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—Original Article—

New cellular imaging of oocytes and preimplantation embryos using Lumitein™: Evaluation of oocyte quality and new information on protein dynamics within the perivitelline space during the one-cell oocyte stage in mice

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Abstract. The extracellular matrix between the oocyte and zona pellucida (ZP) plays an important role in mammalian fertilization and preserves the specific environment of the perivitelline space (PVS) during the development of a preimplantation embryo after fertilization. In this study, we applied a highly sensitive luminescent protein dye, Lumitein™, to observe the hydrophobic status of proteins in oocytes and preimplantation embryos. Lumitein™ is widely used for detecting denatured proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lumitein™ fluorescence was detected primarily in the PVS and degenerated first polar body of fresh normal metaphase II (MII) oocytes but much less within the ZP and ooplasm, which suggested a hydrophobic PVS environment in the MII oocytes. Unexpectedly, abnormally-shaped fresh or aged oocytes showed stronger fluorescence in the PVS, which reflected oocyte quality. Interestingly, 10 h after fertilization, the fluorescent signal in the PVS temporarily increased in a patched pattern that appeared and then disappeared by the two-cell stage. After the two-cell stage, the decreased fluorescent signal was maintained throughout the development of the preimplantation embryo. These results suggest new protein dynamics in the PVS during the one-cell stage of the oocyte. Thus, cellular imaging of oocytes and preimplantation embryos using Lumitein™ provides new information on protein dynamics.
Key words: Mouse, Oocyte, Perivitelline space, Preimplantation, Protein dynamics Preimplantation

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The perivitelline space (PVS) between the oolemma and the zona pellucida (ZP), an extracellular matrix (ECM), provides a specific environment during the fertilization and development of the preimplantation embryo [1]. Unfertilized oocytes contain cortical granules, and following sperm penetration, the cortical granules release their contents through exocytosis into the PVS [2, 3]. This cortical reaction results in the diffusion of several proteinases into the ZP that digest ZP2, a ZP protein, which prevents the binding of additional sperm to the ZP and blocks polyspermy [4–7]. In addition, some of the cortical granules remain in PVS to form a new ECM, referred to as the cortical granule envelope (CGE), which is made of electron-dense granules [1, 2]. The CGE is maintained throughout preimplantation development and is only lost after blastocysts hatch from the ZP [2, 8].

After oocyte activation, several types of proteins are also secreted into PVS, including tissue plasminogen activator [9], N-acetylglucosaminidase [10], peptidylarginine deiminase [11], lactate dehydrogenase B chain, arginine deiminase type-6, ubiquitin carboxyl-terminal hydrolase isozyme L1, phosphatidylethanolamine-binding protein 1, heat shock protein 90-alpha, protease serine 1, trypsinogen 7, trypsin 4, trypsin 10, and superoxide dismutase 1 [12]. Thus, the types and quantities of proteins in the PVS of live embryos dynamically change from metaphase II (MII) oocytes to zygotes following oocyte activation. These proteins might provide a specific environment during the development of the preimplantation embryo. In the absence of a ZP, the zona-free (ZF) environment affects the preimplantation development of the ZF fertilized embryos. ZF mouse embryos that are transferred into recipient mice at one-, two-, and four-cell stages do not implant [13]. In addition, the removal of the ZP from fertilized eggs reduces DNA methylation at the two- and four-cell stages [14]. However, the roles of proteins in the PVS during embryonic development after fertilization remain unclear.

Small fluorescent probes have become valuable tools for fluorescence-based live-cell imaging, which enables the visualization of a variety of cellular processes in live cells, including the dynamics of specific cellular organelles, changes in microenvironments, and numbers of biomolecules of interest [15]; however, there are few small fluorescent probes that can visualize the cell surface and ECM [16]. Lumitein™ Protein Gel Stain 100X (Biotium, Hayward, CA, USA; U.S. Patent, US9193746) [17], a highly sensitive noncovalent fluorescent dye, is widely used to detect denatured proteins in the gels used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). In this study, we applied Lumitein™ to visualize the protein dynamics in the PVS of oocytes and embryos.

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Materials and Methods

Animals

Oocytes and sperm were collected from female and male Institute of Cancer Research (ICR) mice that were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were approved by the Animal Experimentation Committee at the University of Yamanashi, Japan and were conducted in accordance with the committee's guiding principles.

Collection of oocytes

Mature oocytes were collected from the oviducts of 8- to 10-week-old female mice that had been induced to superovulate with 5 IU pregnant mare serum gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) followed by 5 IU human chorionic gonadotropin (hCG) (ASKA Pharmaceutical) 48 h later. Cumulus–oocyte complexes (COCs) were collected from the oviducts ~16 h after hCG injection. To collect small denuded MII oocytes, COCs were placed in HEPES-buffered CZB medium and treated with 0.1% bovine testicular hyaluronidase (Sigma-Aldrich, St Louis, MO, USA). After several minutes, the COCs were washed twice and placed into a droplet of CZB medium for culture.

In vitro fertilization and subsequent embryo development

Sperm collected from the caudal epididymis of mature ICR males over 10 weeks old were allowed to disperse in human tubal fluid (HTF) medium and were preincubated for 1 h at 37°C with 5% CO_2. For *in vitro* fertilization (IVF), the collected COCs were placed with the sperm in HTF medium for 3 h. Oocytes fertilized *in vitro* were cultured in the CZB medium for 96 h to examine their subsequent development. For rotenone treatment, 10 mM rotenone stock solution (TCI, Tokyo, Japan) was diluted into 5 μM rotenone with CZB medium. The two-cell embryos were transferred into CZB medium containing 5 μM rotenone. After 24 h, the embryos were stained with Lumitein™.

Staining with Lumitein™

Lumitein™ was diluted with CZB medium to prevent any cytotoxicity from the full concentration of the dye. The Lumitein™ Protein Gel Stain 1 × used in this study was diluted 400 times with CZB to 0.25 ×. Oocytes and embryos were placed separately in the medium for 30 min at 37°C with 5% CO_2 in the presence of Lumitein™, fixed with 4% paraformaldehyde for 10 min at room temperature, washed five times with PBS, and transferred into a glass-bottom dish (MatTek, Ashland, MA, USA) for observation. Serial images were taken using fluorescence confocal microscopy (FV-1200, Olympus, Tokyo, Japan). We applied Lumitein™ to visualize the denatured or hydrophobic surface-exposed proteins in the oocytes and preimplantation embryos. We observed the stained oocytes using confocal microscopy with excitation by a laser line at 559 nm and emissions between 575 and 675 nm. The intensity of Lumitein™ fluorescence was quantified using the digital images of sections using Image J (https://imagej.nih.gov/ij/).

Statistical analyses

Fluorescent intensities were compared using the Student's *t*-test, and differences among the experimental groups were considered significant when $P < 0.05$. The chi-square test analyzed the resulting data on embryonic development.

Results

Staining fresh normal MII oocytes with Lumitein™

Lumitein™ is a widely used luminescent dye designed for detecting denatured proteins in SDS-PAGE gels. Lumitein™ is commercially provided as Lumitein™ Protein Gel Stain 100X and generally used at 1X for protein gel staining. To avoid any artificial staining by fixation with Lumitein™, we stained, washed, and fixed oocytes before observing them under a confocal microscope. To minimize oocyte damage and avoid any artificial changes in the proteins from the solution with Lumitein™, we stained live oocytes with Lumitein™ diluted from 0.125X to 1X (Fig. 1A) at 37°C for 30 min with 5% CO_2. Although the oocytes stained with Lumitein™ > 0.5X had bright fluorescence, some also showed strong fluorescence in the ooplasm, which indicated oocyte abnormalities, and is described in the next subsection (Fig. 1A). In contrast, either Lumitein™ 0.25X or 0.125X staining was enough to detect Lumitein™ fluorescence in the PVS with minimal fluorescence in the ooplasm; therefore, we used Lumitein™ 0.25X staining for further analysis. Additionally, we confirmed that 1X staining of fertilized embryos developed at a significantly reduced blastocyst rate (Fig. 1B). In a normal fresh MII oocyte stained with Lumitein™ 0.25X, the fluorescent signal was detected primarily in the PVS and degenerated first polar body. In contrast, the ZP and the ooplasm showed extremely low fluorescence. Next, we examined whether fixation affected the fluorescent pattern. A similar fluorescence pattern was observed when cells were stained after fixation, which suggested that fixation did not affect staining patterns, and confirmed lower fluorescent intensity in the ZP and ooplasm of MII oocytes as compared with that of the PVS (Fig. 1C). Thus, we concluded that 0.25X Lumitein™ was enough to visualize fluorescence in the PVS. These results revealed the main subcellular localization of Lumitein™-positive proteins was in the PVS of the MII oocytes, including the degenerated first polar body.

Lumitein™ staining of MII oocytes with abnormal morphology

As mentioned in the previous subsection, fluorescence was detected largely in the PVS as well as the degenerated first polar body in normal MII oocytes. In contrast, a remarkably lower fluorescent signal was observed in the ooplasm and ZP (Fig. 2A); however, morphologically abnormal oocytes, such as those with shrunken or fragmented ooplasm, demonstrated an unusually strong Lumitein™ fluorescence intensity in the PVS and ZP, which suggested distinct changes in protein structure or quantity, or both. Conversely, we observed extremely weak fluorescence in an oocyte with a damaged ZP in the PVS; therefore, we created a ~10-μm diameter puncture using a glass needle and a micromanipulator with piezo-electric elements to examine whether the damaged ZP maintains Lumitein™-positive materials in the PVS. Thirty minutes after puncturing the ZP, the Lumitein™ signal significantly decreased in the PVS (Figs. 2B, C). Thus, the Lumitein™-positive materials could be held in the PVS, depending on the integrity of the ZP. Taken together, the Lumitein™ staining pattern could reflect oocyte and ZP normality.

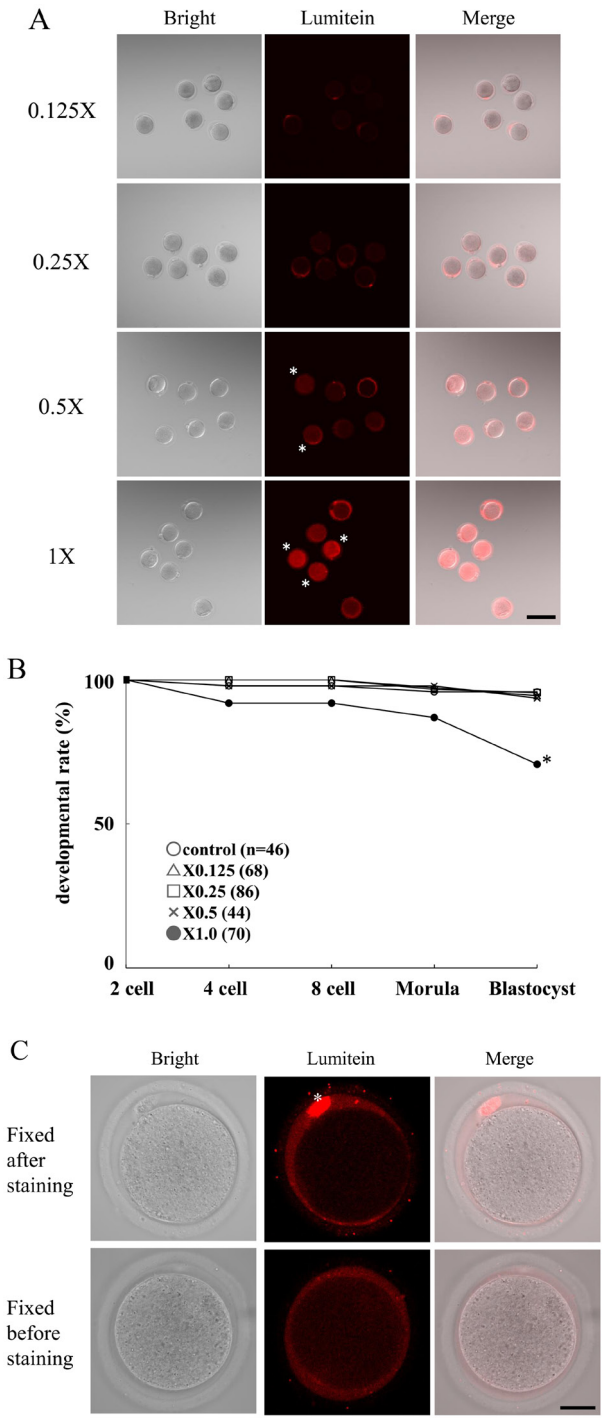

Fig. 1. Optimization of Lumitein™ staining on fresh oocytes. (A) Freshly collected mouse oocytes incubated with CZB medium containing Lumitein™ 0.125X for 30 min at 37°C with 5% CO_2 and fixed with 4% paraformaldehyde. Asterisks (*) indicate oocytes with bright fluorescence in the ooplasm. Scale bar = 100 µm. (B) To evaluate the toxicity of Lumitein™ staining, these fertilized oocytes were subjected to *in vitro* culture for 96 h. * $P < 0.05$. (C) Lumitein™ staining either before or after fixation by 4% paraformaldehyde provided similar images, which implies the impact of fixation before staining on a fluorescence image is minimal. Scale bar = 20 µm. An asterisk (*) indicates the first polar body with strong fluorescence, which is out of focus in the fixed oocyte.

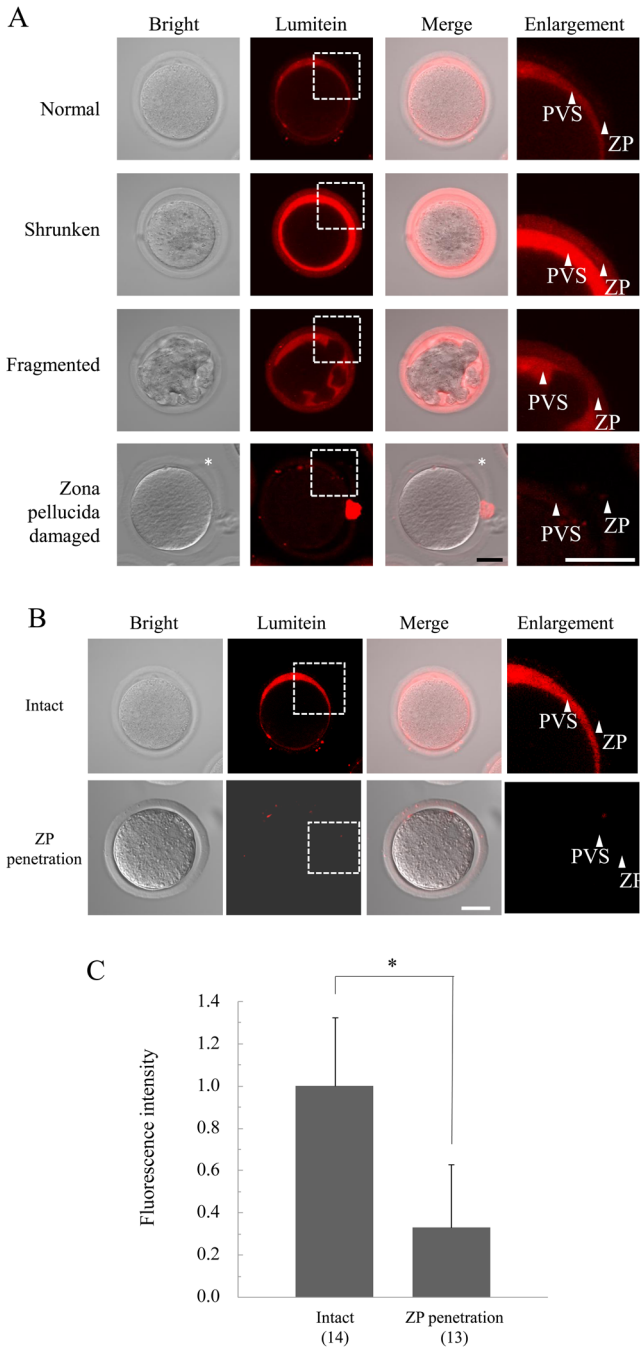

Fig. 2. Distinct Lumitein™ staining patterns in abnormal oocytes. (A) Abnormal oocyte morphologies with distinct staining patterns. Asterisks (*) indicate damaged zona pellucidae (ZP). Scale bar = 20 µm. (B) ZP of a freshly collected oocyte penetrated with a glass needle (~10 µm in inner diameter) and stained with Lumitein™ 0.25X for 30 min. Scale bar = 20 µm. The photos of these oocytes were quantified (C). Fluorescence intensity. The numbers in parentheses indicate sample size. * $P < 0.05$.

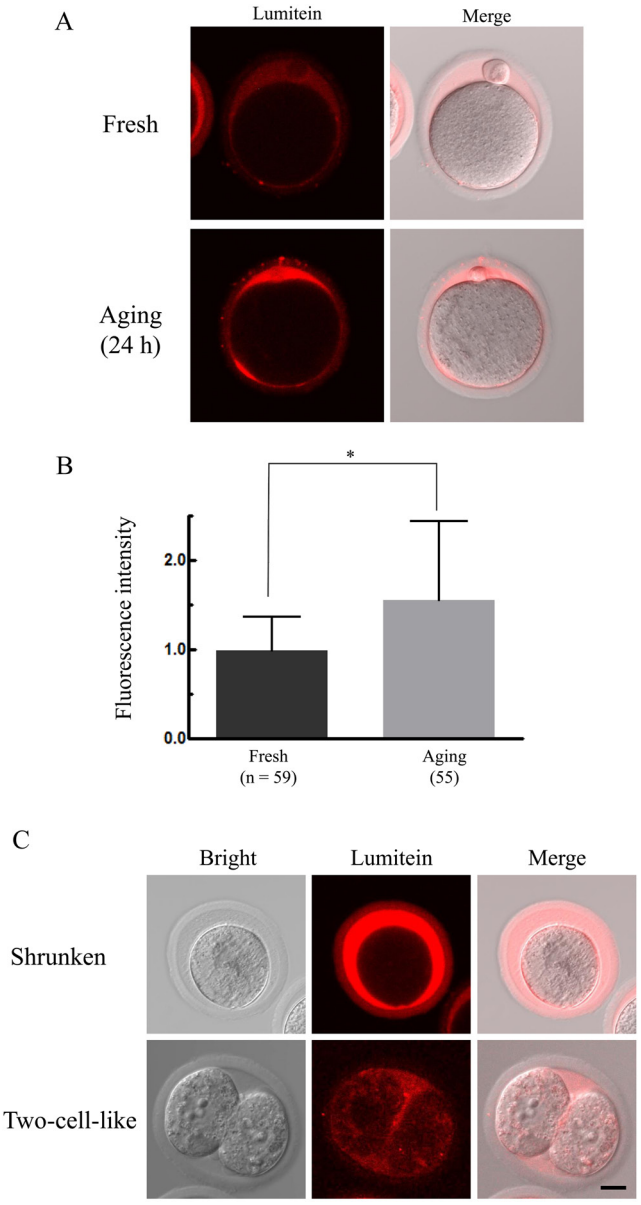

Fig. 3. Distinct Lumitein™ staining patterns in postovulatory aging oocytes. (A) Freshly collected mouse oocytes incubated with CZB medium for 24 h and stained with Lumitein™ 0.25X for 30 min. More fluorescence was detected in the perivitelline space (PVS) of aging oocytes with apparent normal morphology. Photos were quantified. (B) Fluorescence intensity. The numbers in parentheses indicate sample size. * $P < 0.05$. (C) Aging oocytes with abnormal morphologies showing distinct patterns. Aging oocytes with shrunken ooplasm showing more fluorescence in the PVS and zona pellucida (ZP). Two cell-like aging oocytes show strong fluorescence inside the ooplasm. Scale bar = 20 µm.

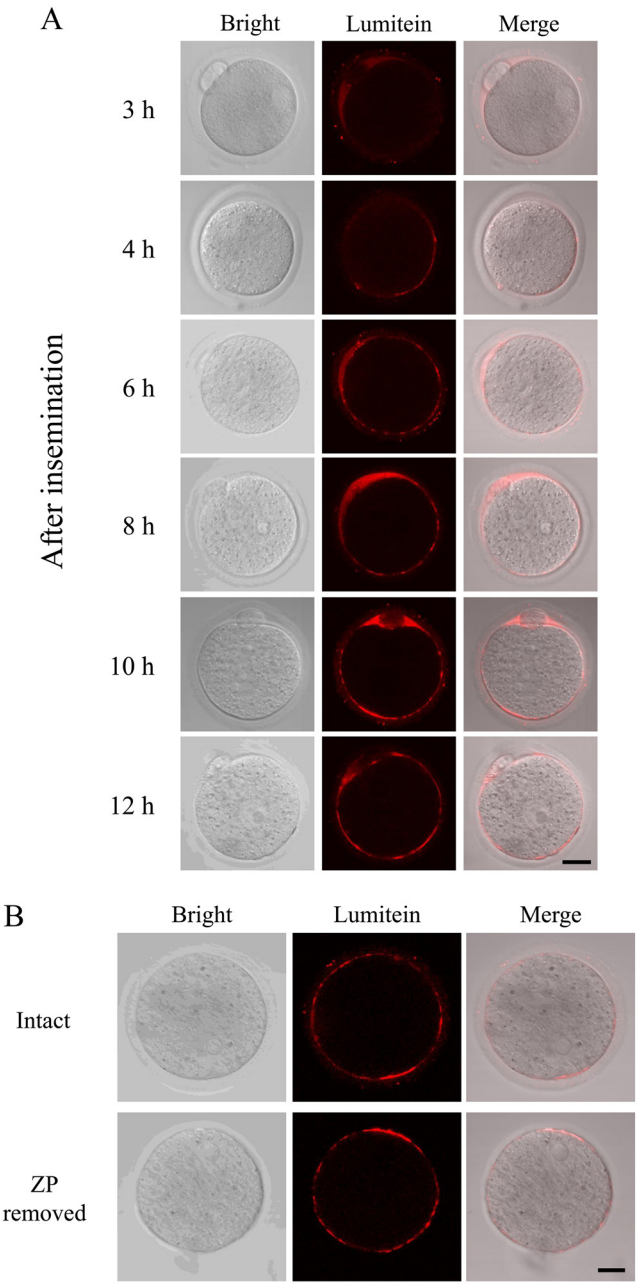

Fig. 4. Dynamic changes in Lumitein™ staining patterns during the one-cell stage after fertilization. (A) Embryos stained with Lumitein™ 0.25X for 30 min from 3 to 12 h after insemination. Perivitelline space (PVS) shows more fluorescence 4 h after insemination. Asterisk (*) marks a degenerated polar body. Scale bar = 20 µm. (B) A discrete signal in the PVS detected after removing the zona pellucida (ZP). (C) Three-dimensional view of sections from 0 (unfertilized) to 24 h after insemination and of the Lumitein™-positive patched structure observed on the embryo surface 10 h after insemination. Scale bar = 20 µm.

Lumitein™ staining of postovulatory aging oocytes with normal and abnormal morphologies

During postovulatory oocyte aging, dynamic cellular and molecular changes occur that result in the loss of oocyte developmental potential [18]. Next, we compared Lumitein™ staining patterns between fresh and 24 h-postovulatory aged oocytes. There was a significant increase in Lumitein™ fluorescence in the PVS staining patterns

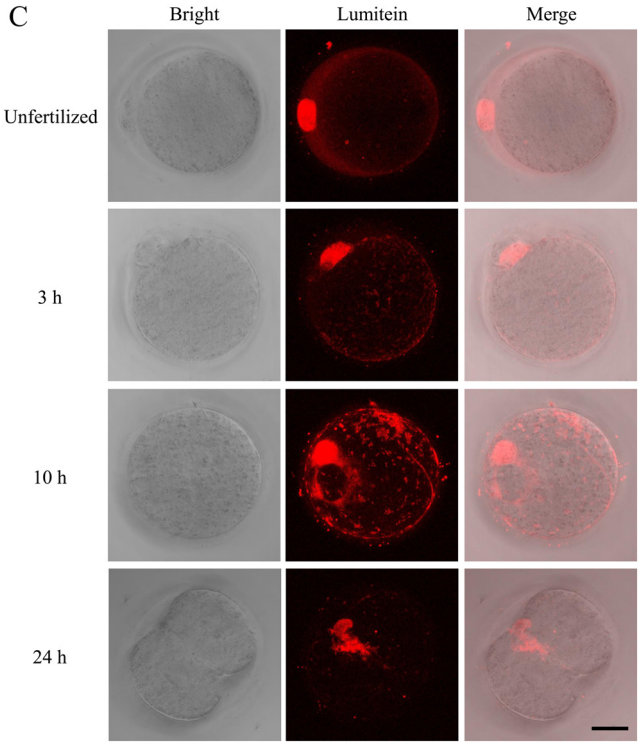

Fig. 4. continued.

between fresh and aged oocytes (Fig. 3A, B). Some aged oocytes showed an abnormal morphology 24 h after being collected [19, 20]. We also observed an increased signal in the PVS and ZP in the shrunken oocytes and the ooplasm of the two-cell-like oocytes (Fig. 3C). Thus, Lumitein™-staining patterns changed with oocyte aging and morphological abnormalities.

Dynamic changes in Lumitein™ staining patterns during the one-cell stage after fertilization

Lumitein™ fluorescence in the PVS displayed strong discrete fluorescent signals 4 h after insemination (Fig. 4A). No change in fluorescence was observed when the ZP was removed with an acidic Tyrode solution, which contrasts with that of the MII oocytes (Fig. 4B). These data suggested that the patched Lumitein™-positive structure was not diffusible and might have been associated with the oolemma (Fig. 4B). Using three-dimensional (3D) images constructed from a series of confocal z-stacks of Lumitein™-stained embryos, a strong patched staining pattern was revealed on the surface of oocytes 10 h after IVF; however, most of the staining pattern disappeared by the two-cell stage 24 h after insemination (Fig. 4C). The temporal appearance of Lumitein™-positive materials on the oolemma suggested that a dynamic reorganization of proteins might occur in the PVS.

Lumitein™ staining of in vitro fertilized embryos after the two-cell stage

We stained the fertilized embryos from the one-cell to blastocyst stages with Lumitein™. After the two-cell stage, a low level of

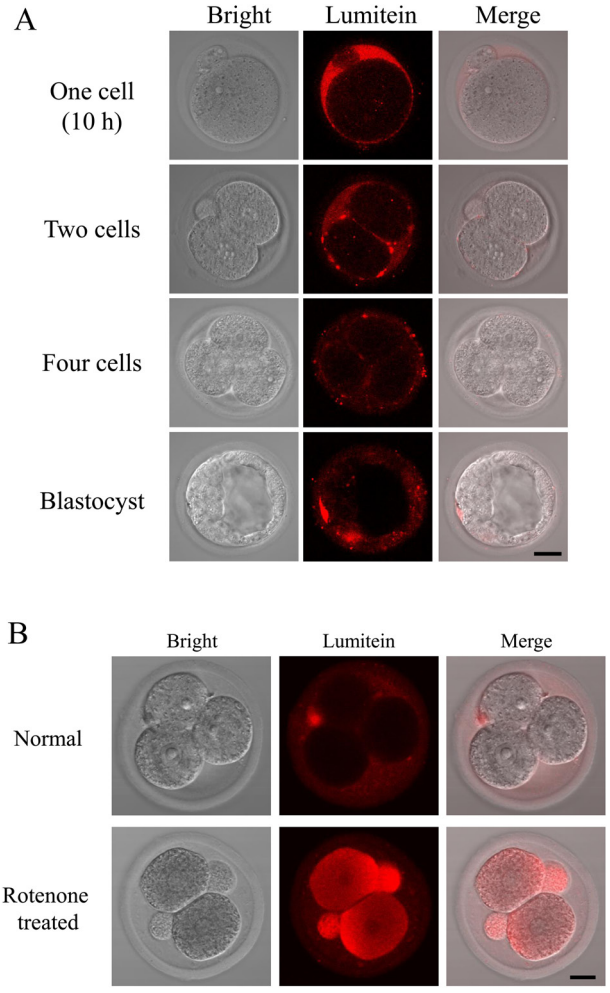

Fig. 5. Lumitein™ staining patterns in preimplantation embryos after fertilization and in degenerated embryos. (A) One-cell stage to blastocyst stage stained with Lumitein™ 0.25X. Scale bar = 20 µm. (B) Two-cell stage embryos were cultured in the presence of 5 µM rotenone for 24 h and stained with Lumitein™. Scale bar = 20 µm.

Lumitein™ fluorescence was detected in the PVS, and occasionally a high level of fluorescence was observed in the degenerated first or second polar body, or both (Fig. 5A). Interestingly, more fluorescent signals were observed at the blastocyst stage in the cytoplasm of the trophectoderm but not inside the blastocoel or inner cell mass. Finally, we examined how cell degeneration could affect the Lumitein™ staining pattern given that the degenerated polar bodies showed strong fluorescence. After IVF, we treated two-cell stage embryos with 5 µM rotenone, a respiratory chain inhibitor, for 24 h, which stopped their development and caused their degeneration. Consistently, strong fluorescence was observed in the cytoplasm of embryos degenerated by rotenone and stained with Lumitein™ (Fig. 5B).

Discussion

Our objective was to observe hydrophobic surface-exposing proteins in oocytes and preimplantation embryos using a highly sensitive luminescent protein dye, Lumitein™ Protein Gel Stain, which was originally designed for detecting small amounts of proteins (≤ 1 ng) using SDS-PAGE gels. In general, Lumitein™ will not stain proteins in nondenaturing polyacrylamide gels but is sensitive to protein hydrophobicity, which was an advantage for this study. However, the product is supplied as a ready-to-use 1X staining solution or as a highly concentrated 100X solution, which includes methanol and acetic acid (safety data sheet for Lumitein™). Therefore, to avoid possible Lumitein™ 1X toxicity to oocytes and embryos, we used Lumitein™ 0.25X or 0.125X to minimize any damage and enable us to observe any changes in fluorescence in the live cells. Using these Lumitein™ concentrations, we observed that in a normal fresh MII oocyte, the fluorescent signal was primarily detected in the PVS and degenerated first polar body. In contrast, the ZP and ooplasm showed relatively low fluorescence, which implied that most proteins within these structures expose more hydrophilic regions on their surfaces. Consistently, the fixation process before Lumitein™ staining did not dramatically affect the fluorescent images, which suggested that fixation does not dramatically change protein structure.

More than one-half of the first polar body is known to degenerate within a few hours after ovulation, and the majority disintegrates during the next 12 h [21]. The degenerated polar body shows apoptotic DNA fragmentation [22]. High fluorescence intensity in the degenerated first polar body might reflect digested proteins that have exposed their hydrophobic surface areas after proteolysis from apoptosis [23].

Fluorescence was significant in the PVS of freshly ovulated MII oocytes, which suggested a hydrophobic environment; fluorescence was reduced by the four-cell stage. Studies have revealed that the PVS is a complex matrix comprised of filaments and granules with proteins [2, 8]. In addition, recent studies have revealed that the PVS is filled with CD9- and CD81-containing exosomes, which play an important role in fertilization [24–26]; therefore, we speculate that a Lumitein™-positive hydrophobic environment of the PVS might represent such proteins on the exosomes. The hydrophobic environment in the PVS might contribute to fertilization processes, including gamete fusion. We also found that penetration of the ZP or damaged ZP could not maintain the Lumitein™-positive materials, which implied its diffusible nature in the PVS. Reportedly, intracytoplasmic sperm injection (ICSI) and IVF embryos show different gene expressions [27]. Because the ICSI method includes penetration into the ZP, the loss of the Lumitein™-positive materials might result in a different environment, which could explain the different gene expressions of ICSI and IVF.

In either the fresh or aging oocytes with abnormal morphologies, more accumulation of Lumitein™-positive proteins in the PVS was at times concomitant with those of the Lumitein™-positive ZP. Thus, once the oocyte physiology is altered by aging or damage, the PVS environment is altered as well. Interestingly, the abnormally shrunken oocytes often had Lumitein™-positive ZPs; however, the mechanism underlying this result remains unclear. Perhaps, the ZP proteins were denatured, or the hydrophobic proteins were accumulated from the PVS. Alternatively, the Lumitein™ signal in the ZP after fertilization or in aged oocytes with normal morphology was not dramatically increased. The murine ZP consists of an intertwined 3D meshwork of filaments formed by three glycoproteins (ZP1, ZP2, and ZP3), which have a conserved polymerization ZP domain (ZP domain) that directs protein fibril formation [28–30]. The cleavage of ZP2 after fertilization is accomplished by ovastacin, an egg cortical granule metalloendoprotease [31, 32], that contributes to blocking polyspermy [4–7]. In addition, the ZP of aged oocytes is hardened and displays a “cobblestone” appearance, which is comprised of tight aggregations of granulofibrillar materials [33]. Regardless, Lumitein™ fluorescence intensity in the ZP was not affected by the digestion of ZP2 protein after fertilization or ZP hardening by aging, which suggested that these processes might maintain the internal hydrophobic regions.

In this study, we first described a Lumitein™-positive characteristic pattern that temporarily appeared only at the one-cell stage. After fertilization, a ruthenium red–positive cortical granule envelope developed in the PVS around fertilized oocytes [2]; however, this envelope lasts only until the blastocyst stage, which is not inconsistent with the behavior of Lumitein™-positive materials. Although the reason for this phenomenon is unclear, one possibility is that the proteins in the oocytes, including membrane proteins, might dynamically reorganize during this timeframe, leading to physiological changes in the embryos. For example, Na+/H+ exchange becomes prominent during the late one-cell oocyte stage by Janus kinase 2 to maintain cell volume homeostasis [34].

To our knowledge, this is the first study that utilizes Lumitein™ to image and analyze the dynamics of protein hydrophobicity in oocytes and embryos. This new and simple staining method would provide a convenient assessment of the ZP, oocytes, and embryos. Although we presume that Lumitein™ fluorescence reflects the amount of the hydrophobic surface-exposing proteins, additional studies should focus on the molecular bases relevant to Lumitein™ fluorescence changes, such as those in aged oocytes and one-cell stage embryos.

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