Japan Society of Histochemistry and Cytochemistry Established 1959

Acta Histochem. Cytochem. 41 (4): 65–82, 2008
doi:10.1267/ahc.08010

Review

Historical Survey on Chromatoid Body Research

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Received March 27, 2008; accepted May 14, 2008; published online July 15, 2008

The chromatoid body (CB) is a male reproductive cell-specific organelle that appears in spermatocytes and spermatids. The cytoplasmic granule corresponding to the CB was first discovered some 130 years ago by von Brunn in 1876. Thirty years later the German term "chromatoide Körper" (chromatoid body) was introduced to describe this granule and is still used today. In this review, first, the results obtained by light microscopic studies on the CB for the first 60 years are examined. Next, many findings revealed by electron microscopic studies are reviewed. Finally, recent molecular cell biological studies concerning the CB are discussed. The conclusion obtained by exploring the papers on CB published during the past 130 years is that many of the modern molecular cell biological studies are undoubtedly based on information accumulated by vast amounts of early studies.

Key words: chromatoid body, research history, P-body function, lysosomal function

I. Introduction

The term "chromatoid body" (CB) is derived from fact that this cytoplasmic granule is strongly stained by basic dyes similar to chromosomes and nucleoli [19, 83, 116]. In mammals, the CB is observed in the cytoplasm from early spermatocytes to late spermatids and its shape is usually spherical. As spermatids develop, the CB assumes various shapes and its size gradually diminishes. It appears first in the juxtanuclear cytoplasm and moves caudally to the cytoplasm at the base of the flagellum [144]. Finally, it is dispersed into a residual body where it is presumably degraded [158, 199]. In the 1960s electron microscopic techniques were introduced to investigate spermatogenesis and the ultrastructure of the CB was clarified as a result. The CB consists of fine granular or fibrous substances with high electron density, has no limiting membrane, and is frequently surrounded by many small vesicles [26, 48, 59, 171, 201, 208]. Histochemical analyses showed that the CB contained RNA, polysaccharides and basic proteins [11, 20, 76, 84, 198, 199, 219, 226]. The CB has never been biochemically isolated with high purity thus far, hence no biochemical approach is complete. A preliminary experiment for the isolation of the CB has been attempted by Figueroa and Burzio [60]. Recent studies using molecular biological and genetic techniques have elucidated that the CBs are a site for storage and processing of reproductive cell-specific RNA [34, 35, 107, 108, 110, 150, 209, 211, 213]. On the other hand, it has been suggested from its aggresome-like features that the CBs are a site for degradation [76]. In this review, studies on the CB from its initial discovery in the 1880s to the latter half of 1950s, as well as subsequent electron microscopic and histochemical studies and more recent molecular cell biological studies are reviewed and discussed. A review concerning early studies was written by Sud [200] in 1961 and several reviews on current aspects of the CB have also been published [109, 146, 152].

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This paper was presented as Takamatsu Prize Lecture in 48th Annual Meeting of Japan Society of Histochemistry and Cytochemistry (Yamanashi).

II. Early Studies (1876–1955)

In 1876 von Brunn [214] first described two CB-like structures as "Protoplasmaanhäufungen" (protoplasmic depositions) in the early spermatid forming acrosome cap

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(Fig. 1a). He concluded that one of them was involved in the formation of the acrosome cap and the other with the creation of flagella. Hermann [83] found in spermatogenic cells of salamander "Nebenkörper" that was sharply stained red by safranine-gentiana and consisted of rings and a core. He also found a similarly stained structure in mouse testis. In 1891 Benda [19] observed cytoplasmic granules that were different from the nucleolus and colored red by safranin staining in spermatocytes of guinea pig. He first used the term "chromatoide Nebenkörper" for these granules. This terminology means that "Nebenkörper" shows a staining pattern similar to chromosomes and nucleolus. He supposed that "chromatoide Nebenkörper" was derived from "Intranclearkörper" that was different from the nucleolus, and was concerned with the spiral (mitochondria of middle piece) formed in late stage of development. The idea that "chromatoide Nebenkörper" was derived from "Intranclearkörper" was also proposed by von Ebner [215] and Lehhossék [116]. Moore [137, 138] studied spermatogenesis of six elasmobranches and used "chromatic body" for the CB. He thought that the "chromatic body" was formed from the debris of nuclear chromatin. Niessing [144] investigated in detail the involvement of centriole and "Sphäre" (currently proacrosomal granule) in spermatogenesis of guinea pig, rat and mouse and referred to the formation of the "chromatoide Nebenkörper" of these animals. He observed that "chromatoide Nebenkörper" of these animals appeared in the juxtanuclear cytoplasm of spermatocytes and suggested that during differentiation of the cells it moved to the cytoplasm at the base of the fla-

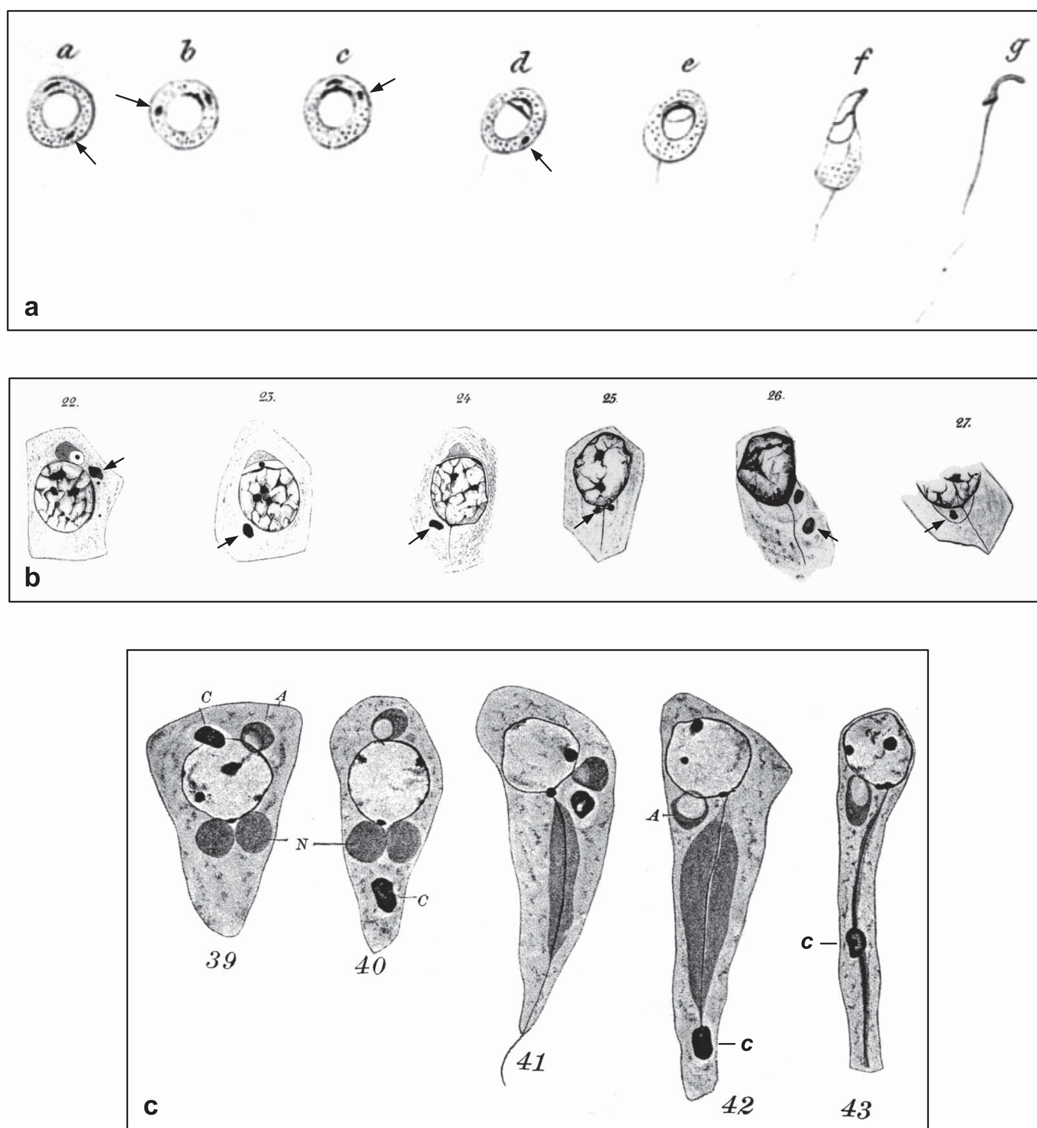

Fig. 1. Chromatoid body (CB) depicted in early papers. **a.** The first description of a CB-like granule of rat spermatids (arrows) by von Brunn in 1876. The granule is absent in late spermatids. **b.** CBs of rat spermatids illustrated by Niessing in 1897 (arrows). Movement of CB during spermiogenesis is shown. Finally, CB enters the residual body at the base of flagellum. **c.** CBs depicted by Wilson in 1913. CBs (c) of an insect, cabbage bug, are surrounded by clear hollow, which seems to correspond to CB vesicles revealed by electron microscopy.

gellum and finally entered into a residual body cytoplasm where it is degraded (Fig. 1b).

Regaud [158] described in detail the structure of seminiferous tubules and spermatogenesis of rat, in which he called the cytoplasmic granules stained black by Heidenhain's iron-hematoxylin and deeply red by safranine "corps chromatoides". He followed the movement of this granule during spermiogenesis and concluded that this granule entered into "corps résiduel" where it was finally degraded. Similar results were also reported in Australian opossum by von Korff [216] who observed that "chromatoide Nebenkörper" first closely attached to the nuclear membrane of spermatocytes and then moved to the neck cytoplasm near the flagellum to form clusters of small granules, and finally disappeared. In 1905 Schreiner and Schreiner [176] reported a study on the development of the hagfish male reproductive organ and summarized the data on the CB published until 1905 using the term "chromatoide Körper" (chromatoid body). They identified these nucleolus-like granules that were strongly stained by iron-hematoxylin from other weakly stained nucleolus which they called "chromatoiden Nucleolen" instead of the term "intranuclearkörper" coined by Benda [19]. According to them, the "chromatoiden Nucleolen" first gathered to form a cluster adjacent to the nuclear membrane that was partially melted away and soon were dispersed to the cytoplasm to form "chromatoide Körper". The "chromatoide Körper" always existed in young spermatids and ultimately entered into the nucleus of sperm. This idea of CB nuclear regression was never proposed thereafter. Duesberg [46] also closely observed the mitosis of spermatocytes and concluded that the CB was derived from the nucleus. In the investigation of the relationships between accessory chromosome (X-chromosome) and the CB of *Pentatoma*, the cabbage bug, Wilson [228] described that the CB of this insect first appeared in the cytoplasm of spermatocytes and later in spermatids, and that it was surrounded by clear vacuolar spaces (Fig. 1c).

In late spermatids, the CB is discarded into the spherical structures located near the flagellum. In addition, after every mitosis of spermatocytes the CB entered either one of two daughter cells and consequently only one daughter cell had the CB and the other did not [21, 230]. This movement was similar to that of the X-chromosome. A detailed study on human spermatogenesis was carried out by von Winiwater [218], who termed the structure corresponding to "corps chromatoides" named by Regaud [158] "corpuscles chromatiques". Although the CB might appear first in the cytoplasm of spermatocytes, its true origin was unclear. It existed in spermatids at the late stage and finally entered into the "corpus résiduel" where it was absorbed as described by Regaud [158]. Gatenby and Woodger [67] also reported the appearance of CB from early to late spermatids of many species. Fasten [57] studied the spermatogenesis of freshwater crayfishes and recorded the CB. The CBs first appeared in the cytoplasm of pachytene spermatocytes, disappeared in secondary spermatocytes and after that never appeared. It was stained strongly like chromosomes and surrounded by clear vacuoles, and very similar to that observed in the insect, the cabbage bug [228]. When the silver nitrate staining method was used for visualization of Golgi apparatus, various names were given to granules that were presumed the CBs from the authors' illustrations and the description for the CB was confused [66, 71, 239].

Light microscopic studies on the CB carried out from 1876 to 1950 were summarized as follows: this structure was first called "chromatoide Nebenkörper" [19], because it was stained red by safranine like nucleolus and dividing chromosomes. Many authors [116, 132, 144, 216] used this name. The current name chromatoid body (CB) was derived originally from the German term "chromatoid Körper" coined by Schreiner and Schreiner [176] and was widely accepted. Two ideas were proposed for the origin of the CB: one was that the CB appeared from the beginning in the cytoplasm of spermatocytes or spermatids [56, 57, 67, 83, 144, 176, 214, 216, 218, 228], and the other was that the CB came out of the nucleoplasm and then moved to the cytoplasm [19, 46, 116, 138, 215]. Ideas as to the fate of CB can be summarized as follows: either the CB exists in late spermatids and form a part of the acrosome or the axis of the flagellum [19, 83, 214], or the CB enters the bag-like structure which is formed in the cytoplasm at the base of the flagellum, and is degraded there [144, 158, 228]. People who supported the latter idea assumed that the CB had no important role in spermiogenesis; this was due to the limited resolution of light microscope and the immaturity of biological knowledge. However, some illustrations, especially those of the cabbage bug depicted by Wilson [228] were noteworthy. He described that the CBs of this animal were surrounded by clear vacuolar space. This fact was confirmed later by electron microscopy, namely, that the CB was surrounded by small vesicles at a certain stage of development.

III. Observations with Electron Microscope (since 1952)

In the 1950s, it became possible to observe biological specimens with electron microscope. The rat testis was first examined [223], followed by Challice [32] who observed the spermatogenesis of rodent. However, these authors did not refer to the CBs. Burgos and Fawcett [27] observed in cat spermatids an irregular mass of osmiophilic granular material in the cytoplasm near the Golgi complex and assumed that it corresponded to the "chromatic body" or "accessory body" of light microscopy. It was composed of closely aggregated dense granules of unknown origin. They described that it migrated back to the caudal pole of the nucleus later in the development of the spermatid, but they did not conclude that it participated in the formation of any of the structural elements of the middle piece. Minamino [133] also observed similar structures in rat spermatids and regarded them as segments of the Golgi complex. More detailed observations on the ultrastructure of rat CB were carried out by Sasa [174]

who described that the CB was composed of clusters of granular osmiophilic material and contained vesicles in the matrix, and consequently that it exhibited a honeycomb-like structure. The similar results were reported in rat again [26] and in mouse [65]. When testis was fixed by potassium permanganate instead of osmium, the typical structure of the CB was not detected [136]. Instead, they observed clusters consisting of small vesicles near the Golgi complex and they insisted that these clusters were not detectable in osmium-fixed tissue. It is unclear whether these clusters are consistent with the CB. Horstmann [85] first observed human spermatogenesis by electron microscope but did not refer to the CB.

Electron microscopic observations on male and female reproductive cells of various species were carried out by many investigators from the 1950s to the 1970s and several dense structures were noted. André and Rouiller [8] termed them "nuage" (meaning "cloud," in French). These dense materials gathered in clusters and embedded in the space among the mitochondria was commonly detected in the reproductive cells of the following species: annelids [45], crustaceans [236], insects [124, 178], myriapods [82], ascidians [86, 98], teleostei [232, 238] and amphibians [3, 15, 128, 227]. The presence of "nuage" was also confirmed in the following mammals: guinea pig [1, 59], hamster [59, 147, 202, 224], rabbit [143], chinchilla [59], macaque [59], mouse [59, 91], rat [26, 48, 59, 70, 167, 237] and human [28, 151, 221]. Full-fledged electron microscopic study on the CB of several mammals was performed by Fawcett and coworkers [59], who investigated the origin of CB during spermatogenesis and its topographic relationships to other organelles of the germ cells. They found no evidence to support a nuclear origin of the CB. Instead, it seemed to arise from the dense interstitial material that accumulated in the mitochondrial clusters of spermatocytes. In spermatids, the CB established an intimate but transient relationship with groups of nuclear pores, suggesting some kind of interaction between the CB and nucleus. As spermatids developed, the CB migrated to the caudal pole of the nucleus and formed a ring around the base of the flagellum together with the annulus. Finally, the CB diminished in size and disappeared late in spermiogenesis apparently by disaggregation and dispersal of its subunits. They found no clear morphological evidence indicating that the substance of the CB contributed directly to the formation of any of the structural components of the mature spermatozoon. In addition, they threw doubt on sharing of the CB through the cell division of primary spermatocytes to spermatids that have at least one CB. These authors newly described a chromatoid satellite that was located near the CB and whose electron density was lower than that of the CB. It was smaller than the CB and composed of loosely associated fine fibers. The origin and destiny of the chromatoid satellite were unclear. Eddy [47] applied a staining method for DNA and digestion by pronase and RNase to resin-embedded sections to clarify whether the CB contained DNA, proteins and RNA. He refuted the results of Daoust and Clermont [41] and Sud [198, 199] showing the existence of RNA in the CB. Susi and Clermont [201] studied the structural changes of the CB and its polysaccharide content and showed that the CB was a membrane-unbound organelle, consisting of dense fine fibrous material and surrounded by small vesicles containing glycoprotein stained by periodic acid-chromic acid-silver methenamine method.

Eddy [47] investigated the form and distribution of the "nuage" in germ cells and found that it was present in primordial germ cells in the gut epithelium, in germ cells in indifferent gonads and in germ cells in the sexually differentiated fetal, neonatal and adult rat gonads. He proposed the idea that the CB was the developed form of the structures belonging to the "nuage". A similar idea was also proposed by Russell and Frank [171], who characterized six different types of "nuage" in rat spermatocytes on the basis of their form, distribution and association with other organelles. All types of "nuage" behaved in a dynamic way and were concerned with the CB. They observed two types of CB. The first type consisted of spherical particles with a diameter of 70–90 nm. These particles appeared in mid-pachytene spermatocytes together with small vesicles and disappeared during the first meiotic division of late diplotene spermatocytes. The second type (0.5 μm) was observed in the secondary spermatocytes and spermatids and was called the definitive CB that was formed by coalescence of a different type of "nuage" found scattered throughout the cytoplasm of diplotene spermatocytes. These observations might convincingly explain why almost every spermatid has at least one CB although a pachytene spermatocyte contains one or two CBs. Namely, the different types of "nuage" dispersed throughout the cytoplasm is apportioned almost equally to spermatids and newly forms the CB after cell division.

In the 1970s, almost all of the ultrastructural descriptions of the CB seemed to be published. The fine structures and fluctuations of the CB during spermatogenesis revealed by EM are summarized as follows: 1) The CB is one of a family of "nuages" observed specifically in both male and female germ cells. 2) In the spermatogenic cells, it is composed of irregular aggregations of dense fibrillar material without any limiting membrane and surrounded by small glycoprotein-containing vesicles. 3) It appears first in early spermatocytes and reduces or disappears during first meiotic division. But it appears again in the cytoplasm around the Golgi apparatus of the secondary spermatocytes and spermatids and then moves to the caudal cytoplasm where it remains until step 16 spermatids. 4) Its number per cell is one or more in spermatocytes but only one in spermatids.

IV. The Origin and Fate of the CB (1889–1980)

The origin of CB has been argued since its discovery. As mentioned above, employed method was only a light microscopy by which most investigators followed the structures showing staining attitude for some dyes such as iron-hematoxylin or safranine. These dyes did not specifically stain the CB but other structures such as chromosomes,

nucleolus and some cytoplasmic granules, so that each investigator differently interpreted the results obtained. Largely two ideas were proposed on the origin of CB; the CB originated from nuclear components and it existed from beginning to the end in the cytoplasm. As mentioned, Benda [19] and Regaud [158] thought that the CB derived from the nucleolus. Other people [2, 46, 83, 116, 215] proposed that the CB derived from "intranuclearkörper" other than the nucleolus. On the other hand, many investigators insisted that the CB was formed in the cytoplasm [56, 57, 67, 144, 214, 216, 218, 228]. The latter idea contained the suggestion that the CB was formed from the preexisting structures such as centriole and acrosome. However, Wilson [228] and Pollister [156] denied such an idea by their studies on the formation of cabbage bug CB and proposed that the CB in these insects appeared directly in the cytoplasmic matrix. Many authors who observed the CBs of various animals by electron microscope supported the cytoplasmic origin of CB [47, 48, 58, 59, 156, 171, 198, 199, 201, 228] whereas many other authors insisted on its nuclear origin [2, 19, 39, 46, 83, 116, 137, 138, 158, 176, 186, 189, 190, 215]. Söderström examined the incorporation of [^{3}H] uridine by rat CB [190] and showed the existence of intranuclear product in the CB. An image suggesting the direct interaction of nuclear membrane with the CB was also shown [191]. In addition, Comings and Okada [39] suggested that in mouse the CB derived from the nucleolar material of pachytene and diplotene spermatocytes. The authors suggested the cytoplasmic origin and also observed that in pachytene spermatocytes and early spermatids dense material similar to the "nuage" embedded in the space among clustered mitochondria located in the cytoplasm very near the nuclear membrane where several nuclear pores gathered [47, 48, 58, 59, 171, 201]. However, they did not recognize any image suggesting the exchange of substances between the nucleus and the "nuage". On the contrary, people holding to the nuclear origin theory observed dense material similar to the CB and intermitochondrial "nuage" gathered at the nuclear pore and noted the existence of similar material in the corresponding nucleoplasm, suggesting the supply of some sort of nuclear material to the CB [191]. However, Söderström [189] suggested that the "nuage" and the CB were separate organelles although their functions were somehow related to each other. In spermatids of a certain grasshopper, Werner and coworkers [225] observed that the CB grew in the cytoplasm facing clustered nuclear pores regardless of intermitochondrial "nuage". Thus, it is likely that transfer of certain materials takes place from the nucleus to the CB. Kotaja and coworkers have directly evidenced this issue [108]. The ideas proposed for the origin and fate of the CB so far are summarized in Figure 2.

Since no CB was found in the spermatozoa people assumed that it had disappeared after it had finished some kind of role, formed some other structure as its component or changed to some other structure. For example, von Brunn

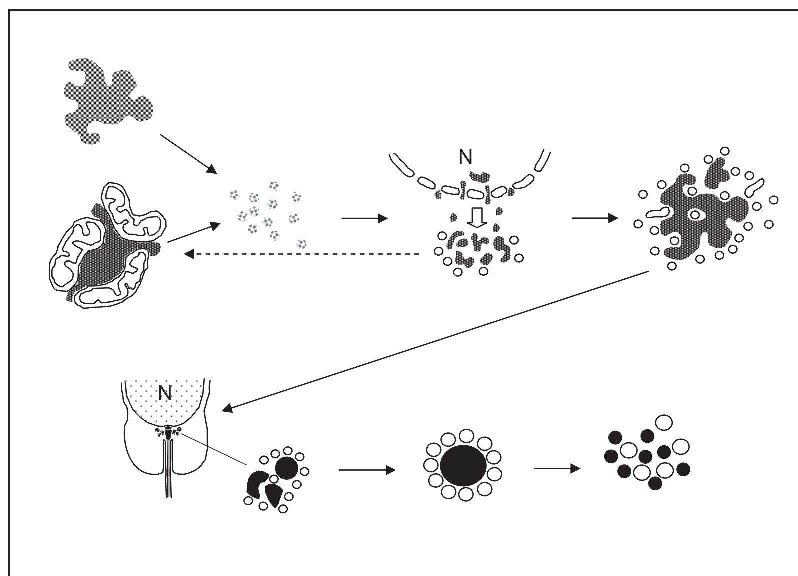

Fig. 2. Formation of CB. The CB and intermitochondrial cement ("nuage") appear in pachytene spermatocytes. Immediately after the second meiotic division, the CB is dispersed in the cytoplasm as small dense vesicles. At the same time, the intermitochondrial cement also disappears. During steps 1 and 2, the vesicles assemble again to form the CB near the nucleus. At this stage the CB receives nuclear materials such as mRNA and is surrounded by small clear vesicles and tubules, which are positive for lysosomal membrane proteins, LAMP1 and LAMP2. Communication of the CB with the intermitochondrial cement is not clear but ATP-dependent DEAD-box RNA helicase MVH (mouse VASA homolog) is detected in both structures. During steps 3 and 6 the CB moves slowly toward the cytoplasm at the caudal pole of nucleus. At steps 7 and 8, the CB reaches the base of flagellum, where it is enclosed compactly by small vesicles and its matrix becomes denser. Gradually the CB decreases in size and finally disappears.

[214], Hermann [83] and Benda [19] thought that it formed acrosome or flagellum, and von Molle [217] suggested that the CB was concerned with formation of manchette. Currently, nobody supports such ideas; instead it is widely accepted that the CB is finally broken down after completion of its role. Niessing [144] and Wilson [228] suggested that as the spermatids maturate, the CB moves to the caudal cytoplasm at the basis of flagellum, enters into the bag-like structure and is finally degraded. Deusberg [46] observed that rat CB was reduced at late spermiogenesis to form several fragments and disappeared into the cytoplasm. In addition, the CBs of rat [14, 158, 159], opossum [216] and human [218] entered into the "corps résiduel" (residual body) and disappeared. According to electron microscopic observations, the CBs moved from rostral to caudal cytoplasm, where they formed "ring centrioles" together with the annulus. As the annulus migrated to the caudal part of the developing middle piece, its size reduced and disappeared [59, 201].

V. Contents of the CB

The clarification of the kinds of substances contained in the CB is one of the important clues for the physiological role of this organelle in spermatogenic cells. Daoust and Clermont [41] first showed that staining of the CB by pyronine was erased by digestion of the sections with ribonuclease and by treatment with trichloroacetic acid and suggested that the CB contained RNA and arginine-rich basic proteins. Sud [198, 199] supported this result, which showed that staining of rat and snake CB by methyl green or safranine was removed by extraction with trichloroacetic acid and by ribonuclease digestion. However, Eddy [47] could not detect RNA either after digestion of Epon sections by ribonuclease or staining with indium trichloride. No accumulation of poly(A)-containing mRNAs in the CBs of spermatids was detected by electron microscope radioautography [139]. However, Kotaja *et al.* demonstrated by *in situ* hybridization using oligo(dT) that poly (A)+-mRNAs were accumulated in the CBs but ribosomal RNAs were not [107]. Biggiogera and coworkers [20] detected no DNA in the CB by immunoelectron microscopy using anti-DNA antibody.

Table 1 lists the substances that have detected in the CB so far. Forty-four proteins, microRNA (miRNA) and mRNA including unspecified RNA, and two metal ions have been detected. The CB is composed of fine fibrillar materials and in many cases it is surrounded by numerous small vesicles (Fig. 3). These small vesicles are closely associated with the CB from early to late stage of CB formation [6, 59, 188]. In Table 1, substances detected in these vesicles are also listed. In addition, there are two types of protein distribution pattern in the CB: 1) distribution of proteins in all areas of the fibrillar matrix, and 2) localization of proteins to the boundary between the matrix and the cytoplasm [76]. These delicate distribution patterns seem to be related to the function of these proteins in the CB. As shown in the Table 1, 29 proteins have been detected in the CB matrix, three on the surface of the CB and twelve in the vesicles surrounding the CB. In the following sections, we will consider the proteins and RNAs detected in the CB, discuss the following three points: 1) the components for the formation of CB, 2) the RNA silencing pathways in the CB, and 3) the degradation of unnecessary proteins occurring in spermatogenic cells during spermatogenesis.

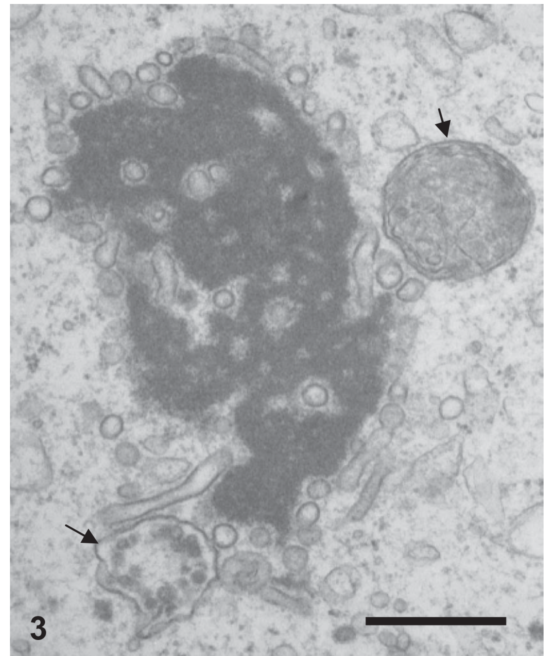

Fig. 3. Typical CB of step 3 spermatid observed by electron microscope. The CB consists of electron dense matrix and is surrounded by small clear vesicles and tubules. The CB has no limiting membrane. Frequently, multivesicular bodies are located near the CB (arrows). Bar=0.5 µm.

VI. Components for the Formation of the CBs

One of the unique phenomena in male germ cell development is the conspicuous condensation of sperm chromatin, which drastically inhibits the transcriptional activity at late stages of germ cell maturation. For this reason, the storage and translation of mRNAs for spermatid and spermatozoa proteins are exquisitely controlled [23, 100, 101]. It is well known that in the germ cells of some animals dense fibrous material accumulates into a cytoplasmic structure called germ plasm containing electron dense granules (polar granules), which is required for germline formation [49]. In *Drosophila melanogaster* and *Caenohabditis elegans*, the polar granules are localized in the cytoplasm at the posterior pole of the egg [125, 126]. After fertilization of the egg, the germ plasm forms pole cells which are essential for function in the specialization of germline lineage and the initiation of embryonic development [87, 88, 197]. In *Drosophila* polar granules, several components such as Oskar [52], Vasa [78, 79, 114], Nanos [115, 220], Aubergine [61, 77, 145]

Table 1. *Components of the CB*

Localtion	Component	Function	References
Matrix	Ubiquitin	Targeting signal for proteasome degradation	76
	Hsp70	Function in protein folding machinery	76
	Actin	Contractile protein	76, 219
	LDH	Dehydrogenation of 2-hydroxybutylate	76
	Enolase	Conversion of 2-phosphoglycerate to phosphoenoylpyruvate	76
	Histocompatibility antigen	Determination of acceptance or rejection of a tissue graft by the immune system	80
	F1α	ATP synthase subunit α, ATP synthesis	76
	F1β	ATP synthase subunit β, ATP synthesis	76
	COX1	Cytochrome C oxidase subunit 1; electron transport in mitochondria	76
	PHGPx	Phospholipid hydroperoxide glutathione peroxidase; removing lipid hydroperoxides from biological membranes	76
	Cytochrom C	Component of the electron transfer chain	84
	Histone H2B	Chromatin condensation	76
	Histone H4	Chromatin condensation	226
	Acetylated histone H3	Regulation of transcription	76
	Acetylated histone H2B	Regulation of transcription	76
	MVH	DEAD-box RNA helicase; essential for spermatogenesis	34, 146, 204, 209
	MIWI	Argonaute/PIWI family RNA-binding protein; *Miwi*-/-blocks afterward development of early round spermatid and disturbs CB condensation.	43, 73, 107, 109
	GW182	RNA-binding protein, component of P-body	107
	GRTH	Gonadtropin activated RNA helicase; essential for spermatogenesis; *Miwi*-/-blocks afterward development of early round spermatid and decreases markedly CB size.	211
	p48 and p52	Germ-cell-specific RNA-binding protein	149
	RanBPM	RanGTP-binding protein; involvement in microtubule nucleation; binding to MVH	181
	MTR-1	Tudor protein; involved in the assembly of snRNPs	34
	snRNP	Essential component of the splisome complex, functioning in pre-mRNA processing	20, 141, 151
	AGO2 and AGO3	Argonaute proteins; components of RISC in RNAi and miRNA pathway	107
	Dicer	Cytoplasmic endonuclease processing miRNA and siRNA	107
	Dcp1a	Decapping enzyme; component of p-body	107
	RNA		41, 60, 193, 198, 199, 219
	miRNA	Sequence-specific silencers of mRNA	107
	mRNA	Translation template	107, 175
	E2	Ubiquitin-conjugating enzyme	76
	Ca^{2+}, Mg^{2+}		7, 166
Surface	p52	26S proteasome subunit	76
	PA700	Lids of proteasome; proteasome activator	76
	Vimentin	Intermediate filament; cytoskeleton	76
Vesicles	LAMP1 and LAMP2	Lysosomal membrane glycoproteins	76
	Acid phosphatase	Lysosomal enzyme; hydrolysis of orthophosphoric monoester	11
	Cathepsins B, D, H, L	Lysosomal protease; hydrolysis of proteins	76
	LAP	Leucine aminopeptidase	76
	DNase	Lysosomal enzyme; hydrolysis of DNA	76
	RNase	Lysosomal enzyme; hydrolysis of RNA	76
	NADPase	Golgi-lysosomal enzyme; hydrolysis of NADP	205, 208
	CMPase	Golgi-lysosomal enzyme; hydrolysis of cytidine monophosphate	205, 208
	Polysaccharides		111

and Tudor [13, 17] have been identified. Formation of polar granules is impaired by mutation of any one of the maternally acting genes, such as *Oskar*, *Vasa*, *Tudor*, and *Aubergine* [22, 77, 94, 96, 207]. As mentioned above, the polar granules have been categorized into "nuage" seen in germ cells and the CB of male mammalian haploid germ cells is classified into "nuage" [48].

During late spermiogenesis, the chromatin of the hap-

loid spermatids highly condenses and virtually all transcription activity ceases [190, 192]. The mRNAs coding proteins required in late spermatids are transcribed in the earlier stage of spermatogenesis, stored in the cytoplasm and translated when spermatids need them. Such delayed translation of many mRNAs occurs in post-meiotic cells [101, 102, 175, 203]. It is suggested that the CB stores RNA and proteins for terminal differentiation of the sperm cells [81, 153, 193]. Thus, the CB is thought to contain mRNA and protein and plays a crucial role in post-transcriptional control during spermatogenesis. Recently, several proteins involved in RNA metabolism have been reported to localize in CBs as summarized in Table 1. They are ATP-dependent RNA helicase of the DEAD (Asp-Glu-Ala-Asp)-box protein family, of which VASA is the best-characterized component of germ plasm in *Drosophila* [79, 114, 117]. DEAD-box RNA helicases play important roles in RNA metabolism [162]. In yeast, they are involved in mRNA export [117, 184], pre-mRNA splicing [196], translation initiation [33, 155], RNA decay [38, 134] and ribosome formation [42]. Based on conserved structure, many homologue genes to *Drosophila vasa* have been isolated in various animal species such as *C. elegans* [165], planarian [182], *Xenopus* [103], zebrafish [149, 234], mouse [62] and rat [104]. VASA (mouse Vasa-homologue, Mvh) protein is localized in the CBs from early spermatocytes to differentiating spermatids [146, 181, 209, 211]. Male mice homozygous for a targeted mutation of Mvh exhibit a reproductive deficiency [204]. In addition, when knock-in ES cells, in which *GFP* or *lacZ* was expressed from the endogenous Mvh, are transplanted into testicular tubules, they can produce functional germ cells *in vitro* [210].

In *Drosophila* egg, *oskar* gene is a key component in pole plasm assembly and is required for the stepwise assembly of posterior pole plasm together with four other genes of *vasa*, *pipsqueak*, *tudor* and *valios* [22, 30, 51, 99, 177, 184]. In addition, Staufen protein is required for both *oskar* RNA localization to polar granules and its translation. Localization of *oskar* RNA permits translation into Oskar protein that subsequently regulates its own RNA localization through a positive feedback mechanism [163]. Oskar protein interacts with Vasa and Tudor proteins and is a component of polar granules, the germ-line-specific RNP structures [24, 94, 127]. Localization of Oskar protein to "nuage" and polar granules has been studied using GFP-tagged versions of Vasa and Aubergine to characterize and track "nuage" particles and polar granules in live preparations of ovaries and embryo [186]. Oskar protein nucleates the formation of polar granules from cytoplasmic pools of components shared with "nuage" and appears to stabilize at least one shared component, Aubergine, suggesting Oskar-dependent formation of polar granules [186]. Localization of Oskar protein in "nuage", including the CBs of male germ cells is unclear so far.

Recently, *Tudor* domain containing *1/Mouse tudor repeat-1 (Tdrd1/Mtr-1)* has been isolated and its product, Mtr-1 protein is present in the cytoplasm of prospermatogonia, spermatocytes, and round spermatids, and predominantly localizes to the CBs [34]. MTR-1 co-localizes with Sm proteins which are components of snRNPs in the CBs located in the perinuclear region [20, 34, 141], suggesting that MTR-1 functions in assembling snRNP into cytoplasmic granules including the CBs in germ cells. *Tudor* is genetically downstream of *vasa*, and encodes a component of the *Drosophila* "nuage" [17, 69]. Genetic study shows that the activity of *tudor* is required during oogenesis of *Drosophila* for the determination and/or formation of primordial germ cells and for normal embryonic abdominal segmentation [22]. In *Drosophila* embryos from *tudor*-null mothers, the polar granules are greatly reduced in number, size, and electron density, suggesting that *tudor* is dispensable for somatic patterning, but essential for pole cell specification and polar granule formation [207]. Amikura and coworkers [5] show that in *Drosophila tudor* mutant females, the localization of mitochondrial large and small rRNA (mtrRNA) is reduced, although the polar granules are maintained and that Tudor protein is colocalized with mtrRNA closely between mitochondria and polar granules, suggesting that Tudor mediates the transport of mtrRNAs from mitochondria to polar granules.

Molecular function of Mtr-1 protein is still unclear like *Drosophila* Tudor. It may function in assembling several proteins or other cellular components into macromolecular complexes such as "nuage" in the cytoplasm [34]. *Tdrd1/ Mtr-1* mutants show strong reduction of intermitochondrial cement (one type of "nuage"), but the CBs are apparently not affected [35]. Recently, Chuma *et al.* have shown that Mtr-1 protein is associated with intermitochondrial cement but not with the CBs, suggesting that the CBs likely have an origin independent of or additional to intermitochondrial cement [35]. Drosophila, *valios* (*vls*) gene has been identified to encode the Vls protein, which localizes to "nuage" and pole plasm in oocyte, is involved in the assembly of these structures, and binds to Tudor and methyltransferase Capsuléen [10]. In *Drosophila*, *aubergine* is required for posterior body patterning and significantly enhances the translation of *oskar* in the *Drosophila* ovary [229]. In addition, *aubergine* is required for pole cell formation independently of its initial role in *oskar* translation, related to eukaryotic initiation factor-2C, eIF2C, and co-localized in polar granules with Vasa and the product of *Maelstrom*, a *Drosophila spindle*-class gene [61, 77].

Intercommunication of the CBs with nuclear materials has being suggested by many investigators since their discovery [2, 19, 39, 46, 83, 116, 137, 138, 158, 176, 186, 189, 190, 213, 215]. Immediately after the second meiotic division, the CB is dispersed in the cytoplasm as small granules. During step 2, the small granules coalsece with each other to form a typical CB structure and move rapidly to make transient contact with the nuclear envelope [152]. Electron microscopic observations reveal material continuities between the nuclear materials and the CBs [191]. These rapidly moving CBs have been suggested to collect gene products from the nucleus and to be involved in nucleo-

cytoplasmic RNA transport [152]. Kotaja *et al.* have found that MIWI, an RNA-binding protein, which is discussed later, interacts with KIF17b, a testis-specific kinesin, which shuttles between nuclear and cytoplasmic compartments and is concentrated in the CBs of round spermatids [108]. It is suggested that PIWI-binding RNAs are transported through nuclear pores with the help of KIF17b to the CBs and stored there until they are needed [108]. Thus, recent studies are turning the light of molecular explanations on the observations performed hundred ago.

VII. RNA Silencing Pathways in the CB

RNA silencing or RNA interference (RNAi) occurs in a wide variety of eukaryotic cells and is triggered by double-stranded RNAs (dsRNAs) that vary in length and origin. dsRNAs induce the silencing of cognate genes, by which gene expression is regulated at both the transcriptional and posttranslational levels [4, 119, 131]. Long dsRNA molecules and microRNA (miRNA) are first processed by the RNase III-like enzymes Drosha and/or Dicer. In *Drosophila*, Dicer-2 converts long dsRNA into 21- to 22-nucleotide (nt) small interfering RNAs (siRNAs). The siRNAs and miRNA are incorporated into RNA-induced silencing complexes (RISCs), which elicit decay or translational repression of complementary mRNA targets. Studies of the miRNA and RNAi pathways in somatic cells have rapidly progressed but those in germ cells have only just started [107, 108, 183]. Several miRNA are known to be testis-specific and expressed in testis much more than in other organs [16, 235].

RISC contains a member of the PIWI/Argonaute (AGO) protein family [43, 113], which is characterized by a central PAZ (PIWI/Argonaute/Zwille) domain, Mid and a C-terminal PIWI (P-element induced wimpy testis) domain [29, 31, 75, 131]. The PAZ domain recognizes siRNA and miRNA [118, 194, 233] and the Piwi domains of human and *Drosophila* can cleave mRNAs fully complementary to siRNA or miRNAs [120, 130, 135, 157]. In *Drosophila*, four Argonaute paralogs (AGO1, AGO2, PIWI, or Aubergine) play essential roles in RNA silencing [55, 160]. Mammalian Piwi proteins are restricted to the germline and are important for male germline development, particularly spermatogenesis [43, 112, 113]. In mice, there are four Ago members (AGO1-4) and three Piwi members (MIWI, MILI/PIWIL2, and MIWI2/PIWIL4). MILI is expressed from spermatogonia to pachytene spermatocytes, whereas MIWI is observed from mid-pachytene spermatocytes to early round spermatids. Recently, Piwi-interacting RNAs (piRNAs) were isolated from mouse testes independently by four groups [12, 68, 72, 222]. Two distinct populations of piRNAs have been found: the first group of piRNAs is 29–31 nucleotides (nt) in length and associated with MIWI proteins; the second group is slightly shorter piRNAs (26–28 nt in length) and preferentially associated with MILI protein. The distinct roles of these two RNAs are unclear so far. As described above, RISC components such as MIWI, Ago-proteins and Dicer, have been detected in the CB of mouse pachytene spermatocytes and round spermatids [73, 107, 108]. RNA-binding and RNA-processing proteins such as MVH [204, 209], and components of RNA decay pathway and the miRNA pathway such as miRNA are also detected in the CBs [107]. In addition, other RNA-binding proteins, p48, p52 and GW182, and decapping enzyme, DCP1a, are present in the CBs [107, 148]. GW182 and DCP1a are also components of processing bodies (P-bodies) in many non-germline cells. P-bodies are subcellular ribnucleoprotein (RNP) granules that are hypothesized to be the sites of mRNA degradation, mRNA translational control, and/or mRNA storage [18, 25, 40, 89, 90, 121, 123, 180, 206, 212].

P-bodies have been first discovered by Sheth and Parker [180] in yeast. Related structures had been found in mammalian cells [18]. Mammalian homologues of a number of yeast P-body proteins have been identified in mammalian P-bodies [9, 40, 54]. They are Stau, RCK/Me31B/Dhh1p (DEAD-box RNA helicase), Xm1p (exoribonuclease), RAP55/Tral/Scd6p, Edc3p, Lsm1-7p, Dcp1p/Dcp2p (decapping enzymes), Ago 1, Ago 2, elF4E, elF4E-T, PABP and GW182, which are key components of the RISC and act in the RNAi pathway [9, 53, 121, 122, 179, 180]. Several components of P-bodies are also found in the CBs, whereas no ribosomal RNA is present in both bodies [107]. These facts correlate well with the current hypothesis on the function of P-bodies [25, 37, 97, 122, 164, 206]. It is supposed that P-bodies store temporarily a pool of translationally repressed mRNA-RNP particles that are released to translation when the protein is needed [164]. The functional analogy between P-bodies and CBs is increasing. Elucidation of piRNA function might open the way for the solution of the CB roles in spermiogenesis. Considering the presence of several separate processing pathways in the CB, Kotaja and coworkers have proposed that the CB might function as a sorting center that determines the destiny of mRNAs [107].

On the other hand, the relationships between intermitochondrial “nuage” and CB have not been clearly explained so far. Furthermore, it is also unclear how the RNA-binding and RNA-processing proteins are concerned with the six different types of “nuage” and two types of CBs found in adult rat testis by Russell and Frank [171]. The elucidation of these problems might contribute to clarify the roles of “nuage” and CBs in spermatogenic cells. This type of study is now possible because we could label those structures with CB markers such as MVH and others (see Table 1).

VIII. Relationship of CB to the Lysosomal System

The relationship of the intracellular vacuolar system including Golgi apparatus and multivesicular body to the CB has been reported by enzyme cytochemical studies. Activities of acid phosphatase [11], nicotinamide adenine diphosphatase (NADPase) [208] and cytidine monophosphatase (CMPase) [205, 208] were detected in small vesicles sur-

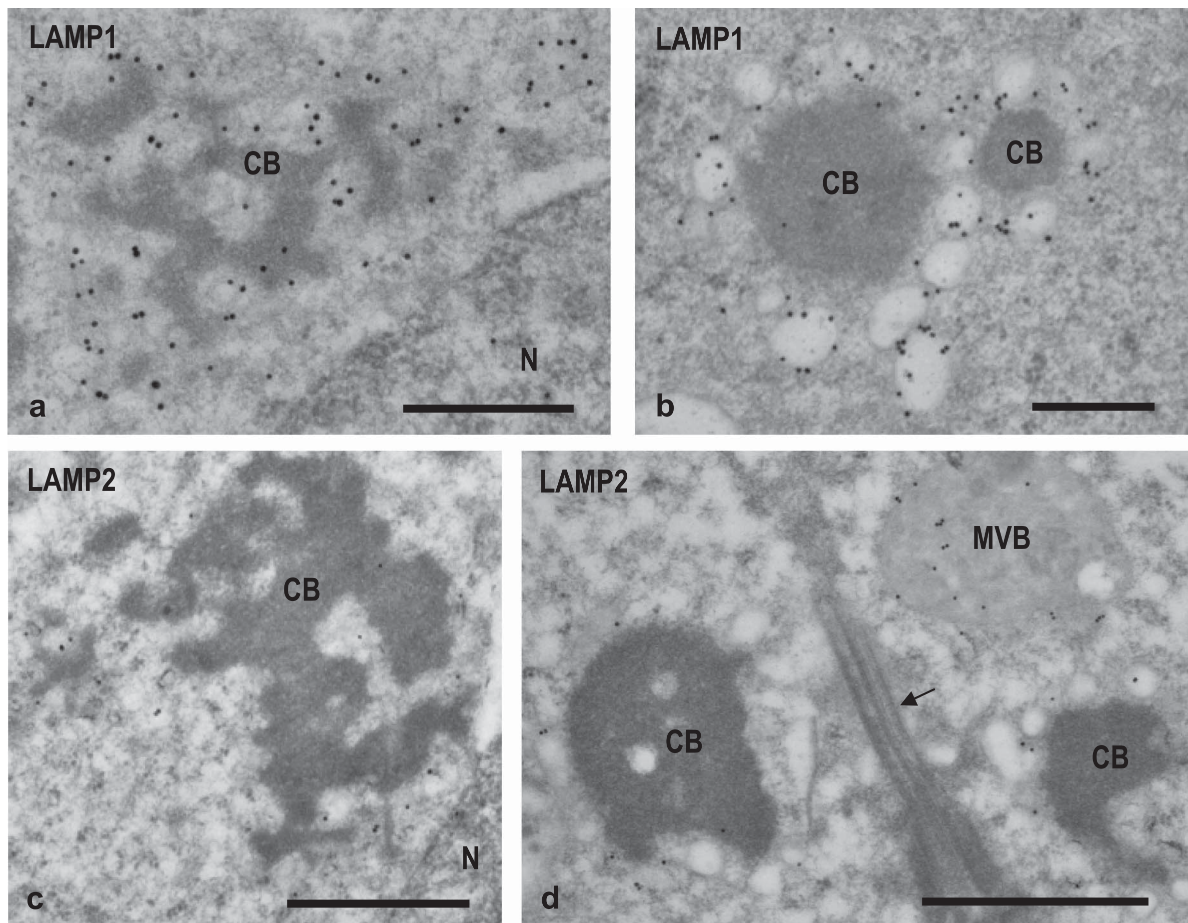

Fig. 4. Immunoelectron microscopic localization of lysosomal membrane proteins, LAMP1 and LAMP2 in small vesicles surrounding the CB. **a.** CB (CB) of step 1 spermatid. Gold particles are associated with the vesicles. N: nucleus. Bar=0.5 µm. **b.** CBs (CB) of step 10 spermatid. LAMP1 signals are present in small vesicles surrounding CB. Bar=0.5 µm. **c.** CB (CB) of step 2 spermatid. Weak gold label is seen in vesicles surrounding CB. N: nucleus. **d.** CBs (CB) of step 8 spermatid. Gold labeling is noted in the vesicles and multivesicular body (MVB). Arrow indicates developing flagellum. Bar=0.5 µm.

rounding the CB (CB-vesicles). These enzymes are also distributed in the trans Golgi network, Golgi vesicles and multivesicular bodies so that the CB-vesicles seem to belong to the endosome-lysosmal pathway. No activity of thyamine pyrophosphatase, the Golgi marker enzyme, was detected in the CB-vesicles [205], suggesting that the membrane of CB-vesicles might not be directly derived from the Golgi apparatus. We have detected seven lysosomal enzymes and two lysosomal membrane proteins (LAMP1 and LAMP2) in the CB-vesicles from early to late spermatids [76] (Fig. 4). In the spermatids, LAMP1 and LAMP2 were localized to multivesicular bodies and lysosomes in addition to the CB-vesicles, but never in developing acrosomes [76]. Similar results were shown by fluorescent microscopy of LAMP1 and LAMP2 [140]. These results suggest that CB-vesicles belong to endosome-lysosome pathway. The CB-vesicles are closely associated with the CB from the beginning of CB formation to just before its disappearance (Fig. 3), indicating that the CB is closely related to lysosomal function. It is quite unclear why CB-vesicles can specifically attach to the CB. Although it is unclear whether proteins and RNA are actually digested in the CB-vesicles, it is likely that proteins and RNA detected in the dense fibrillar matrix of the CB are transferred into the CB vesicles to degrade.

It has been shown that cells have an aggresomal pathway by which aggregates of misfolded proteins or mutated proteins are transported to the area around the microtubule organizing center where they form large aggresomes [63, 64, 93, 106]. Furthermore, a diverse array of human disease, including amyloidosis and neurodegenerative disorders, are caused by the accumulation of misfolded proteins due to an impaired degradation system. The resulting cellular depositions are very similar to the aggresomes [92, 95, 129, 142, 161, 173]. Thus, misfolded or unnecessary proteins are assembled to small aggregates and transported to aggresomes where abnormal proteins are degraded by the ubiquitin-proteasome system or eventually by autophagy [64, 105, 231]. The aggresomes are characterized as follows: they are located around the pericentriolar area, contain ubi-

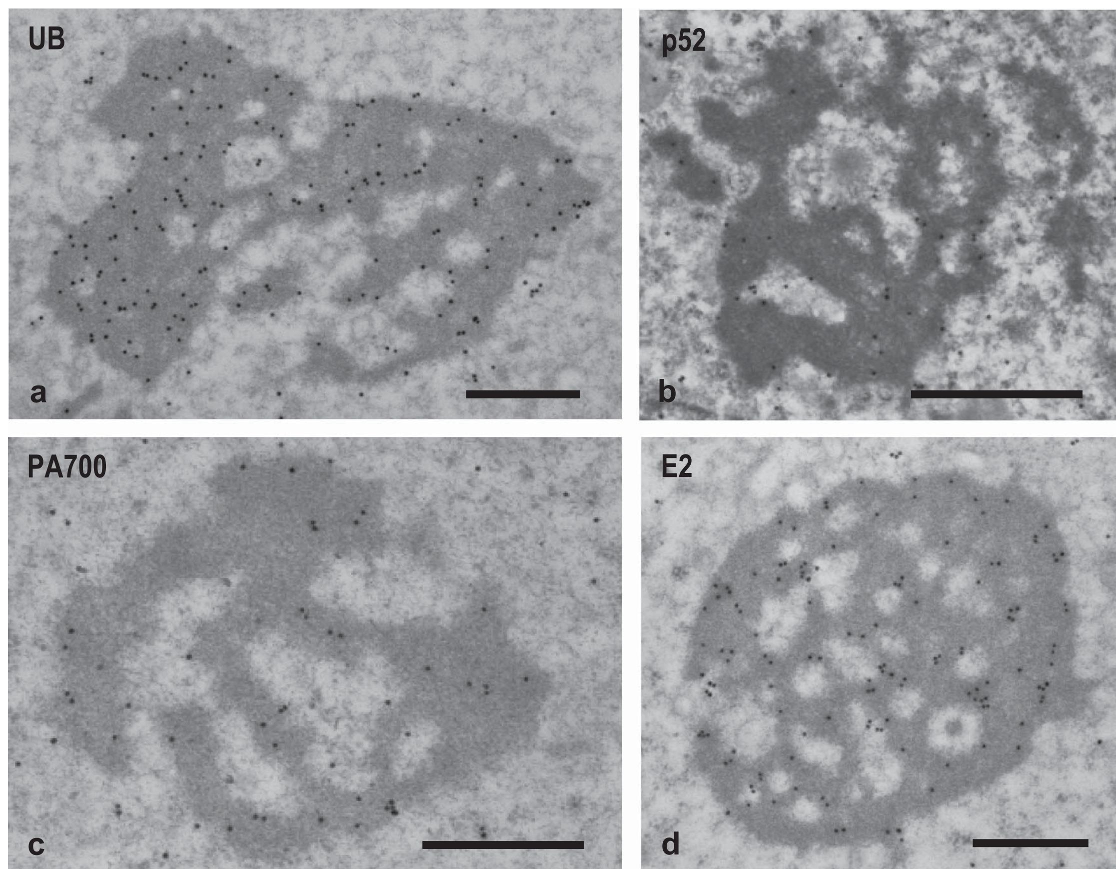

Fig. 5. Immunoelectron microscopic localization of proteins relating to ubiquitin-proteasome proteolytic system. **a.** Ubiquitin signals in the CB of step 3 spermatid. Bar=0.5 μm. **b.** Localization of proteasome subunit p52 in the CB of step 1 spermatid. Note that gold particles are seen on the surface of dense matrix of the CB. Bar=0.5 μm. **c.** Gold labeling for proteasome activator PA700 in the CB. Most of the gold particles are located on the surface of dense material. Bar=0.5 μm. **d.** Gold labeling for ubiquitin conjugating enzyme, E2 in the CB. Gold particles are present on the CB matrix. Bar=0.5 μm.

quinated proteins, chaperone proteins such as Hsp70 and Hsp40 and abnormal proteins, and are surrounded by vimentin filaments and proteasomes. These aggresomal markers are mostly found in the CB as shown in Table 1, suggesting that the CB has an aggresome nature as an intracellular degradation system for unnecessary proteins. Many of proteins accumulated in the aggresomes are ubiquitinated [63, 64, 93, 106]. Polyubiquitin-conjugated proteins are recognized by PA700, lids of proteasome [44], and are degraded by 20S proteasomes [36]. Thus, polyubiquitinated proteins gathered to the aggresomes are degraded by proteasomes. The presence of E2, ubiquitin conjugating enzyme, and strong ubiquitin signals in the CB suggests that unnecessary proteins are gathered and polyubiquitinated. Although which proteins are polyubiquitinated in the CB is unclear, it is likely that the polyubiquitinated proteins are degraded by proteasomes detected at the surface of the CB (Fig. 5b). Thus, it is supposed that two degradation pathways, a lysosomal system and a ubiquitin-proteasome system, are associated with the CB.

Considering our accumulated data on the CB, the physiological function of the CB might be summarized as shown in Figure 6. Haploid gene products binding with ribonucleoprotein particles are transported to the cytoplasm and collected into the CB that contains testis-specific PIWI/Argonaute family member (MIWI), RNA-binding and RNA-processing proteins such as MVH (mouse VASA homolog), and components for RNA decay pathway and miRNA pathway such as miRNA, Dicer and Ago proteins. On the other hand, the CB contains nuclear proteins such as histones, mitochondrial proteins such as F1α and β, COX1 and PHGPX, and cytoplasmic proteins such as actin, LDH and enolase. In addition, ubiquitin signals are detected in the CB [76]. Thus, many proteins derived from different compartments are assembled in the CB. Some of these proteins are probably ubiquitinated and degraded by proteasomes associated with the surface of the CB. The other proteins are broken down in the vesicles (lysosomes) attached to the CB.

It was reported that over 75% of the cytoplasm was eliminated in the late spermatids to provide spermatozoa [169, 195]. This elimination is supposed to be performed by the fluid pump appearing in stage of spermatid elongation

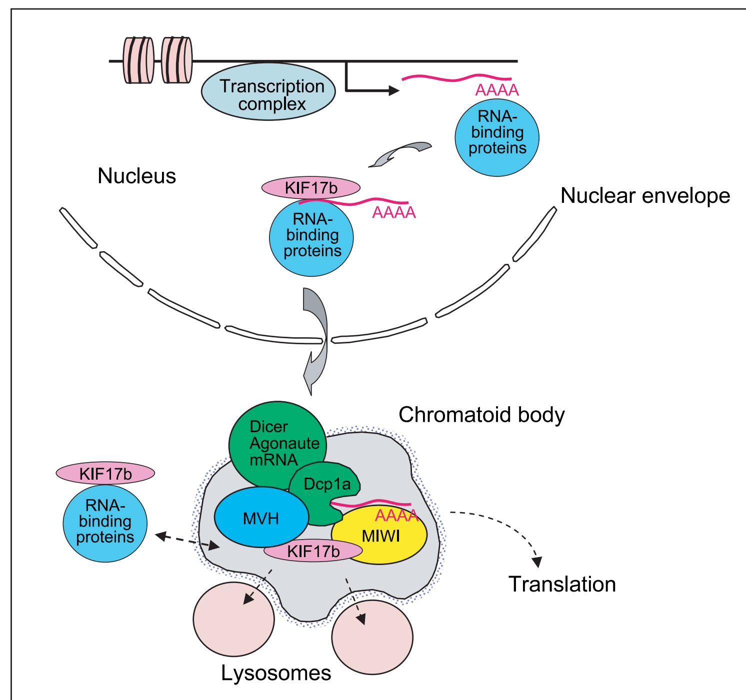

Fig. 6. Hypothetical model for the function of CB. Haploid gene products are assembled into the ribonucleoprotein particles containing RNA-binding proteins. The mRNA-protein complex is then transported through the nuclear pore into the cytoplasm with the help of kinesin KIF17b. In the cytoplasm, moving CBs make frequent contact with the nuclear pores and collect mRNAs. KIF17b interacts with the testis-specific PIWI/Argonaute family member, MIWI. CB contains RNA-binding and RNA-processing proteins such as MVH (mouse VASA homolog), and components of the RNA decay pathway and the miRNA pathway such as miRNA, Dicer, decapping enzyme Dcp1a and Argonaute proteins. Stored mRNAs are released to the cytoplasm by appropriate stimulus and translated. The complex of RNA-binding protein and KIF17b may be reused. The CB also contains various proteins derived from different subcellular compartments such as the cytosol, mitochondria and nucleus, and enzymes for ubiquitination such as E2, that are not depicted here. Some of them are likely polyubiquitinated and degraded by proteasomes located on the surface of the CB (area shaded by dots). Other proteins are degraded by lysosomes (small vesicles) closely attached to the CB. Thus, in post-meiotic male germ cells, the CBs seem to have a P-body-like role on the one hand and an aggresome-like role on the other hand.

and by the special structure termed tubulobulbar complexes appearing in late spermatids [168, 170, 172]. This idea was supported by Guttman and coworkers [74]. This enormous elimination of the cytoplasm including unnecessary proteins and organelles might be performed by the mobilization of all degradation systems. Thus, in post-meiotic male germ cells, the CBs seems to play a P-body-like role on the one hand and an aggresome-like role on the other hand.

IX. Conclusions

This review article surveys the studies on the CBs of various animals published in the past 130 years. Early light microscopic studies published from 1876 to 1955 have described the behavior of CBs stained mainly by safranine or iron hematoxylin during spermiogenesis. These dyes stain DNA, RNA and basic protein, this caused some confusion among researchers as they sought to explain the structures and their origin. However, the relationship between CBs and the nucleus and the movement of CBs during spermiogenesis are largely consistent with the conclusions obtained by electron microscopic observations. After electron microscopic studies started in 1955 the fine structures of the CBs and related granules, including “nuage”, were described in detail. Histochemical studies performed at the same time revealed some of the content of the CBs. On the other hand the CBs have never been successfully isolated, hence no biochemical studies on the CBs have been published. Since 1995 some proteins in the CBs were identified, making molecular cell biological study on the CBs possible, with the similarity of CBs to P-bodies proving to be very useful in elucidating the role of CB in spermatogenesis. The mystery surrounding the CBs since their discovery over one hundred years ago is now being slowly unraveled.

X. Acknowledgements

Our studies contributing to this review were supported in part by Grants-in-aid (14580693) and (17570158) and the Center of Excellence (21COE18999997) program from the Ministry of Education, Culture, Sports, Science and Technology Japan.

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