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Mineral-bearing vesicle transport in sea urchin embryos

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ABSTRACT

Sea urchin embryos sequester calcium from the sea water. This calcium is deposited in a concentrated form in granule bearing vesicles both in the epithelium and in mesenchymal cells. Here we use *in vivo* calcein labeling and confocal Raman spectroscopy, as well as cryo-FIB-SEM 3D structural reconstructions, to investigate the processes occurring in the internal cavity of the embryo, the blastocoel. We demonstrate that calcein stained granules are also present in the filopodial network within the blastocoel. Simultaneous fluorescence imaging and Raman spectroscopy show that these granules do contain a calcium mineral. By tracking the movements of these granules, we show that the granules in the epithelium and primary mesenchymal cells barely move, but those in the filopodial network move long distances. We could however not detect any unidirectional movement of the filopodial granules. We also show the presence of mineral containing multivesicular vesicles that also move in the filopodial network. We conclude that the filopodial network is an integral part of the mineral transport process, and possibly also for sequestering calcium and other ions. Although much of the sequestered calcium is deposited in the mineralized skeleton, a significant amount is used for other purposes, and this may be temporarily stored in these membrane-delineated intracellular deposits.

1. Introduction

In less than three days after fertilization sea urchin embryos develop a calcium carbonate skeleton consisting of two spicules (Millonig, 1970; Okazaki and Inoue, 1976; Politi et al., 2006; Wilt and Benson, 1988). This rapid mineralization process involves massive sequestering and transport of calcium and carbonate ions to the forming spicule. The calcium ions are sequestered from sea water, whereas the carbonate ions are both sequestered from sea water and are the product of metabolic processes (Sikes et al., 1981). Calcium enters into the embryonic body cavity (blastocoel), probably in the form of ions in solution or possibly nano-sized clusters (Decker and Lennarz, 1988; Hwang and Lennarz, 1993; Nakano et al., 1963; Stumpp et al., 2012; Tellis et al., 2013). The first appearance of calcium carbonate in a concentrated form is observed inside both epithelial cells and primary mesenchyme cells (PMCs) (Beniash et al., 1999; Decker and Lennarz, 1988; Hwang and Lennarz, 1993; Okazaki, 1975; Vidavsky et al., 2014; Wilt et al., 2008). These calcium carbonate deposits consist of nanospheres 20–30 nm in diameter, and are located in an intracellular environment inside membrane delineated vesicles (Beniash et al., 1999; Vidavsky et al., 2014). The blastocoel separates the epithelial tissue from the tissue containing the PMCs. The focus of this study is on the blastocoel, in particular on the dense network of filopodia that connects the epithelium with the PMCs. We use *in vivo* calcein labeling to track calcium deposits and movements, *in vivo* Raman spectroscopy to characterize the mineral deposits and the novel method of cryo-FIB-SEM to better understand 3D aspects of this filopodial network preserved in a high pressure frozen embryo.

Filopodia are thin cytoplasmic extensions of cells that are composed of actin filaments covered by a plasma membrane. They are flexible and strong, and in the sea urchin embryo they grow and retract at rates of up to 10 μm/min (Jacinto and Wolpert, 2001). PMCs, epithelial cells and secondary mesenchyme cells (SMCs) in the sea urchin embryo extend thick (~1 μm in diameter) and thin (0.2–0.4 μm in diameter) filopodia. These filopodia can extend to lengths of tens of microns and are involved in cell–cell interactions associated with cell movement (thick filopodia), signaling and skeletogenesis patterning (thin filopodia) (Gustafson and Wolpert, 1963; McClay, 1999; Miller et al., 1995). It is known that PMC migration and spicule shape are determined through PMC

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filopodia by VEGF (Vascular Endothelial Growth Factor) signaling from the epithelial cells (Duloquin et al., 2007; Knapp et al., 2012), as well as through interactions with the extracellular matrix (Hodor et al., 2000). PMCs and SMCs come into direct filopodial contact during the transmission of cell fate signaling (Ettensohn, 1992). Filopodial connections between mesenchymal and epithelial cells were hypothesized to be involved not only in cell–cell signaling, but also in nutrient transport (Stumpp et al., 2012). Most significantly for this study, 'calcareous granules' were observed within the filopodial strands by light microscopy and electron dense granules were detected by TEM (Decker and Lennarz, 1988; Okazaki, 1960). Here we investigate the dynamic movement of calcium carbonate granules in the filopodial network, and produce high resolution 3D reconstructions of the blastocoel environment and of the vesicles incorporated within the filopodia.

2. Materials and methods

2.1. Sea urchin embryonic/larval culture

Mature cultured adult sea urchins *Paracentrotus lividus* were produced and supplied by the Israel Oceanographic and Limnological Research Institute. Spawning was induced by injecting 1 mL of 1 M KCl solution into the coelomic cavity. The egg suspension was collected, washed, and kept in sea water containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma–Aldrich, 3810740) at 18 °C. Sperm was kept at 4 °C undiluted. The fertilization was carried out by sperm dilution in sea water and rapid mixing with the egg suspension inside sea water containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma–Aldrich, 3810740). The time of mixing is defined as time 0 post fertilization. The culture was kept at 18 °C with gentle shaking (100 rpm). The developmental stage of the embryo was determined from the spicule and embryo characteristic morphology, using a light microscope with normal and polarized light. When the desired prism or pluteus (Giovanni, 1973) developmental stage was reached, which occurs approximately 42–49 h post fertilization, the samples were imaged *in vivo* or high pressure frozen without any processing, as described below. The research involving sea urchins is approved by the Israel Oceanographic and Limnological Research, National Center for Mariculture.

2.2. Calcein labeling

Calcein, 20 μM (Sigma–Aldrich, 154071484), was dissolved in sea water containing 30 mg/L Penicillin G sodium salt (Sigma–Aldrich, 69578) and 15 mg/L Streptomycin sulfate salt (Sigma–Aldrich, 3810740) and filtered in a 0.22-μm sterile Corning filter system. For continuous labeling, developing embryos were immersed from time 0 in calcein-labeled sea water at 18 °C with gentle shaking (100 rpm). At the prism or pluteus stage, the embryos were washed with sea water and examined. For pulse labeling, the embryos were transferred to 20 μM calcein in sea water for 45 min at the prism or pluteus stage, or at 26 h post fertilization and until the pluteus stage was reached. After the labeling, the embryos continued to grow in unlabeled sea water.

2.3. Sample mounting for microscopy and spectroscopy experiments

1 μL of the embryo suspension was put between a glass slide and a glass coverslip, using Vaseline as a spacer to confine the embryos spatially in a hydrated environment.

2.4. Fluorescence microscopy and image processing

The fluorescence images and movies were obtained using a Deltavision system (Applied Precision) with X100 NA 1.4 and X60 NA 1.42 oil immersed objectives and confocal spinning disc system (Zeiss Observer Z1 spinning disk Yokogava CSU-x1) with an X63 NA 1.4 oil immersed objective. In the Deltavision system, calcein was excited at 490 nm and the emission was detected at 525 nm. In the confocal spinning disc, calcein was excited at 494 nm and the emission was collected at 514 nm. For Fig. 7A, 20 time points, at 10 s intervals are shown. For Fig. 7B, 81 time points, at 15 s intervals are shown. Images were taken with a photometrics cool SNAP HQ2 CCD from Roper Scientific. Image analysis and processing were conducted using Image J (US National Institutes of Health, Bethesda, Maryland, USA) and Adobe Photoshop CS 4 Extended (Adobe systems, San Jose, CA). Particle tracking was conducted using the MTrackJ program (Meijering et al., 2012) in 4D.

2.5. Correlative fluorescence imaging and Raman spectroscopy

The Raman setup is based on a commercially available confocal Raman microscope (Alpha 300, WITec, Ulm, Germany) equipped with a Helium Neon (HeNe) laser (633 nm) for excitation and a piezoscanner (P-500, Physik Instrumente, Karlsruhe, Germany). The spectra were acquired with a thermoelectrically cooled CCD detector (DU401A-BV, Andor, UK) placed behind a grating (600 g mm^{-1}) spectrograph (UHTS 300; WITec, Ulm, Germany) with a spectral resolution of 3 cm^{-1}. The red laser beam (633 nm) was focused through a 100× oil immersion (Nikon, NA = 0.9) microscope objective. A pinhole size of 50 μm was used. The ScanCtrlSpectroscopyPlus software (version 1.38, Witec) was used for measurement and WITec Project Plus (version 2.10, Witec) for spectra processing. The in-house implementation of the fluorescence modality on the above mentioned confocal Raman microscope relies on the addition of two dichroic mirrors (Thorlabs, USA) and a CCD camera (Infinity3-1URM, Lumenera Corporation, Canada) in the imaging arm of the microscope, as well as two LEDs for dual excitation at 480 and 380 nm in the illumination arm. The dichroic mirrors were carefully chosen so as to not hinder imaging using Raman microscpectroscopy at 633 nm. Details of a similar setup can be found elsewhere (Bennet et al., 2014).

2.6. Sample preparation for cryo imaging

Embryos and larvae at the prism or pluteus stage were high-pressure frozen: 10 μL of the embryo suspension were sandwiched between two metal discs (3-mm diameter, 0.1 mm and 0.05 mm cavities) and cryo-immobilized in a high pressure freezing device (HPM10; Bal-Tec).

2.7. Cryo-FIB-SEM 3D imaging

Serial FIB milling and block face SEM imaging under cryogenic conditions is a recently developed cutting edge technique for high resolution 3D visualization of unstained and unprocessed samples (Schertel et al., 2013). The samples are first high-pressure frozen omitting any fixation or staining and then these native frozen samples are processed in a FIB-SEM microscope under cryogenic conditions. Thin (down to 10 nm) slices of material are removed in a serial manner by FIB milling and then each newly exposed surface is imaged by SEM using InLens secondary electron (SE) detection. Water-rich areas such as cytoplasm or extracellular space show a brighter grey level in the InLens SE image than lipid-rich structures such as membranes. The image contrast is most likely obtained due to local differences of surface potential as discussed in (Schertel