Spontaneous self-assembly of DNA fragments into nucleus-like structures from yolk granules of fertilized chicken eggs: Antoine Béchamp meets Bong Han Kim via Olga Lepeshinskaya

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1. Introduction

In the history of biology, there have been some scientists to be regarded as heretics: Antoine Béchamp (Béchamp, 1912), Gaston Naessens (Davies, 1991), Wilhelm Reich (Wilehlm, 1979), Olga Lepeshinskaya (Lepeshinskaya, 1954) and Bong Han Kim (Kim, 1965a,b). They have been neglected as they insisted the fundamental unit is not a cell but microparticles. Their microparticles had different names respectively: microzyma, bion, somatid, living substance and sanals. However they had shared with the same idea on the alternative pathway to form new cells independent from cell mitosis via assembly of DNA containing microparticles. Among them our attention was focused on Bong Han Kim’s work since 2002 up to recent days. Firstly we have investigated the realization of anatomical structures corresponding to acupuncture meridian system as Bong Han Kim insisted: primo vessels (PV) and primo nodes (PN) floating in blood vessel (Lee et al., 2004, 2008), bovine heart (Lee et al., 2011), lymphatic vessel (Johng et al., 2007; Lee et al., 2006), brain ventricle and brain venous sinuses (Lee et al., 2010, 2012a). Very recently one of authors, BC Lee has noticed the system consisting of PV and PN, primo vascular system (PVS), contain and may transport DNA containing microparticles (Lee et al., 2013a).

Based on our previous works, we have demonstrated the assembly and fusion of DNA containing microparticles even though we could not prove the fused DNA structures play a role as
cells (Lee et al., 2012b,c, 2013b). Interestingly recent days, some papers reported that DNA nanoparticles assembled into 3D structures; however, they assembled under artificial condition, not in a living body (Ke et al., 2012; Sudhof, 1995). At this point, we conjectured that DNA fragments from a living body could assemble under certain conditions. From reference studies, we noticed that yolk granules themselves contained genomic DNA fragments, but their functions remained a mystery. Moreover, one pioneer, Olga Lepeshinskaya, considered yolk granules as living substances to make new cells. From our previous works and references, we proposed a working hypothesis that DNA fragments in yolk granules have a key role in the assembly of nuclei.

In order to investigate our working hypothesis, as in previous work we made a special chamber in which a microscope was inserted with a temperature-control system for simultaneous incubation and observation of DNA fragments. Specimens were taken from homogenated blastoderm and area opaca of fertilized chicken eggs as they have many clusters of yolk granules. For tracing the DNA signals of the specimens, we applied two vital DNA staining dyes, acridine orange and Hoechst 33258. We also used partial phase contrast microscopy to observe not only phase contrast images but also bright-field images of the yolk granules. Finally, we used deoxyribonuclease I (DNase) to confirm that those DNA signals were from real DNA molecules. In a series of experiments, we were able to trace detailed image changes in the DNA signals over the incubation time. Thus, we suggest the possible assembly of DNA that may be released from yolk granules. We also discuss similarities and differences between the work of the first pioneer, Olga Lepeshinskaya and our work on yolk granules.

2. Materials and methods

2.1. Setup for observation

For these experiments we made a special setup for observation and incubation for specimens. The detail description was made in previous our work (Lee et al., 2013b).

2.2. Preparation of specimens from fertilized chick eggs

Fertilized fresh eggs (each about 60 g) were obtained from Nonghyup Company (Deajeon, Korea) for this study. The eggs were incubated in an automatic digital incubator (Boochoaechonguk, Deajeon, Korea), which kept the eggs at a relatively constant temperature of 37.2 °C to 38.0 °C. In order to specify the exact specimen, we isolated the blastoderm or the area opacas from fertilized eggs at different stages. We also manually homogenated them by using a rubber stick. All procedures to make the specimens were performed on a clean bench to avoid contamination.

2.3. Observation of incubating specimens

1–2 drops of 0.01% acridine orange or Hoechst 33258 in phosphate-buffered saline (PBS, pH = 7.4) was added to the above-prepared specimens. After that we loaded about 20 μl of them into the Vaseline walls on the slide glass as illustrated in Fig. 1. Before loading the prepared specimens, we checked that the incubating system as shown in our previous work (Lee et al., 2013b) was working at the optimal temperatures, about 37.5 °C. Then, we observed the changes in the yolk granules for several hours by using a (CCD). In order to exclude optical artifacts, we took all pictures at the same ISO 1600 to compare the changes in the yolk granules over the incubation time. Finally, in order to confirm the effect of DNase (1 mg/ml, deoxyribonuclease I, from bovine pancreas with a specific activity of 2200 Kunitz units/mg, SIGMA), we separated the same specimen taken from the area opaca of the fertilized chicken egg into a control specimen (no DNase) and a test specimen (with DNase). In this setup, we used the maximum working concentration of DNase, 1 mg/ml, to check whether the nucleus-like signals were really from assembled DNA molecules. After 2 h of incubation, we examined the changes in the nucleus-like structures in the control and the test specimens by using Hoechst 33258 staining.

3. Results

With the special setup used in our previous work (Lee et al., 2013b), we were able to cultivate and observe the changes in the yolk granules taken from fertilized chicken eggs. With preliminary data, we first made a working hypothesis as indicated in Fig. 1. As illustrated in Fig. 1, we conjectured that yolk granules release DNA materials as they have DNA fragments.

Fig. 2 shows fluorescence images of gradual changes, over a period of 23 h, in the DNA signals from yolk granules clusters (YGC) taken from the area opacas of fertilized eggs stained with acridine orange. Before incubation, all YGC had no DNA signals or slight DNA signals. Over the incubation time, the intensity of the DNA signal gradually increases with increasing cluster diameter. The first (A) image shows that no DNA signals emerged, after which the signal was gradually intensified at 1, 2 and 23 h of incubation. The colors of the yolk granules also changed from red fluorescence to green. Another representative change was that in two YGCs initially with no DNA signals, DNA signals gradually emerged and became stronger over the incubation time.

In order to specifically trace the changes in the DNA signals, we applied Hoechst 33258 as shown in Fig. 3. The pattern of the changes in the DNA signals for the specimens stained with Hoechst.
33258 was the same as that for the specimens stained with acridine orange in Fig. 4. In the first image (A) of YGCs before incubation, only one slight DNA signal is observed. After a 1-h incubation, another slightly emitted DNA signal emerges; however, other hard-to-see DNA signals are thought to be present. After additional 1-h incubation, almost all observed signals show distinctive structures with bright DNA signals.

Fig. 4 shows partial phase contrast and fluorescence images of the gradual changes, over a 23-h incubation, in the DNA signals of YGCs stained with acridine orange. The partial phase contrast images of the YGCs suggest that liquid-like material (LLM) emerged around the YGCs. Noticeably, the LLM changed color over the incubation time from transparent to yellowish; new LLM also appeared. The LLM under partial phase contrast microscopy correspond to the LLM under greenish fluorescence microscopy. Fluorescence microscopic data demonstrated the existence of gradually intensified DNA signals: (1) Change from no DNA signals to strongly assembled DNA signals via weak DNA signals from yolk granules. (2) From the LLM outside the yolk granules, some weak DNA green signals emerged and were gradually intensified during an 1-h incubation.

Finally after 14 h of incubation, the DNA signals were much stronger in certain structures.

Fig. 5 shows partial phase contrast and fluorescence images of the gradual changes, over a 4-h incubation, in the DNA signals from YGCs stained with Hoechst 33258. The phase contrast images of the YGCs (A) suggest the presence of a LLM around the YGCs. Fluorescence images demonstrate that the DNA signals gradually intensify over a 4-h incubation.

In order to confirm that nucleus-like structures are real DNA molecules, we examined the changes in the nucleus-like structures after application of DNAse to the same specimen as a control. As shown in Fig. 6, we took phase contrast and fluorescence images of the nucleus-like structures around the yolk granules clusters (YGC) stained with Hoechst 33258 after a 2-h incubation. The phase contrast images of the control specimen, A and B, and of the test specimen, C (DNAse treated), shared the same characteristics. Fluorescence images, however, were decisively different in that the control specimen, A and B, showed distinctive DNA signals whereas the DNAse-treated specimen, C, showed no fluorescence from the YGCs. In order to confirm the effect of DNAse, we observed all

Fig. 2. Fluorescence images of the gradual changes, over 23 h, in the DNA signals from YGCs taken from the area opaca of fertilized eggs stained with acridine orange. The first image (A) shows YGCs before incubation in which there are no DNA signals (two dotted circles and three arrows) and slight DNA signals (small greenish spots). The two dotted circles show that the DNA signal intensities gradually increase as the YGCs increase in diameter. The three arrows indicate DNA signals as follows: from no DNA signals (A) to gradually intensified DNA signals at 1.2 and 23 h of incubation. The colors of the yolk granules also changed from red fluorescence to green. It is noticeable that some YGCs had no DNA signals (e.g., two dotted circles); however, over incubation time, DNA signals emerged. All scale bars are 35 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 3. Fluorescence images of the gradual changes, over 6 h, in the DNA signals from YGCs taken from area opaca of fertilized eggs stained with Hoechst 33258. The first image (A) shows YGCs before incubation in which there is only one DNA signal (dotted circle). After 1 h of incubation (B), slightly emitted DNA signals (yellow arrows) and hardly seen DNA signals (white arrows) exist. Additional 1-h incubation produced stronger DNA signals from weak DNA signals (yellow arrows in B) and from very weak DNA signals (white arrows in B). After 6 h of incubation, all DNA signals show distinctive structures, except one (dotted arrow) which seemed not to be formed. All scale bars are 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
Fig. 4. Partial phase contrast (upper panel) and fluorescence (lower panel) images of the gradual changes, over 23 h, in the DNA signals from YGCs taken from area opacas of fertilized eggs stained with acridine orange. Phase contrast images of the YGCs suggest the presence of liquid-like materials (LLMs) indicated by dotted lines around the YGCs. Noticeably, the color of the LLM changes over the incubation time from transparent to yellowish; a new LLM also appeared, as indicated by the green arrow in (B). These LLMs under partial phase contrast microscopy correspond to the greenish fluorescence indicated by the green arrow in the lower panel. The lower panel shows gradually intensified DNA signals: (1) The three thin arrows indicate a progression from no DNA signals (A) to strongly assembled DNA signals (C) via weak DNA signals from yolk granules. (2) In the LLM outside the yolk granules, as indicated by the dotted circle, a weak DNA green signal, which gradually intensified (B) over a 1-h incubation, exists. Finally, after 14 h of incubation, DNA signals are stronger and have a condensed structure, as indicated by the dotted circle in (C). All scale bars are 25 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 5. Partial phase contrast (upper panel) and fluorescence (lower panel) images of the gradual changes, over 4 h, in the DNA signals from YGCs taken from area opacas of fertilized eggs stained with Hoechst 33258. The phase contrast images of the YGCs (A) suggest the existence of LLMs, as indicated by the dotted lines around the YGCs. The fluorescence images in the lower and the upper panels show gradual intensification of the DNA signals over a 4-h incubation. All scale bars are 7 μm.
specimens on the slide which showed no DNA fluorescence in which we demonstrate C of Fig. 6 as representative example.

4. Discussion

Taking all results, we suggest that yolk granules clusters may have a pivotal role in making new nuclei during the very early stages of egg fertilization via spontaneous self-assembly of DNA molecules. Biologically these yolk granules clusters have been named “periblasts” whose functional points related with nuclei still remain unsolved (Yamamoto, 1982), therefore, we in this report considered them as cluster form of yolk granules in the fertilized chicken eggs.

Through a series of experiments, as shown in Table 1, we reach the tentative conclusion that an alternative pathway to making new cells independently from cell mitosis may exist: (1) The yolk granules release liquid-like material (LLM) in which spontaneous self-assembly of DNA into nucleus-like structures (NLSs) occurs. (2) As the DNA molecules from yolk granules assemble, the color of the yolk granules changes from red fluorescence to green which share similar implication with previous work on yolk granules (Fausto et al., 2001). (3) By using DNase treatment, we confirmed that nucleus-like structures are really assembled DNA molecules.

Is our finding that DNA fragments spontaneously assemble into nuclei in the very early stage of egg fertilization absolutely novel? Before our finding, the first pioneer to recognize the importance of yolk granules was Olga Lepeshinskaya (Lepeshinskaya, 1954). She insisted that yolk granules were living substances that made new cells, as we conjectured, but she thought that the yolk granules themselves became the new cells. Contrary to that, we contemplate that yolk granules with genomic DNA fragments function as sources for assembling DNA fragments to form nuclei. Also, she did not notice that the yolk granules released LLM-containing DNA material whereas we found the emergence of LLM under partial phase contrast microscopy. We also observed newly formed DNA signals from inside the LLM. At this point, one may wonder if DNA molecules can be released from other types of cells beside yolk granules as we have insisted interestingly life scientists have noticed the release of DNA molecules from lymphocytes and cancer cells, even from bacteria (Chen et al., 2005; Skvortsova et al., 2006; Pisetsky, 2012), but the functions of the released DNA molecules, named extracellular DNA (eDNA), remain a mystery. Thus, we suggest that the LLM released from yolk granules and the eDNA released from cells may have the same biological functions.

On the other hand, a paper reported that DNA sequences of genomic DNA in yolk granules were heterogeneity (Lijun et al., 1998). Thus, theoretically different yolk granules could release

<table>
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<th>Number</th>
<th>Incubation time (h)</th>
<th>Observation time (h)</th>
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different DNA fragments which might be assembled to form NLs containing all the DNA information needed for the nucleus in a cell. However, we did not confirm that the NLs play the role of a real nucleus or cell. Interestingly, Bong Han Kim, the pioneer who revealed the anatomical reality of acupuncture meridians, also insisted that the first event in making new cells was to form NLs, from which spontaneously cytoplasm was formed. On the other hand, one hot issue in modern science, synthetic biology, depends on DNA assembly by various artificial approaches (Ke et al., 2012; Sudhof, 1995); ironically, several pioneers have already predicted that spontaneous assembly of DNA fragments occurs in living creatures (Bechamp, 1912; Davies, 1991; Wilehlm, 1979; Kim, 1965b).

At this point, one may be curious whether the spontaneous self-assembly of DNA can only occur in very early stage of egg fertilization? Our previous works suggest that DNA fragments can grow into bigger ones in a concentric pattern (Lee et al., 2012c). Very recently, we showed evidence that DNA fragments in microvesicles combine spontaneously into specific structures which share the same morphology with normal cells (Lee et al., 2013b). In a study on that, we noticed that the microvesicles containing DNA signals could be taken from various tissues from different species (Lee et al., 2013b). Based on our previous works and those of other pioneers, we believe that spontaneous self-assembly of DNA fragments can occur in all kinds of species. To understand this novel phenomenon, we suggest that the building of a new genetic theory beyond the semi-conservative replication as Watson and Crick suggested may be necessary (Watson and Crick, 1953).

For more important works for clinical application with this novel phenomenon, we would like once again to introduce then insights of three pioneers, Antoine Béchamp in the 1880s, Olga Lepeshinskaya in the 1950s and Bong Han Kim in the 1960s: Antoine Béchamp was the first pioneer to suggest that tiny particles named microzyma could assemble to give rise to new cells (Bechamp, 1912; Lepeshinskaya, 1954; Kim, 1965b). Olga Lepeshinskaya insisted that these tiny particles could assemble into new cells (Lepeshinskaya, 1954), and Bong Han Kim suggested a novel system corresponding to the acupuncture meridian system through which DNA fragments named Sanals flowed (Kim, 1965b). Thus, we suggest that our findings of evidence for the spontaneous self-assembly of DNA may usher in a universal life paradigm with a novel genetic principle in the form of the primo vascular system, a putative acupuncture meridian system: Antoine Béchamp meets Bong Han Kim via Olga Lepeshinskaya.

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References