A Timetable of the Early Development Stage of Silkies Embryo

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ABSTRACT : The early embryos are obtained in different time after the former egg had been laid, and the aim of the present study was to observe the development law of chicken early embryo.The embryo development has been divided into the two periods according to morphology of blastodisc. Cleavage period, from 5.5 h (0 h uterine age) to 15.5 h (10-10.5 h uterine age) after the former egg had laid, formation blastodisc of 6-7 layers cell. Later blastocyst period, from 17.5 h (12-12.5 h uterine age) to area pellucida formation after the former egg had been laid. The first division took place at 5 h (0 h uterine age), morular at 11.5 h (6-6.5 h uterine age), and blastocyst at 15.5 h (10-10.5 h uterine age) after the former egg had been laid. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 6 : 800-805)***

Key Words : Silkies, Early Embryo, Development, Morular, Blastocyst

INTRODUCTION

From cleavage stage to primitive streak determinate stage of chicken embryo. only Eyal-Giladi & Kochavs had been studied. provided us primary learning about the process of chicken early embryonic development in uterine tube. After that. most of researchers devoted to study the development of embryo from the blastula stage and older, fewer focused on the blastula stage onward. Up to now, there were no unified morphologic criteria and integrate systematic study on developmental morphology and histology of chicken early embryo (from the first cleavage to primitive streak) in many published papers. However. with the rapid development of chicken biotechnology, it appears more and more important to further comprehend on systematic development of chicken early embryo with different time before the egg laid (in oviduct). Accumulated information also suggested that chicken embryo is a kind of ideal model to analysis formation and process of avian gastrulation. Therefore. the aim of the present study is to deal with morphological transformation of chicken spermovium or embryo according to a temporal development order, demonstrate the dynamic of cell group and its distinct development affairs. provide powerful theoretical and practical evidence on developmental biology and genetic operation of chicken embryo.

MATERIALS AND METHODS

Experimental chicken

Subject of 60-70 hens in laying peak about 28 weeks of age were randomly selected from the Silkies population conserved in the Institute of Poultry Science of Jiangsu Province and fed on the same diet and circumstance. The nutrient composition of diet: Metabolizable energy (MJ/kg). 11.5; Crude protein. 15.0%; Ca. 3.0%; Available P (AP). 0.45%; Methionine. 0.37%; Methionine+cystine. 0.6%; Lysine, 0.9%.

Experimental methods

Hens were kept in individual cages and artificial in seminated. Prior to a planned extraction of egg. the hens were observed and the extract time of laying was recorded for each hen. Started to collect the spermoviums or embryo from oviduct or uterus at different time after laying the previous egg. i.e. 4. 5. 5.5. 6. 6.5. 7.5, 8.5. 9.5. 10.5. 11.5. 12.5. 13.5, 15.5. 17.5, 19.5. 21.5. 22.5 and 23.5 h. or slaughter the hens. and taken 3 spermoviums or embryos each time.

The egg was open into a culture dish. the embryo was prefixed in Rossman's fluid for 10 min. the germ was then dissected out. then put embryo into warm (40°C). 0.75% physiological saline to cleaned from adherent yolk. freed of the vitelline membrent. postfixed in Rossiman's for 20 min. After the fixation embryos were dehydrated in a graded series 70%, 80%, 90%. 100% of alcohol resp, transparency in n-butanol overnight. Mounting with a few drops neutral balsam were added to prevent the thinned-out germs from curling, observed the morphological variety of embryo cleavage. recorded the number of cleavage spheres and cleavage times. photographed through microscope as promptly as possible.

RESULTS AND ANALYSIS

In this experiment 60-70 eggs was collected which included all developmental stage. The stage was defined according to distinct morphological events and time of

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embryo development in oviduct and uterus. The time of embryo development was calculated as the interval between the laying of the former egg and the extraction of the studied egg. The uterine age was calculated as the interval between the laying of the former egg and the extraction of the studied egg minus 5-5.5 h. Two periods were defined and described: the first is cleavage period, the second is later blastocyst period. Roman letters have been used in order to distinguish them from Hamburger-Hamilton.

Cleavage Period

This period the cytoplasmic mass of the spermoviums or embryo is cleaving very fast. the time span between stage 0 and Ⅵ being about 15.5 h.

Stage 0 (4 h after the former egg had been laid) : 4 h after the former egg had been laid (0 h uterine age): The spermoviums at this stage had entered slender of oviduct, yolk was wrapped by a layer of thick egg white, and there wasn't any cleavage furrow on germinal disc.

Stage Ⅰ(5-6.5 h after the former egg had been laid) : i) 5 h after the former egg had been laid (0 h uterine age): At this stage the greater part of embryo entered slender of oviduct. A cleavage furrow appeared in the central or encentral region of germinal disc. For it's a kind of extreme yolk egg, division only limited in region where karyomere existed, the others of yolk hadn't any division. The cleavage furrow took on longitudinal fissure at this time, which through central part of germinal disc formed two partially separated cleavage spheres.

ii) 5.5 h after the former egg had been laid (0-0.5 h uterine age): At this stage, a greater half of embryo stay in slender of oviduct, the other parts still in uterus. It was surrounded by a layer of flaccid shell membrane, the albumen inside membrane got very viscous. The whole germinal disc being a large cell, the diameter was about 1.350 μm. In central region of the disc the second division took place, its cleavage furrows vertical by the first cleavage furrows just as cross. The whole germinal disc divided into four incomplete cleavage spheres.

iii) 6 h after the former egg had been laid (0.5-1 h uterine age): The greater part of embryo had entered uterus. The germinal disc had been cleavaged three times, eight cells could be seen. Cells lay in the same plane, only to see cleavage furrows, but between cells are not complete independence. Therefore, cleavage spheres were all opened, the original morpha of cells didn't form yet. And the cytoplasm at this stage contains large vacuoles.

iv) 6.5 h after the former egg had been laid (1-1.5 h uterine age): The embryo at this stage entered uterus completely, the shell didn't form yet. The diameter of whole germinal disc was about 1.800 μm, in its central region, four times of divisions could be seen and 13±3 cleavage spheres formed. The cell was rather irregular, its diameter

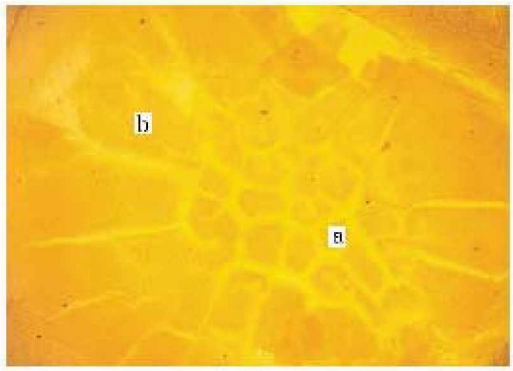

Figure 1. The irregular blastomeres. (×40)
a. The closed blastomeres in central.
b. The open blastomeres in perpheral. (7.5 h)

was about 16-20 μm, still lay in the same plane. At this stage, the cell group in central region was enclosed by vertical cleavage furrows, and began to separate from the yolk with elongated furrows. The continual mitosis formed many irregular cells. The cleavage furrows extended from central part to peripheral region.

Stage Ⅱ(7.5h after the former egg had been laid. 2-2.5h uterine age) : The whole germinal disc had about 40 blastomeres on the upper surface, indicating cells had had fifth or sixth mitotic divisions. In central region about 20 blastomeres are small, the diameter about 15-20μm. Cleavage furrows were distinct and extended to everywhere. furrows between the peripheral blastomeres were indistinct. All the blastomeres distributed a cell layer and rather irregular, some being quadrate, some being trapezoid, and others being triangle, stayed in the same plane. The cleavage furrows spread out to the edge of the germinal disc. (Fig.1)

At stage Ⅰ-Ⅱ, we do not refer to each division as a separate stage, as the rate of divisions was very high, five to six divisions during the first 7.5 h (from the first cleavage to stage Ⅱ).Therefore, we included in stage Ⅰ (5-6.5 h after the former egg had been laid), the whole range of cleaving patterns in which all cells were still open peripherally, or even those with one to two centrally located and laterally closed cells. Additional Stage Ⅰ germinal discs are shown to demonstrate the variability of cleavage patterns from a very orderly one to another with a single circumscribed cell, indicating asynchrony of the mitotic divisions.

Stage Ⅲ(8.5-9.5 h after the former egg had been laid) : i) 8.5 h after the former egg had been laid (3-3.5 h uterine age): On the upper surface a central group of 64-80 laterally closed blastomeres were seen, and 6-8 divisions during this stage. The central group blastomeres being minor cell, the

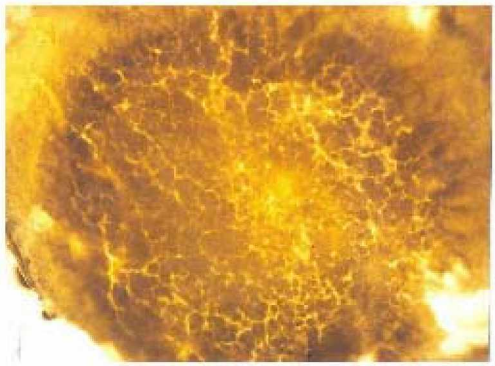

Figure 2. All blastomeres were closed. (×40)
Similar to morula.11.5

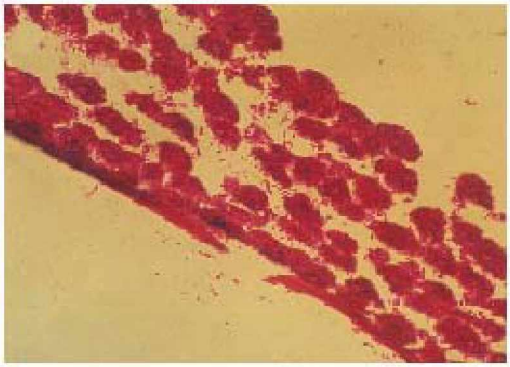

Figure 3. 6-7 lays of cells formed in central region. (×100)
15.5 h.

diameter about 9-15 μm, and the others were larger, and its shape were irregular. The whole germinal disc's diameter was about 3,300 μm. The central blastomeres separated from the lower surface yolk, formed a layer of integral cells, which were called central cells. From its margin cleavage furrows radiated to the edge of the germinal disc obviously. The margin blastomeres of germinal disc were still linked out with yolk, which were called peripheral cells.

ii) 9.5 h after the former egg had been laid (4-4.5 h uterine age): There were about 160±40 blastomeres in germinal disc at this stage. The diameter of cleavage region was 2,600 μm. The blastomere being regular. The central groups were smaller, about 10-12 μm; the margin groups were larger, about 16-20 μm. And various forms were shown. The divisions were not only horizontal but also in vertical plenum. During cleavage the cytoplasmic disc shrinks horizontally and thickens vertically.

Stage Ⅳ (10.5-12.5 h after the former egg had been laid) :

i) 10.5 h after the former egg had been laid (5-5.5 h uterine age): On the upper about 215±35 closed blastomeres existed at this stage. The size of cells was obviously different, divided into three zones. Cells in central part were smaller, about 6 μm, cells in secondary edge were larger and had irregular forms, about 11 μm; cells in extreme edge region were the largest. On non-stained condition, of 750 magnified, germinal disc had full of yolk granule, and cytoplasm in whole germinal discs separated completely. From this stage, larger single cleavage sphere could be obtained for embryo operations.

ii) 11.5 h after the former egg had been laid (6-6.5 h uterine age): Generally, the germ at this stage was similar to that at 10.5 h. In the central part of germinal disc had division regularly, the diameter of cells was about 4-7 μm. The margin cells were larger, there were about two layers in central cells, compact, well-distributed, just likely to morula stage of mammalian embryo. (Figure.2) It is suggested that some phenomena of chicken embryo during development process were similar to development of mammalian embryo.

iii) 12.5 h after the former egg had been laid (7-7.5 h uterine age): About 350±50 cells at this stage. The whole germinal disc's diameter was about 3,000 μm. The germinal disc has a shrinking than stage Ⅱ .The development of early embryo was likely to the early-blastula of mammalian embryo. The cells in central region were smaller, the diameter about 5-6 μm, while the diameter of secondary edge cell about 8-10 μm, and cells in extreme margin region were the largest. The blastomere could be seen clearly.

Stage Ⅴ (13.5 h after the former egg had been laid, 8-8.5 h uterine age) : A cleavage is much more advanced, about 900±100 cleavage spheres at this stage, closed blastomere occupy equally large areas both on the upper and lower surface .The central region got thicker, and the diameter of cells was about 3-5 μm and rather regular; the diameter of cells in secondary edge was about 6 μm. After division, cell in extreme region was about 12 μ m irregular. At this stage, more single-cellular blastomeres could be obtained for embryonic operation. It showed that, development of chicken embryo was different from mammalian, and had its own distinct trait.

Stage Ⅵ (15.5 h after the former egg had been laid, 10-10.5 h uterine age) : About 2000 blastomeres, the peripheral region of germinal disc was thinner, while its central region got thicker obviously, the entire cytoplasmic mass of the germinal disc is cleaved, both on the upper and lower surface. The cells are small and form an epithelial sheet of uniform thickness, about 6-7 layers of cells formed. (Figure.3) The diameter of cells central region was about 3-5 μm , cells in margin region was about 10 μ m. At this time, there weren't any obvious distinguish between area pellucida and area opaca (Figure.4). At this stage, the

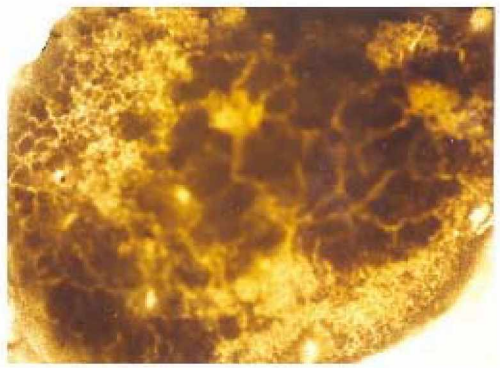

Figure 4. The small closed blastomeres. (×40)
Similar to blastocoed. 15.5 h.

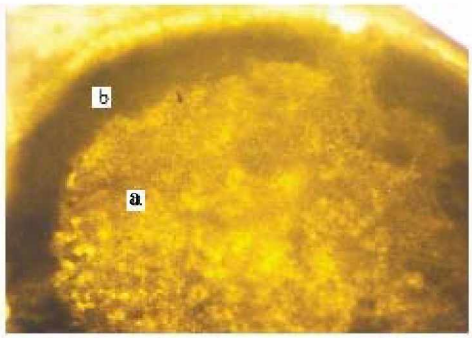

Figure 5. Formation of area pellucida and area opaca. (×40)
a. Area pellucida, b. The area opaca. 21.5h

embryos by cross slicing. seven layers of cells in cytoplasm and epiblast which was made up of 3 layers could be seen. Cell layers began to separated from lower yolk, and blastocoele formed between them. The process of embryos morphogenesis is similar to early blastula of mammalian.

Later blastocyst period

Stage Ⅶ (17.5 h after the former egg had been laid, 12-12.5 h uterine age) : At this stage, the germinal disc began to form area pellucida and area opaca. The cell of the upper surface became much smaller as a result of intensive dividing, it is difficult to distinguish the individual cells. The area pellucida already extended to both sides of bottom, formed a sicklelike region, the diameter of that region was about 2,450 μm. The whole marginal region of germinal disc began change into a width of 400 μm area opaca. But the border between the area opaca and the area pellucida is not yet a sharp one, especially at the anterior end. In this stage, observed the embryo by cross slicing, three layers of cells linked closely in epiblast and there were 8 layers of cells in whole embryo. From this stage onward the diameter of the germ increases with progressive development.

Stage Ⅷ (19.5 h after the former egg had been laid, 14-14.5 h uterine age) : The whole germinal disc divided into area pellucida and area opaca could be seen clearly, although the process is not yet completed , a width of 400 μm area opaca, and a width of 3,900 μm area pellucida. There were two layers of cells in area pellucida, and the size of cells at this stage seemed more uniformity than that of 17.5 h, its diameter was about 2 μm, and its transparent degree enhanced obviously. However, there was still larger cells distributed unequally to whole germinal disc where closely compact to yolk layer.

Stage Ⅸ (21.5-22.5 h after the former egg had been laid) :
i) 21.5 h after the former egg had been laid (16-16.5 h uterine age): The area pellucida and area opaca were distinguished from each other clearly, there was only a thinner layer of cells between them. The width of area opaca was 600-700 μm. Cells appeared equally in size by division, the diameter was about 1.5-2 μm, all the cells took on sphere (Fig. 5).
ii) 22.5 h after the former egg had been laid (17-17.5 h uterine age): The germinal disc at in this stage is similar to that of at 21.5 h. The formation of the area pellucida has been completed .The cells of central region seemed uniformity, and didn't differentiation yet. At this term, the embryos are suited to obtain smaller blastomere to make chimera.

Stage Ⅹ (24-26 h after the former egg had been laid, a freshly laid egg about 19-20.5 h uterine age) : The diameter of germinal disc was about 3,950-4,000 μm, a width of area opaca was 600-650 μm, there was a clearly width of 350 μm single cells demarcated border between area opaca and area pellucida. At this stage, the cells in central part of area pellucida had increased 2-3 layers. The cells became smaller and uniformity, the diameter about 1-1.5 μm. And there was abundant lipid droplet in cells. However, in this stage, not only end the formation period of the area pellucida in vivo, but is also the beginning of formation period of epiblast and hypoblast.

A summary of the timetable of embryo development stage is presented in Table 1.

DISCUSSION

Morpha record during the process of embryo development

Much work has been done on the development

Table 1. A timetable of embryo development stage

Stage	Development time	Uterine age	Distinct development affair
	4.0 h after former egg laid	0 h	The spermoviums entered slender of oviduct
	5.0 h after former egg laid	0 h	A cleavage furrow has appeared
	5.5 h after former egg laid	0-0.5 h	The second division took place
	6.0 h after former egg laid	0.5-1 h	The disc cleavaged 3 times, 8 cells could be seen
	6.5 h after former egg laid	1-1.5 h	The spermoviums entered uterus
	7.5 h after former egg laid	2-2.5 h	Cells had had the fifth or sixth division
I	8.5 h after former egg laid	3-3.5 h	The spermoviums took place 6-8 times of divisions
(Cleavage period)	9.5 h after former egg laid	4-4.5 h	The blastomeres amount to 120-200
	10.5 h after former egg laid	5-5.5 h	Size of cells changed obviously, divided into three zones
	11.5 h after former egg laid	6-6.5 h	Similar to that at 10.5 h
	12.5 h after former egg laid	7-7.5 h	The germinal disc had a shrinking
	13.5 h after former egg laid	8-8.5 h	A cleavage is much more advanced
	15.5 h after former egg laid	10-10.5 h	Central region of disc got thicker
II	17.5 h after former egg laid	12-12.5 h	Begin to form area pellucida and area opaca
(Later blastocyst	19.5 h after former egg laid	14-14.5 h	The division of disc continued
period)	21.5 h after former egg laid	16-16.5 h	The area pellucida and area opaca distinguished clearly
	22.5 h after former egg laid	17-17.5 h	Similar to the time at 21.5 h
	24-26 h after former egg laid	19-20.5 h	The end of formation area pellucida and the beginning of formation epiblastand hypoblast

regulation of early embryos of mammalia. i.e. Cao Gui-Fang and other investigators (1998) not only studied development law of early embryo on goat and observation by electro-microscope. but also researched the variational regularity of bodily form after organ differentiation on goat. Distinct feature of the blastocyts were observed in the trophoblast. and discerned ultrastructural of bovine blastocyts developed (Ohboshi et al., 1995). Furthur investigation evaluated some factors in bovine embryonic development from one-cell to blastocyst (Ohboshi et al., 1996). It's revealed the development to morula and blastocyst of rat embryo was capable of utilizing exogenous fatty acid (Yahia et al., 1999). However, on avian, relative research by Blount (1990) were only seen about the development pictures of spermovium on pigeon. For the cleavage furrow of pigeon germinal disc was obvious, Blount fixed germinal disc and stained to show cleavage furrows and photographed it. At the present study, germinal disc was fixed by Rossman's after gaining from yolk and observed by microscope. We obtained series of microphotos on development of chicken early embryo in oviduct by micrography, recorded systematically process of morpha changes of chicken embryo development, provided useful references for further research on chicken embryo.

The time of cleavage

The germinal disc was a little transparent, white and round region before cleavage, its diameter was about 3 mm. The protoplasm of cytoplasm lower contacted with yolk closely, and wasn't any obvious border, the original cleavage only took place on central region of cytoplasm. Gradually, cleavage extended to margin regions. Each direction of cleavage was always vertical by macro-axis of mitotic spindle. the latter cleavage furrows were vertical by the former furrows (Cao. 1998).

Patterson and Olsen's researchs have showed: the quite accurate correlation between the time of elapsed from the laying of the former egg and the number of blastomeres of the next egg. The more time embryo stays in oviduct or uterus, the more the number of blastomere. For cleavage is continuous while embryos pass oviduct and uterus, the time of embryos stay in vivo is different. result in the development degree of chicken embryonic is various, i.e. freshly laid eggs, some remain blastula stage, some in the stage of epiblast and hypoblast formation. Therefore, if only taking fresh egg for experiment material will effect on the result of test. This result suggested that record the temporal regularity of hen's laying is necessary while embryo engineering can be conduct. We also demonstrate that from 17.5 h after the former egg had been laid to the freshly egg laid is the formation period of area pellucida. before the 17.5 h is cleavage preiod, which same as the results of Eyal-Giladi (1976). When chicken spermovium pass oviduct, two important morpha changes happened, one is cleavage, the other is formation area pellucida. In present results, it's found that chicken germinal disc has took place two divisions during 5.5 h after the former egg had been laid. at that time, a greater half of embryo stayed in slender of oviduct, the other parts still in uterus. It's different from other researcher's results. Patterson held that the time of first cleavage was about 6h after the former egg had been laid. Olsen believed that it should at 5.5 h.Besides, the time of the first cleavage took place when egg full into conjunction part of oviduct and uterine (i.e. glandula-shell part) during 5.5-6.5 h after the former egg had been laid.(Eyal-Giladi et al., 1976, Bellairs et al., 1978, Eyal-

Gilad et al., 1976. Fabian B. et al., 1981, Kochav S. et al., 1980. Eyal-Giladi and Kochav et al., 1971) We think that difference is attribute to take different breeds of stocks. Although the time and position of the first cleavage are all different, the end time of cleavage, about 15.5-17.5 h after the former egg had been laid, is synchronization. It also suggested that, the cleavage stage of European chicken germinal disc is shorter than that of Chinese native chicken. We deduced it may be related to speed of chicken growth and development.

The speed of cleavage

In the present study, it's found that, cleavage ratio of chicken spermovium is regular from cleavage beginning to 6.5 h, interval between the two division is about 0.5 h. 16 blastomeres formed during the first four divisions. The fifth cleavage tend to irregular, this result accord with that of Emanuelsson, he claimed that the first five cleavages are regular. However, the results were different from Patterson and Blount, Eyal-Giladi and Kochav (1976). In most cases, they regarded as the third cleavage, and sometimes the second cleavage, is already irregular. Olsen's reports showed that, the time longer of continuous cleavage of chicken breed, the first five cleavages tended to regular. The cleavages of Silkes agree with the finding of Olsen. While the time shorter of continuous cleavage of chicken breed, the cleavage ratios start to change at the third cleavage. We had observed, from the fourth cleavage start, cleavage region extended from central part to margin, and blastomeres are unequal in size. On 64 cell period, cells divided into upper and lower layers, and there's a crevice between cell layers and yolk. At follower stage, central cell layers increase continually, but cell layers near to margin region decrease and the cells contact to yolk. Jiang Xi-Dong (1983) reported that the size of whole germinal disc do not vary while the numbers of cell increased with cleavage continued. However, in this present study, results are different from Jiang Xi-Dong's. At cleavage period, the diameter of germinal disc increased with cell division while cells maintain undifferentiaton. In this period, the main active of cells is to division, so those cells have much ability of plasticity and differentiation potency. It's the best term to make transgenic chimeric chicken.

Formation area pellucida

The result of cleavage is to form thicker germinal disc which made up of 5-6 layer cells before formation area pellucida, this point accord with result of Eyal-giladi and Kochav (1976). Hereafter, the central part of germinal disc gets thinner gradually, margin region remains still. However, it's obvious that area pellucida and area opaca distinguished from each other before egg laid. Formation area pellucida maybe related to determine of embryonal axis which be effected by gravitation (Kochav, 1997). Formation area pellucida attributed to central region of germinal disc got thinner gradually (Eyal-Giladi et al., 1976). Fabian and Eyal-Giladi(1981) suggested that only because of cells of germinal disc in central region lose continually. Although we don't yet have sufficient data to discuss the reason for formation area pellucida, one conclusion could affirm that the central region of germinal disc get thinner continually lead to formation area pellucida directly. When cleavage till this period, there's crevice cavity between cells layer and yolk, that's the sub-germinal cavity, which mark the blastula stage of embryo development is begin, and this stage is also the best term to culture embryo stem cells. Because on general condition, freshly eggs always in different development stages, they are not the best materials for obtain embryo stem cells.

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