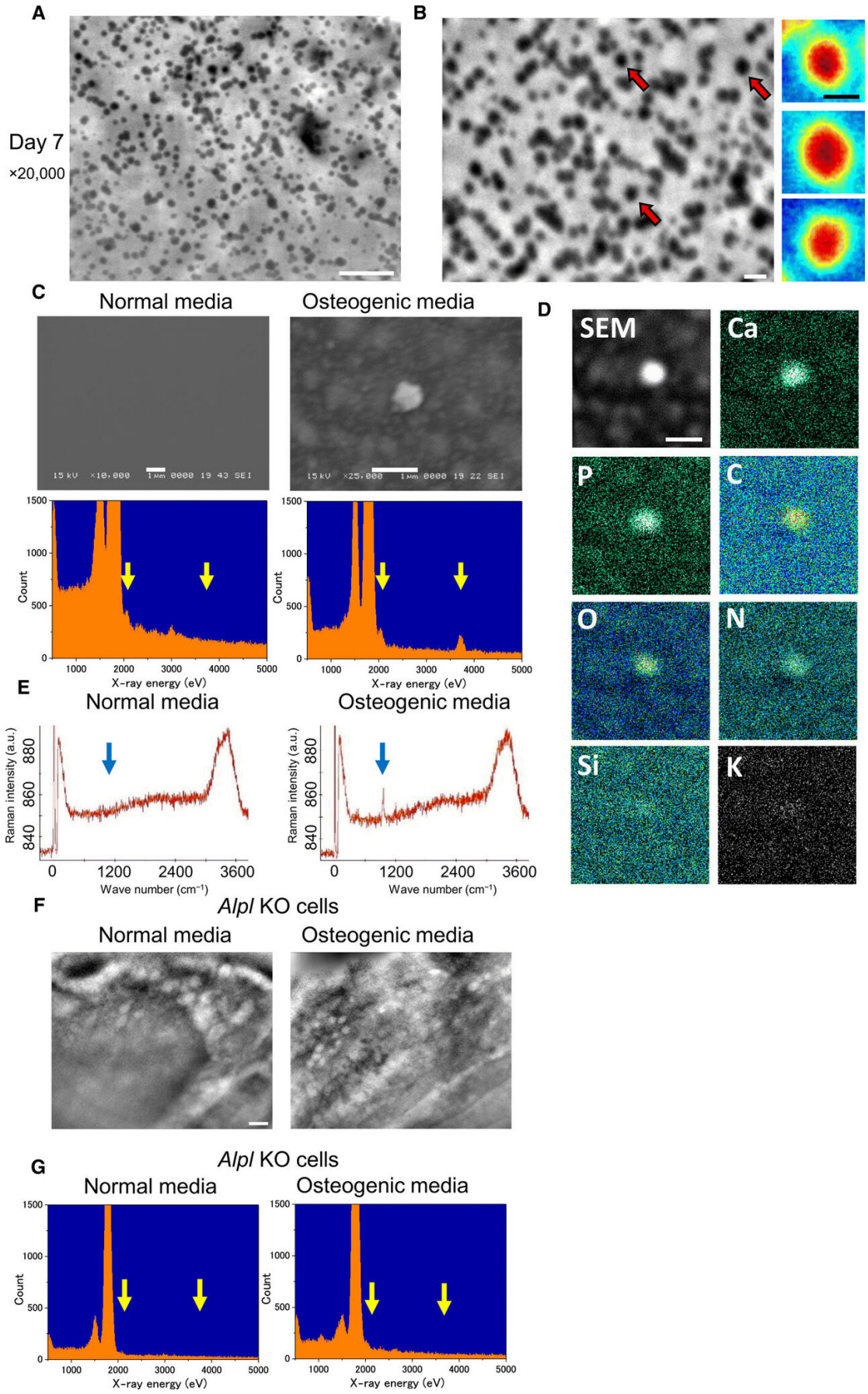
Nanoscale observation of live osteoblasts in culture media, using the SE-ADM system. (A) Representative high-resolution SE-ADM images of osteoblasts

cultured with or without osteogenic media for 2 days. Black particles were evident only when cultured in osteogenic media (right, square in the bottom). (B) Representative high-resolution SE-ADM images of osteoblasts cultured with or without osteogenic media for 7 days. There are many black particles when cultured in osteogenic media (right). (C) Representative high-resolution SE-ADM images of the SiN film after cell removal. In normal media, no particles are observed (left). The image of the film after removal of cells cultured in osteogenic media shows many clear black particles dispersed in the whole area (right). (D) Comparison of particle images during 4 to 10 days of culture in osteogenic media. The particle sizes gradually increased. (E) Distribution of particle size measured during 4 to 10 days of culture in osteogenic media. Approximately 900 to 1100 particles per time point were measured and plotted as a histogram. (F) Representative high-resolution SE-ADM images of osteoblasts cultured with osteogenic media for 7 hours. (G) MVBs have clear gray envelopes. (H) Cut images of various MVB sizes, including particles. (I) Comparison of MVBs with or without a gray envelope. (J) Schematic view of intracellular formation and transport of MVB in mineralizing osteoblasts. Scale bars, 1 μm in (A) to (C) and (F); 500 nm in (G); 200 nm in (D, bottom), (H), and (I). Credit: Science Advances, doi: 10.1126/sciadv.aax0672

To overcome these limitations in the present work, Iwayama et al. used a new microscopic system known as scanning electron-assisted dielectric microscopy (SE-ADM). The method had previously achieved nanoscale resolution and high-contrast imaging for mammalian cells in aqueous media without staining. The scientists used the same technique (high resolution SE-ADM) to explore the possibility of viewing MVs in intact osteoblasts to understand biogenesis of MV trafficking. For the osteoblast cell line they used murine (mouse) osteoblastic cell line KUSA-A1, with high osteogenic capacity in vitro and in vivo. After cell culture under adequate conditions, Iwayama et al. observed the cells with SE-ADM to identify normal intracellular structures. The scientists observed MVs to align with collagen fibrils after 4 to 10 days of cell growth in osteogenic media and the secreted particle size increased due

to fusion or particle growth, with their sizes consistent with previous reports to suggest they were indeed MVs.

Upon further examination with SE-ADM, they noted the involvement of the lysosomal pathway to transport and secrete intraluminal MVs in a similar process to exosomes. Interestingly, both exosomes and MVs are categorized as extracellular vesicles with similar sizes; they are both secreted by osteoblasts and have shared functions during cell-cell communication.


A
Day 7
×20,000
B
C
Normal media
Osteogenic media
Count
X-ray energy (eV)
D
SEM
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E
Normal media
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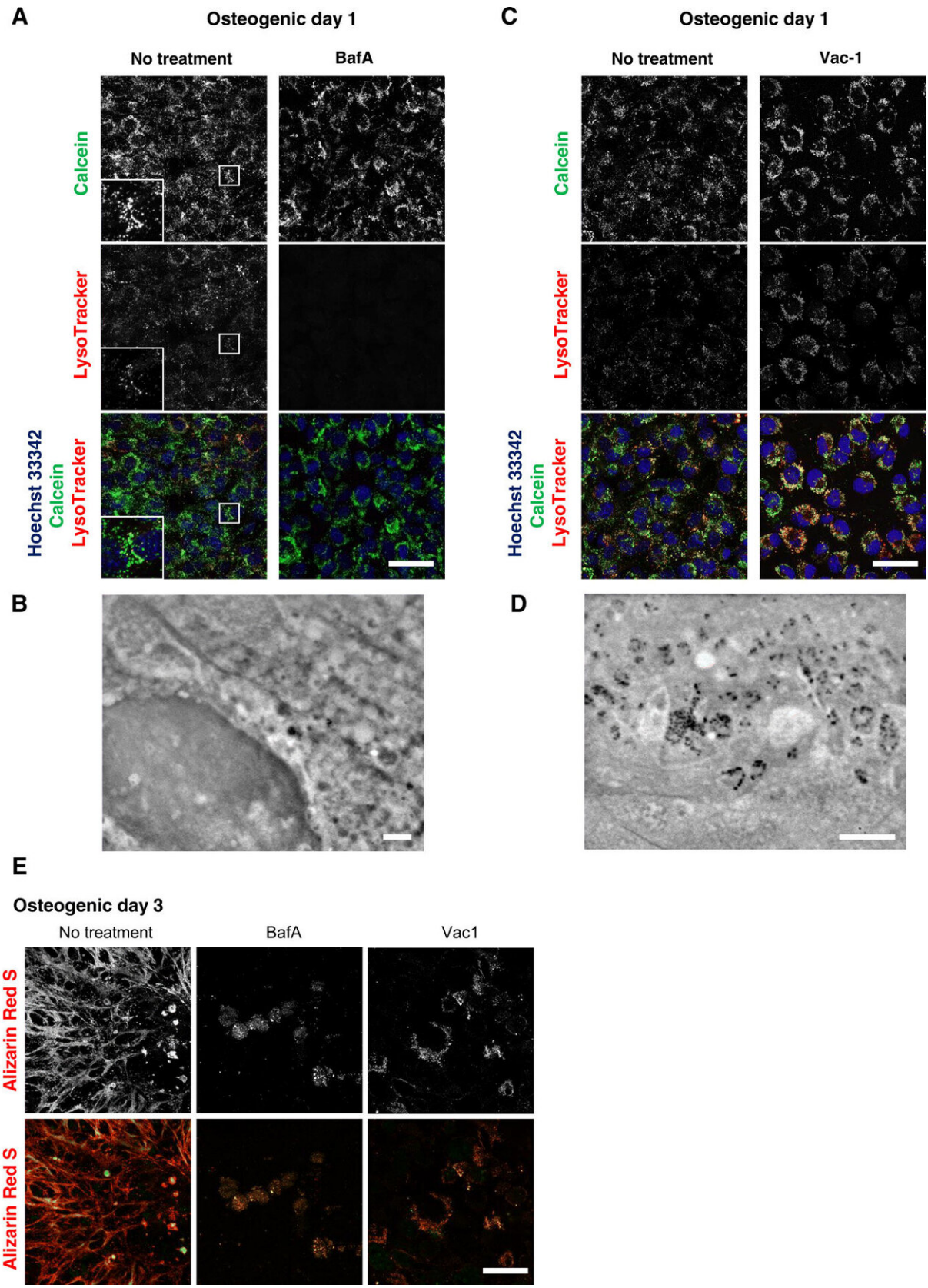
Raman intensity (a.u.)
Wave number (cm⁻¹)
F
Alpl KO cells
Normal media
Osteogenic media
G
Alpl KO cells
Normal media
Osteogenic media
Count
X-ray energy (eV)

Characterization of mineral containing vesicles. (A and B) High-resolution particle images before (A) and after (B) removal of cells cultured in osteogenic media for 7 days. Pseudocolor maps of enlarged particle images indicated by red arrows are shown on the right side of (B). Particles show very smooth structures without crystals. (C) Scanning electron microscopy (SEM) images and EDX spectrometric analysis of particles on a SiN film. SEM image on the left side exhibits the SiN film after removal of cells cultured in normal media, which shows no particles, and EDX spectrometric data show no peaks of phosphorus and calcium. In contrast, the SEM image and EDX spectrometric data on the right side show particles and sharp peaks of phosphorus and calcium after culture in osteogenic media. (D) Analysis of particle elements using EDX spectrometric maps. Particles contained phosphorus, calcium, carbon, and nitrogen. (E) Raman spectra obtained from osteoblasts cultured with or without osteogenic media for 23 days. Sharp peak of 960 cm−1 was evident only in osteogenic media (right side). a.u., arbitrary units. (F) Comparison of SE-ADM images of Alpl knockout (KO) osteoblasts in normal and osteogenic media. Particles completely disappeared in osteogenic media. (G) EDX spectrum of particles from Alpl KO osteoblasts on a SiN film. Left-side EDX spectrometric data exhibit the SiN film after removal of cells cultured in normal media, which show no peaks of phosphorus and calcium. Moreover, particles in osteogenic media of right-side data show no peaks in phosphorus and calcium. Scale bars, 1 μm in (A), (C, top), (D), and (F); 200 nm in (B); 100 nm in (B, right). Credit: Science Advances, doi: 10.1126/sciadv.aax0672

In the next step, Iwayama et al. examined if these particles were MVs containing calcium and/or phosphate. For this, they cultured the cells in osteogenic media for 7 days and observed them using SE-ADM to record very smooth structures without crystal facets. This suggested that the MVs did not crystallize but remained amorphous as also recorded in a previous study. When the scientists examined the MVs on a SiN (silicon mononitride) film, they observed sharp peaks corresponding to

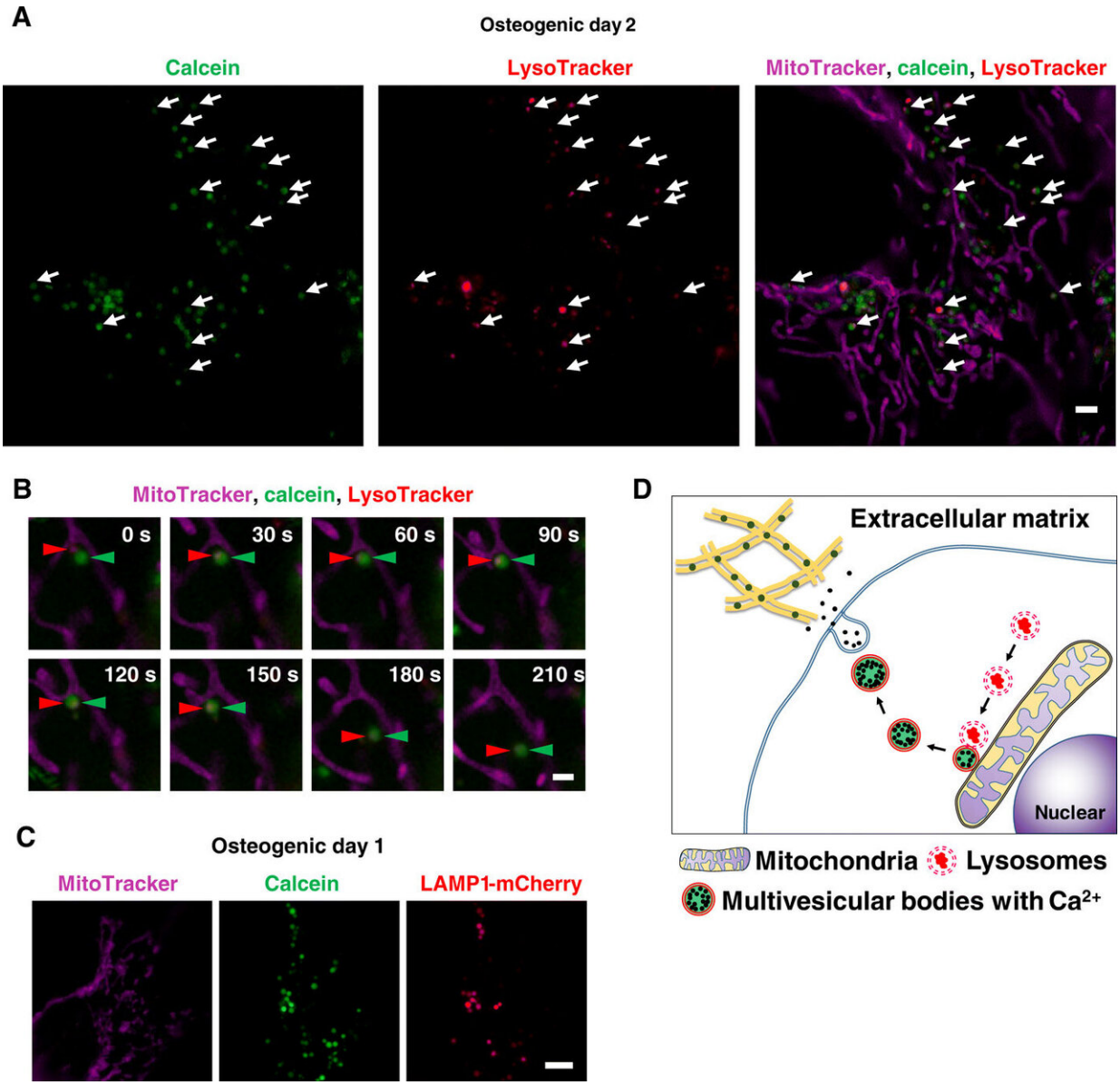
phosphorous, calcium, carbon and oxygen elements. They confirmed the findings using Raman spectroscopy to show the presence of calcium phosphate within MVs.

The scientists also investigated the effects of hypophosphatasia a medical condition encoded by the Alpl (alkaline phosphatase) gene, wherein osteoblasts do not undergo mineralization in vitro. For this, they edited the genome of osteoblast cells using the CRISPR-Cas9 genome editing technology to generate Alpl knockout osteoblast clones. When Iwayama et al. examined the knockout clones using high-resolution SE-ADM, they did not observe MVs, which was further confirmed using spectrometric analysis due to the absence of phosphorous and calcium peaks.


A
Osteogenic day 1
No treatment
BafA
Calcein
LysoTracker
Hoechst 33342
Calcein
LysoTracker
C
Osteogenic day 1
No treatment
Vac-1
Calcein
LysoTracker
Hoechst 33342
Calcein
LysoTracker
B
D
E
Osteogenic day 3
No treatment
BafA
Vac1
Alizarin Red S
Calcein
Alizarin Red S

Lysosomal inhibitors block mineralization. (A and C) Confocal live imaging of 50 nM BafA-or 10 μM Vac-1–treated osteoblasts. Cells were cultured in osteogenic media containing BafA or Vac-1 and stained with Hoechst 33342 and LysoTracker Insets show higher magnification and boxed area of each channel. (B and D) SD-ADM images of BafA- or Vac-1–treated osteoblasts. Cells were cultured in osteogenic media containing BafA or Vac-1. (E) Alizain Red S staining performed without fixation. Cells were cultured in osteogenic media containing BafA or Vac-1 and stained with Alizain Red S. Representative confocal images. Scale bars, 50 μm in (A), (C), and (E); 2 μm (B) and (D). Credit: Science Advances, doi: 10.1126/sciadv.aax0672

After directly observing the production and secretion of MVs using SE-ADM, the scientists further investigated the involvement of lysosomes in intracellular trafficking of MVs to observe live osteoblast mineralization. They cultured the cells in calcium-containing osteogenic media and stained them with LysoTracker to detect the intracellular components of interest. Iwayama et al. located the calcein-fulfilled vesicles matched with lysosomes to suggest the biogenesis of MVs within lysosomes after their fusion with calcein^{+} vesicles. The scientists followed the experiments with loss-of-function and functional inhibition studies to further deconstruct the pathways and examine intracellular mechanisms of action during live cell mineralization in vitro.



Super-resolution live imaging of calcium containing vesicle transports via lysosomes. (A) Snapshot of time-lapse SRM images of calcein-labeled osteoblasts. Cells were cultured with calcein and stained with Lysotracker and MitoTracker. White arrows indicate colocalization of lysosomes and calcein-positive vesicles. (B) Close-up of time-lapse SRM images of calcein-labeled osteoblasts. Red arrowheads indicate lysosome, and green arrowheads indicate calcein. Once lysosomes fused to calcein-positive vesicles adjacent to mitochondria, they started to move toward extracellular space. (C) Representative SRM image of LAMP1-mCherry–expressing cells. Cells were

transfected with LAMP1-mCherry plasmid, cultured with calcein, and stained with MitoTracker. Calcein-positive vesicles matched to LAMP1-mCherry–positive lysosomes. (D) Schematic view of lysosomal involvement in transportation of calcium in mineralizing osteoblasts. Scale bars, 2 μm in (A), 1 μm in (B), and 10 μm in (C). Credit: Science Advances, doi: 10.1126/sciadv.aax0672

Scientists had previously reported the involvement of mitochondria during mineralization due to the presence of electron-dense calcium and phosphorous-rich granules in osteoblast mitochondria. This was observed with a modified cryotechnique. Furthermore, reports also suggest the direct contact of lysosomes and mitochondria with functional significance. When Iwayama et al. stained cells with LysoTracker together with MitoTracker and observed the intracellular components under N-SIM structured illumination super-resolution microscopy (SRM). They observed the presence of most calcein-fulfilled vesicles next to mitochondria and matched with lysosomes. During SRM-time lapse imaging, the scientists further obtained views of intracellular transport of LysoTracker containing vesicles fused to static calcein vacuoles adjacent to mitochondria to validate their hypothesis.

In this way, together with observations of other SRM systems and additional cell lines, Tomoaki Iwayama and colleagues proposed a mineralization mechanism. Wherein lysosomes played a central role in intracellular MV biogenesis and trafficking within osteoblasts. It was reasonable to involve lysosomes for osteoblasts to transport amorphous calcium phosphate without crystallization during its transport in the cytosol. The scientists aim to conduct further experiments to understand the regulatory molecules for MVs and investigate if MVs and exosomes have similar constitutions and mechanism underlying their generation, secretion and function. The SE-ADM strategy used in the present work

can be installed into existing scanning electron microscopy apparatus at a low cost. The work developed in the study will offer non-invasive, high-resolution imaging at the nanoscale applicable to all scientific fields.

More information: Tomoaki Iwayama et al. Osteoblastic lysosome plays a central role in mineralization, *Science Advances* (2019). DOI: 10.1126/sciadv.aax0672

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