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M. Dvořák

The Differentiation of Rat Ova During Cleavage

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With 62 Figures

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1. Introduction and Notes on Materials and Methods

Milan Dvořák

The study of embryogenesis is increasingly concerned with the problems of the earliest stages of development. Mammalian ova, unlike those of lower vertebrates and invertebrates, have been largely neglected by researchers studying the problems of early embryogenesis, although they represent a unique object for investigation of the first differentiation processes. This is due to the fact that obtaining a great number of mammalian ova from the period of fertilization and, mainly, that of cleavage is connected with a number of technical difficulties which are further multiplied when the specimens are prepared for electron microscopy and ultracytochemistry. The possibilities for using segmenting mammalian ova in experimental studies are rather limited.

In our laboratory, we have been carrying out systematic research on the fine morphology of segmenting rat ova for years. We have, therefore, decided to present this comprehensive study of the ultrastructure and ultracytochemistry of segmenting rat ova based on our own results. The cleavage of the rat ovum takes place in a short period during the first 6 days of pregnancy and is accompanied by a number of morphologic, biochemical, and functional changes resulting in the formation of the blastocyst, i. e., an ovum capable of implantation. The study includes three main parts. The first, introductory part provides some information on the basic methods (details are given in the particular papers quoted). The second part contains detailed descriptive facts concerning the individual stages of rat ovum cleavage based on our results. Where necessary, this information is augmented by data from the literature which we had no opportunity of obtaining ourselves and which are important from the point of view of the problem studied. The descriptions are given in detail and separately for each stage of ovum development to enable the reader interested in only one stage to find all pertinent information in a concise form. The third part of the study includes discussions in which morphologic findings are compared with biochemical and functional ones. The references cover a broad scope of papers on the early embryogenesis of both mammals and other vertebrates and, in some cases, even some invertebrates.

Notes on Materials and Methods

The basis of this study are more than 2500 rat ova (Rattus norvegicus var. alba) obtained after fertilization and during cleavage at the one-, two-, four-, and eight-cell stages, the morula, and the early and late blastocysts. The ova were obtained by flushing the tuba uterina or the uterus and processed either for electron microscopy or ultracytochemistry.

For electron microscopic investigation, free ova were fixed in various ways, particularly by double fixation with 1.2% glutaraldehyde in cacodylate or phosphate buffer and with 1% OsO_4 in cacodylate or phosphate buffer. A simple fixation in 1% or 2% OsO_4 in phoshate buffer was used only exceptionally. The embedding medium was Durcupan ACM. Ultrathin sections were made by means of ultramicrotomes Tesla BS 490 or Ultrotome III LKB, stained with 1% aqueous uranyl acetate and lead citrate, and examined under the electron microscopes Tesla BS 500 or Tesla BS 613.

In preparing ova for electron microscopic investigation, part of the ova were studied ultracytochemically. In particular, the location of enzymes was studied: alkaline and acid phosphatases, nonspecific esterase, cholinesterase, succinate dehydrogenase, and endogenous peroxidase. Further more, the occurrence of polysaccharides, especially glycogen, and the glycocalyx were

studied ultracytochemically. The ingestion of exogenous proteins of the horseradish peroxidase and microperoxidase type was also investigated.

Alkaline phosphatase was demonstrated by the method based on the Gomori technique with the incubation medium according to Mayahara et al. (1967) and acid phosphatase with the incubation medium according to Ericsson and Trump (1964–1965). To study the location of nonspecific esterase, the modified method according to Hanker et al. (1972) was used and for the proof of cholinesterase, the method according to Karnovsky (1964). Succinate dehydrogenase was studied by the method based on the reduction of ferricyanide with the incubation medium according to Ogawa et al. (1968). Endogenous peroxidase was demonstrated by the method according to Graham and Karnovsky (1966). Polysaccharides (glycogen) were ultracytochemically demonstrated by the method according to Thiéry (1967) and the glycocalyx by staining with ruthenium red according to Luft's (1971) method. Details about the methods used in the case of horseradish peroxidase are given in the paper by Dvořák and Trávník (1975) and in the case of microperoxidase in Dvořák and Trávník (1976). In addition to qualitative data, the present study is based on and augmented by quantitative data on the occurrence of cytoplasmic structures of the segmenting ova which were obtained by the morphometric method according to Weibel (Dvořák et al., 1977).

2. Submicroscopic Structure and Ultracytochemistry of Segmenting Rat Ova

Milan Dvořák and Jitka Šťastná

This section surveys submicroscopic and ultracytochemical changes which the rat ovum undergoes during the process of cleavage and which, to a certain extent, are the expression of the first transformation of a mammalian embryo in the process of differentiation or – taking into account the marked developmental lability even at the end of the cleavage process – are, at least, not insignificant for future differentiation.

Data are given successively in the individual stages of ovum development; the one-cell fertilized ovum, the two-cell ovum, the four-cell ovum, the eight-cell ovum, the morula, and the early and late blastocysts, as they were obtained in our laboratory. The stage of the unfertilized ovum has consciously not been included in the set of developmental stages of the rat ovum. We assume that it belongs, by nature of its topic, to the problems concerning fertilization, which are not dealt with in this study. In this respect, we refer the reader to the recent paper by Gwatkin (1976). As far as references are quoted in this section, they solely concern observations performed on rat ova. They are given only when they appear to be of extraordinary importance or when they differ from our findings, but they are only discussed in exceptional cases.

2.1. One-Cell Ovum

One-cell fertilized ova were obtained by flushing the tuba uterina at 10:00 a.m. - 2:00 p.m. on the 1st day of pregnancy. The average number of flushed ova in the morning hours was lower than in the afternoon, because it is easier to flush ova that have advanced from the abdominal orifice of the tuba uterina toward the uterus. According to our experience, the fertilized one-cell ovum can be obtained at the earliest at 7:00 a.m. on the 1st day of pregnancy in the favorable case. The earlier the ovum is taken, the more often the cells of the corona radiata occur in its neighborhood. At the period

of sperm penetration, the ovum is in the metaphase of the meiotic division which, in the case of fertilization, is finished, and the separation of the second polar body into the perivitelline space takes place. The ovum then undergoes the so-called pronuclear phase (about 2 h after the penetration of the fertilizing sperm). At this stage, the ovum cell contains two pronuclei, a male one and a female one (Fig. 1). The male pronucleus has originated from the sperm proper and can already be identified in the light microscope, as the middle part of the sperm is usually found in its close vicinity. The description further refers to the fertilized one-cell ovum in the interphase at the stage of pronuclei, as no evidence has been obtained that a synkaryon is formed before the first segmenting division in rat ova. At this stage, the ovum is 47 - 50 μm, surrounded by the zona pellucida ($\sim$ 4.5 μm wide), and consists of the male and of the female pronuclei and cytoplasm (Fig. 1).

2.1.1. Nucleus

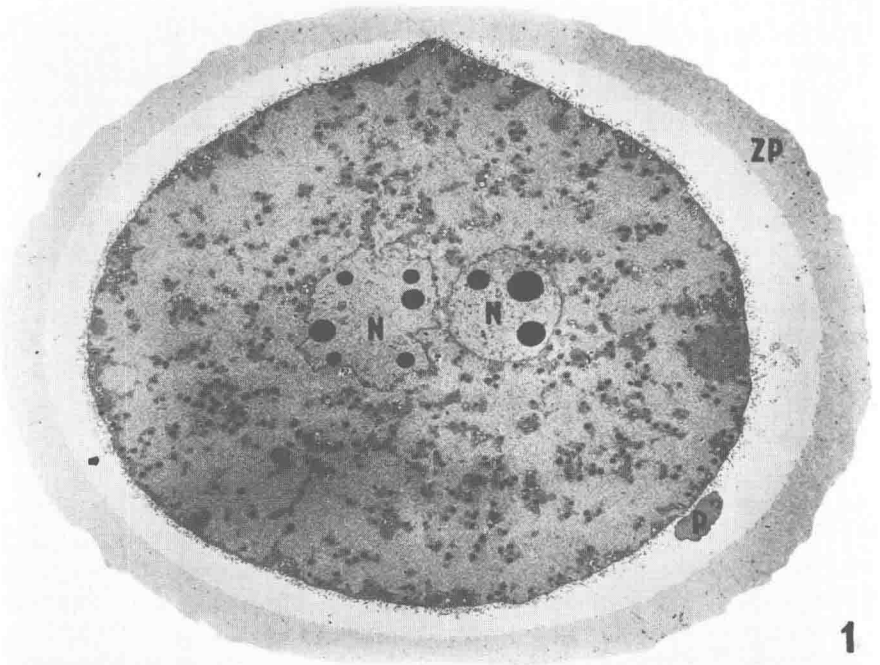
There are a male pronucleus and a female pronucleus in the one-cell ovum (Fig. 1). The volume of the two pronuclei increases, and the volumes of the pronuclei and the number of the nucleoli reach their maximum in about one-half at the pronuclear life span. The volume of the nucleoli increases more rapidly, so that the maximum is reached in about one-quarter of the pronuclear life span. The formation of the zygotic nucleus from the male and the female pronuclei is not known in mammals, perhaps only with the exception of monotremata (Austin, 1961).

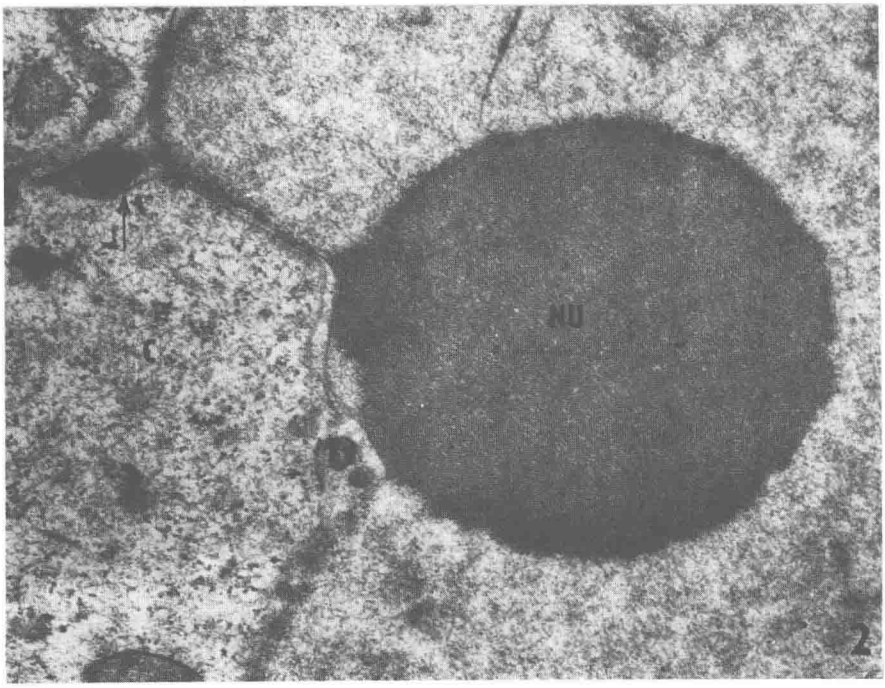
Pronuclei are mostly situated in the cytoplasm excentrically. They reach a size of ca. 15 μm and are characterized by a very similar submicroscopic structure. Their shape is very irregular, owing to the indentation of their surface with deep invaginations and evaginations. Pronuclei of the rat ovum were studied in detail by Austin (1961) under the light microscope. He states that the male pronucleus is about 2.5 times larger than the female one; similar relations are valid for the number and the overall volume of nucleoli.

2.1.1.1. Nuclear Envelope

Pronuclei are limited by two membranes which border the perinuclear space, mostly 15 - 20 nm wide. In some places the perinuclear space is enormously widened due to numerous evaginations of the outer nuclear membrane; these dilatations often exceed 0.5 μm. In the perinuclear space, amorphous fine to flake-like material of low electron density is present. Dense bodies of variable size were often in that space (Fig. 2). They were limited by a membrane which, in some cases, was continuous with an inner membrane of the nuclear envelope. The material of these bodies is morphologically quite identical with that of the nucleoli. The presence of these bodies in the perinuclear space is probably the expression of one of the forms of extrusion of nucleolar material into the cytoplasm which occurs on a large scale at the stage of pronuclei (Szollosi, 1965a; Schuchner, 1970). Sotelo and Porter (1959) state that it is common for the nucleoli of female pronuclei to protrude beyond the surface of the nucleus.

Nuclear pores are irregularly distributed over the surface of the nucleus. Szollosi (1965a) states that the frequency of nuclear pores is very small. In places of their occurrence, the width of the perinuclear space is the same as is usually found in most





Figs. 1 and 2

Fig. 1. One-cell rat ovum. Two pronuclei (*N*) with distinct nucleoli; zona pellucida (*ZP*); polar body (*P*). Fixation glutaraldehyde and OsO_4; embedding medium Durcupan ACM; magnification X 1500

somatic cells (ca. 20 nm). Nuclear pores are, as a rule, filled with filamentous material with the same electron density as the nucleoli of the pronuclei.

Where there are dilatations of the perinuclear space, aggregations of vesicles of smooth endoplasmic reticulum are apparent in the adjacent ground cytoplasm. Similar vesicles in different stages of constriction have been observed in connection with the outer membrane of the nuclear envelope. On the nuclear envelope of the one-cell stage, some enzymes have been proved: acid phosphatase (Šťastná, 1977a), alkaline phosphatase (Šťastná, 1979), and organophosphate-sensitive nonspecific esterase (Trávník, 1977b, 1978).

2.1.1.2. Chromatin

Chromatin is distributed evenly in the nucleus (Figs. 1 and 3). Chromatin only forms minute condensations locally which, however, do not have the character of karyosomes. Mitosis with apparent chromosomes (Fig. 4) will not be discussed in this study.

2.1.1.3. Nucleolus

Nucleoli are prominent nuclear structures at the pronuclear stage (Fig. 3). Their volume is about 10% of the total nucleus volume in the rat at this stage (Austin, 1952). They are quite numerous; in our material, as many as ten nucleoli were found in one section of one pronucleus. Sotelo and Porter (1959) observed 12 - 17 and even more nucleoli in rat pronuclei. Austin (1961) states that the highest number of nucleoli found in the female pronucleus is 17 - 36. Frequently one large nucleolus of 6 μm diameter was observed in the pronucleus, and a great number of nucleoli of about 2 μm diameter and nucleoli or their fragments of considerably smaller size were observed. All nucleoli are compact and formed by the fibrilar component; occasionally nucleoli were also observed which seemed to start to form the granular component (Dvořák, 1974a).

The nucleoli are randomly distributed in the karyoplasm; part of them (nucleoli up to 2 μm diameter) are often located close to the inner membrane of the nuclear envelope (Fig. 2). According to the size and location, Szollosi (1965a) distinguishes three types of nucleoli at this stage; primary, secondary – already described by Austin (1952) – and tertiary nucleoli. According to Szollosi (1965a), tertiary nucleoli are small nucleoli or fragments of nucleolar material attached to the nuclear envelope. The nucleoli are primarily composed of protein material (Austin, 1952); in all three types, RNA was also proved (Szollosi, 1965a). In agreement with Szollosi (1965a) and Schuchner (1970), we found no signs of the presence of perinucleolar chromatin.

2.1.2. Cytoplasm

Cytoplasm is voluminous in the one-cell stage (Fig. 1). The cell organelles are distributed unevenly in the ground cytoplasm. They are principally concentrated in the

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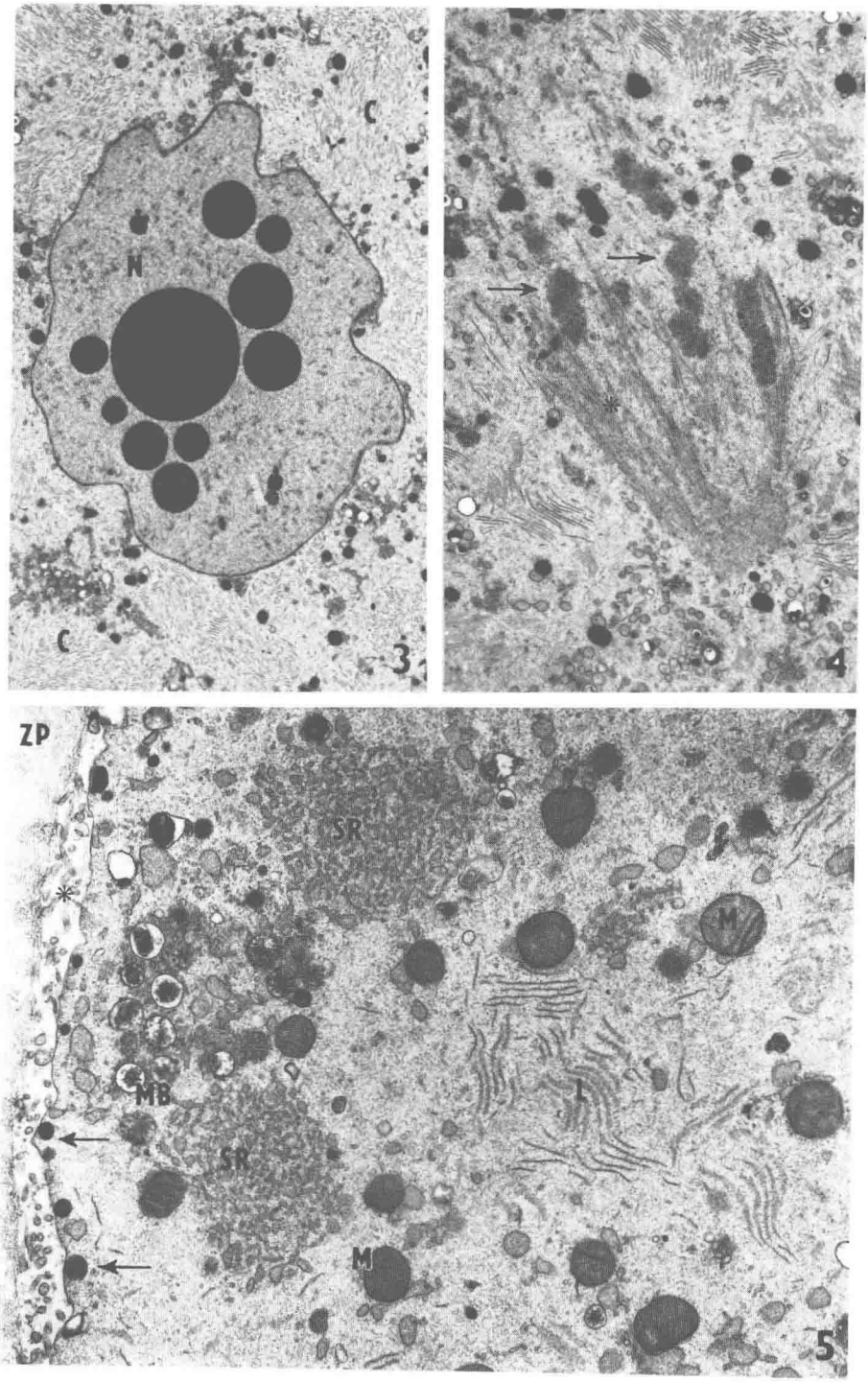
Fig. 2. One-cell rat ovum. Compact nucleolus (*NU*) attached to the nuclear envelope; evagination of the perinuclear space (→) with a dark body; cytoplasm (*C*). Fixation glutaraldehyde and OsO_4; embedding medium Durcupan ACM; magnification X 32,000



Figs. 3–5