EDITORIAL

The nucleolus through the years

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Summary

At present it is clear that nucleoli are multifunctional cell organelles and participate in the cell proliferation, resting state, differentiation, maturation, aging and death. Therefore, they also represent very useful morphological markers of these cell states under physiological, pathological and experimental conditions. One of important nucleolar functions related to the mentioned cell states represents the transcription of ribosomal RNA and assembly of pre-ribosomal particles. Thus nucleoli participate in the creation of the cytoplasmic translation machinery necessary for the cell life and function. Although main nucleolar functions are already known, some of recently reported new additional functions depend on the role of a variety of nucleolar proteins some of which are not participating in the ribosomal biogenesis. It is natural that the role of newly discovered nucleolar proteins is still under discussions and remains to be clarified depending on new methodical approaches. It should be also mentioned that the visualization of some of these proteins and nucleolar localization also are and will be a subject for further studies.

Key words: nucleoli; morphological marker; visualization; ribosomal biogenesis; nucleolar DNA; RNAs; proteins; pathology

INTRODUCTION

Looking at the abundant literature, it was not possible to quote all generally recognized publications dealing with the nucleolus. Therefore, the author expresses a great apology to all nucleolar researchers whose articles were not mentioned. In addition, it is very difficult to distinguish more or less important contributions to the knowledge of the nucleolar morphology, structural organization and functions.

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When I was reviewing the publications on the nucleolus at the onset of the last century, I was just shocked what was known at that time and what was rediscovered at present. When I shall be really retired without playing with the microscope, it will be a real pleasure for me to look after that "old" history of the nucleolar research.

Naturally, my view is strongly influenced by receiving training in biomedical sciences such as cytology, histology, cytochemistry, clinical cytology and clinical medicine, as well as by the possibility to be working with very experienced scientists and clinicians. Moreover, it was for me great pleasure to have a possibility to meet most of present "nucleologists" personally and to be connected with them by a long lasting friendship. At this occasion I have to remember my teachers and friends who already are not with me, Harris Busch, František Heřmanský, and Jan Wolf.

DISCOVERY OF THE NUCLEOLUS

It seems to be likely that shortly after the introduction of the microscope to biological and medical sciences; nucleoli were seen by some microscopists as very compact small bodies in the cell nucleus of a variety of cells (Fontana in 1781, see Stockinger 1953). Therefore, no wonder that Valentin (1836) working with Purkyně used for such body the term "nucleolus". The botanist Schleiden (1838) and zoologist Schwann (1839) called such body "Kernchen" and "Kernkörperchen" (Schwann 1839). All these terms are generally used at present times.

Nucleoli also exhibited different affinity to various staining procedures and microscopic techniques than the nuclear chromatin and exhibited a special structural organization (Montgomery 1899, Ruzicka 1899, Busch and Smetana 1970). Actually, based on the results of such methodical approaches, nucleoli appeared somehow different in various cells. Such differences resulted in their first classification that, however, neglected the differences in their size. That classification was dependent on the nucleolar affinity to basic and acidic dyes introduced for the visualization of main cell components.

Nucleoli stained with acidic dyes were considered to be "real nucleoli" and those stained with basic dyes were believed to be "false". However, in Unna-Pappenheim procedure using two basic dyes – methyl green and pyronin "real nucleoli" were basophilic, i.e. pyroninophylic and methyl green stained the chromatin. Then, much later, this staining result was clarified on the base of a large content of RNA in the nucleolus that contributed to its affinity to the basic dye – pyronin (Brachet 1942). The presence of RNA in nucleoli was also confirmed by UV cytospectrography and related to the cell functional state (Caspersson 1950, Vendrely and Vendrely 1959). Then, according to numerous cytologists and histologists, basophilic nucleoli together with basophilic properties of the cytoplasm due to the presence of RNA were characteristic for proliferating or protein producing cells.

At the onset of the development and use of cytochemistry, nucleoli were identified by basic dyes using the isoelectric point of RNA (Stockinger 1953, Smetana et al. 1969, Ochs 1998). Nucleoli also exhibited different pattern to various extraction procedures, which were clarified and modified to be specific for nucleolar main components much later. Nevertheless, nucleoli were "specifically" extracted by pepsin acidified with HCl at that time (Jorgensen 1913). Further progress of extraction procedures with HCl, NaOH and specific enzymes facilitated to distinguish main nucleolar components with more exact location of structures containing DNA, RNA and certain proteins (Busch and Smetana 1970). It should be also mentioned that the hydrolysis with HCl in Feulgen procedure or before staining with basic dyes also remove RNA in examined specimens.

The functions of the nucleolus were mysterious for a long period of time even after structural and morphological definition of main nucleolar components. However, the different nucleolar size in proliferating or resting cells suggested their important role in various cell states and abnormalities including the malignancy (Koller 1963). In most of proliferating cells the nucleolar size was usually large and increased in terminal stages of the cell cycle just before the mitotic division (Schnedl and Schnedl 1972, Nagel 1976). The nucleolar staining properties and especially nucleolar size were used in both diagnostic cytology or histology and experimental biology since the onset of the last century, even before the clarification of nucleolar functions.

The important discovery of the main nucleolar function was based on several crucial observations leading to the discovery that nucleoli are responsible for RNA synthesis that was influenced by the differentiation or maturation process (Busch and Smetana 1970). At this occasion it should be mentioned that the ribosomal RNA transcription has been established later after successful development of methods for isolation of nucleoli. The analysis of the RNA of isolated and purified nucleolar fraction, in combination with cytochemistry including electron microscopy, resulted in the conclusion that most of this nucleic acid represents the ribosomal or pre-ribosomal RNA (Busch and Smetana 1970).

MOLECULAR ARCHITECTURE

Recent studies using computer assisted RNA image densitometry at the single cell level demonstrated a direct relationship between the nucleolar size and RNA content. The later apparently depends on the nucleolar size (Smetana et al. 2006a). The development of special spreading procedures for nucleic acids facilitated to create the Christmas tree model that represented a direct visualization of genes transcribing the ribosomal RNA (Miller and Beatty 1969, Franke et al. 1976). Some further studies suggested that this model might correspond to ultrastructural organization and distribution of main nucleolar components *in situ* (Jordan 1991, Raška 2003).

The studies on isolated nucleoli also demonstrated that the nucleolar content of DNA coding for rRNA

is relatively small and thus, there must be other RNA's transcribed in the nucleolus. Further studies in this direction agreed with such speculation. Moreover, according to recent studies nucleoli possess other RNA species including messenger, transfer and especially small RNA's. It is known that several small nucleolar RNAs participate in ribosomal RNA processing (Pederson 1998, Olson et al. 2000, Pederson and Tsai 2009). The small RNA telomerase subunit is also "educated" in the nucleolus and the role of other small nucleolar RNA's as well as RNP's also requires a further research.

The presence of a variety of proteins not-participating in the rRNA transcription or processing in the nucleolus is of a great interest and might provide more information on its additional functions (Pederson and Tsai 2009). The recent genomic analysis indicated the presence of hundreds proteins in the nucleolus (Scherl et al. 2002, Staub et al. 2004, Pederson and Tsai 2009). However, the role of most of these proteins should be discovered by future studies for the elucidation of various additional nucleolar functions (Pederson 2002). On the other hand, these studies must exclude a possible contamination of analyzed specimens by extranucleolar components. Moreover, it has been also shown that nucleoli may possess viral proteins associated with nucleolar proteins or nucleic acids and are necessary for the development of certain viruses in infected cells (Busch 1997, Pederson 2010).

A great hope was expected from the recently discovered nucleolar protein – nucleostemin for the possibility to be a marker and control of stem and malignant cell proliferation (Pederson and Tsai 2009). However, using the immunocytochemistry, this protein was also detected in terminally differentiated stage of both normal epithelial and malignant squamous cells. On the other hand, the nucleolar size and signal intensity was significantly larger in malignant cells (Čada et al. 2007).

MORPHOLOGY

After improving methods for the electron microscopy by ultrathin sectioning and fixation as well as "staining" with heavy metal salts it was clear that compact bodies of nucleoli were mostly prominent in the cell nucleus. Using middle and high magnification of ultrathin sections it was possible to see all main nucleolar structures such as more or less distinct trabecular structures – nucleonemata, fibrillar and granular components as well as fibrillar centers

(Bernhard and Granboulan 1963, Busch and Smetana 1970, Figs 1, 2).

The improvement of ultrastructural cytochemistry also contributed to see the perinucleolar and intranucleolar distribution of chromatin. The intranucleolar chromatin was represented by chromatin septa-like structures penetrating into the nucleolar body from the discontinuous shell of the perinucleolar chromatin (Fig. 3). Within nucleolar bodies the intranucleolar chromatin was organized as clusters or solitary filaments (Fig. 3), which were frequently present in fibrillar centers including their periphery (Busch and Smetana 1970, Smetana and Busch 1974).

The spreading procedures for DNA and RNA molecules resulted in the direct visualization of genes transcribing the ribosomal RNA (Miller and Beatty 1969, Franke et al. 1976). Measurements of pre- and ribosomal RNA molecules also contributed to the knowledge of the rRNA processing. At this occasion it must be mentioned again that nucleolus also contains further small molecular RNA species, the functions and structural location of which is a subject of a great interest for a long period of time (Ro-Choi 1997).

Autoradiography and cytochemistry including the immunocytochemistry indicated that fibrillar centers possessed DNA clusters and protein filaments of a low electron density some of which are involved in the ribosomal RNA transcription and processing. After numerous discussions it seems to be established that the periphery of fibrillar centers with adjacent "dense" fibrillar components are sites of the pre-ribosomal RNA transcription (Figs 1, 2, Busch and Smetana 1970, Fakan and Puvion 1980, Raška et al. 1990, Dundr and Raška 1993, Spector 1993, Wachtler and Stahl 1993, Hozák et al. 1994, Medina et al. 2010).

NUCLEOLAR MACHINERY

According to some studies fibrillar centers and adjacent regions with dense RNA containing fibrillar components represent functional compartments – fibrillar complexes (Hozák et al. 1986). Fibrillar components containing the newly transcribed pre-ribosomal RNA are converted – processed to granular components, which actually represent preribosomal particles. The pre-ribosomal RNA is processed in the proximity of the transcription sites (Staněk et al. 2000) and nucleolar regions containing both fibrillar and granular components seen by electron microscopy are also known. The ribosomal
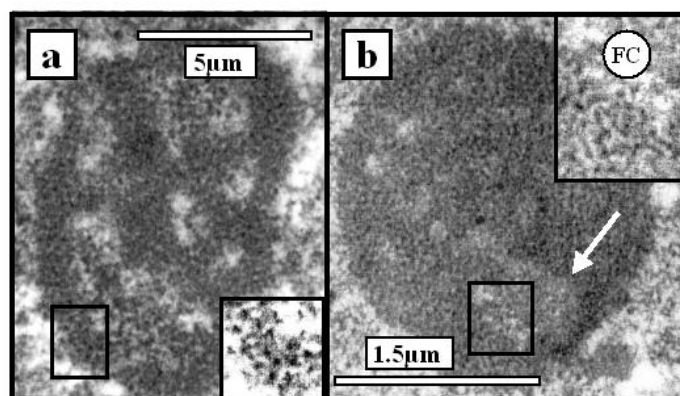
Fig. 1. **A nucleolus with distinct nucleolonemas (a) and a compact nucleolus (b).** Granular components (insert in the Fig. a) and fibrillar components at the fibrillar center (FC) in the insert of the Fig. b. Fibrillar center in the Fig. b – white arrow. The white bars represent 5 μm (Fig. a) and 1.5 μm (Fig. b), respectively.
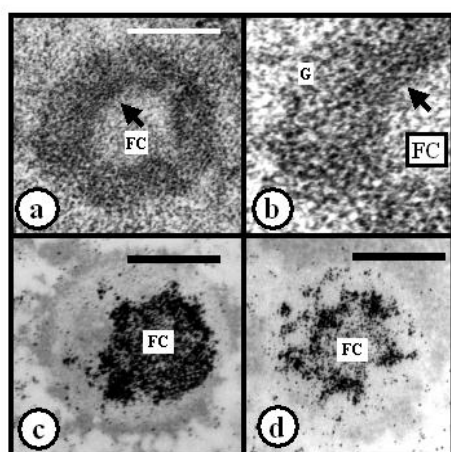

Fig. 2. **Ring shaped nucleoli after osmium fixation and uranyl acetate and lead citrate staining (a, b) or formaldehyde fixation and silver reaction (c, d).** FC – fibrillar center, note the presence of dense fibrillar components at fibrillar centers (a, b). Depending on conditions of the silver reaction the positivity is expressed in the fibrillar center and adjacent region (C) or mainly at the fibrillar center in the region of the ribosomal RNA transcription (d). The thick bars in the figures represent 5 μm.
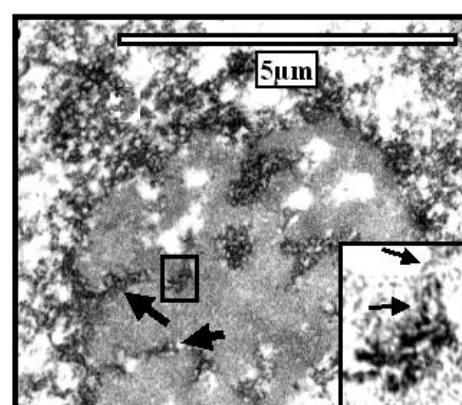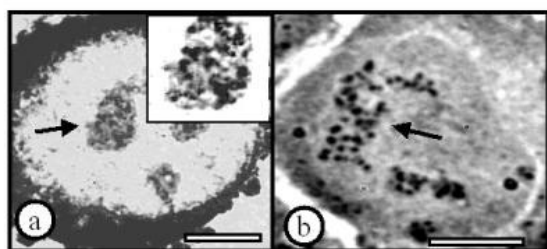

Fig. 3. **Chromatin structures of a nucleolus after RNA extraction are represented by clusters of filaments or filaments emerging from these clusters (insert).** Note the penetration of septa-like chromatin structures from the perinucleolar chromatin into the nucleolar body. The white bar represents 5 μm.

RNA transcription and processing as well as the assembly of pre-ribosomes are very complex events requiring the participation of numerous proteins and small nucleolar RNAs (Busch 1997, Olson et al. 2000).

Some of a large number of proteins are more known including their function in mentioned biosynthetic nucleolar activities. Fibrillar centers and fibrillar components mainly possess fibrillarin and nucleolin; nucleophosmin is characteristic for regions with granular components. RNA polymerase and UBF are stored in fibrillar centers but acting at their periphery or in surrounding regions with fibrillar components (Busch 1997, Ochs 1998). It seems to be also likely that these proteins may translocate within nucleoli depending on their functional involvement in the nucleolar biosynthetic activity. In addition, these proteins are useful markers of nucleolar compartments at the light microscopic level using immunocyto-chemistry. The association of fibrillarin with small nucleolar U3 RNA in the pre-ribosomal RNA process must be also mentioned (Busch 1997).

It must be mentioned that fibrillar centers correspond to silver stained particles in the light microscopy and represent "silver stained nucleolus organizer regions" (Fig. 4). The affinity of fibrillar centers and adjacent regions to the silver in the specific reaction is due to the presence of nucleolar main proteins such as nucleolin and nucleophosmin (Lischve et al. 1979). These and other, silver stained proteins such as RNA polymerase I, upstream binding factor and RNA helicase participate in the ribosomal RNA transcription and processing (Busch 1997). However, it must be mentioned that the silver affinity of proteins and various nucleolar regions depends on the conditions for the silver reaction (Fig. 2, Smetana et al. 1998).

The number of selectively visualized silver stained nucleolus organizer regions counted in the light microscope reflects the state of the rRNA transcription and cell proliferation as well a progress of the cell cycle (Fig. 4, Sirri et al. 2000, Derenzini and Trere 2001). However, the nucleolar transcriptional activities need not to correlate with the total number of ribosomal RNA genes (Haaf et al. 1991).

Fig. 4. **"Active" nucleoli with multiple fibrillar centers, which are unstained in specimens stained for RNA (a), but exhibit a strong positivity after silver reaction (b).** Thick white bars represent 5 μm.

Fig 5. **The translocation of fibrillar centers – silver stained nucleolus organizer regions to the nucleolar periphery in an aging cell.** Silver reaction. The thick black bar represents 5 μm.

The decreased number of silver stained nucleolar regions accompanied by their translocation to the nucleolar periphery was produced in aging and starving cells (Fig. 5, Smetana et al. 2006b). It should be also mentioned that such translocation appeared to be reversible (Smetana et al. 2005). In addition, the increased translocation of silver stained nucleolar organizers was observed leukemic early granulocytic precursors of patients treated with targeted cytostatic therapy and possibly was not followed by a further differentiation of these cells (Smetana et al. 2009).

Nucleolin and nucleophosmin, participating in the RNA processing and assembly of pre-ribosomes, bind silver under defined conditions of the silver reaction, but in different nucleolar regions, which possess fibrillar or granular RNA containing components (Wachtler and Stahl 1993, Ochs 1998).

It has been also shown that both these silver stained proteins as well as other nucleolar proteins translocate from altered nucleoli to the extranucleolar nuclear regions and may play an important role in p53 protein activation (Boulon et al. 2010, Tsuchiya et al. 2011). The translocation of nucleophosmin from the nucleolus to the extranucleolar nuclear regions produced by the RNA transcription inhibitors may indicate the cell sensitivity to such treatment (Chan and Chan 1999). In addition, nucleophosmin acts an apoptotic regulator, "shuttle" and nucleolar localization signal protein for the transport of a variety of proteins to the nucleolus (Busch 1997, Ye 2005).

NUCLEOLAR COMPARTMENTALIZATION

In respect of the present knowledge on the nucleolar compartmentalization (Dundr and Raška 1993), the above-mentioned compartments such as fibrillar centers, dense fibrillar and granular components are related to the ribosomal RNA transcription and processing. However, further studies added to them a rediscovered functional compartment – the perinucleolar chromatin region – related to the transcription of messenger and transfer RNA's, which are subjects of further studies (Pederson 1998).

In earlier ultrastructural studies the proportion of the perinucleolar chromatin was smaller in proliferating and larger in quiescent cells (Unuma et al. 1967). The presence of coiled bodies within nucleoli (Ochs et al. 1994) was also surprising although the mutual relationship of these both nuclear compartments were know for a long time, but started to be discussed because of functional interaction (Scheer and Hock 1999). Such interaction suggests
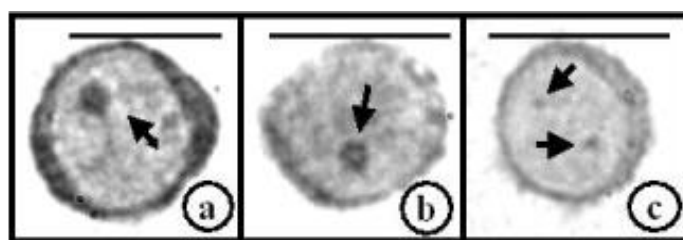
Fig. 6. **Main nucleolar types in immature (a), mature – resting (b) and terminal pre-apoptotic cell (c).** Note that the immature cell contains a large nucleolus with a relatively uniform distribution of RNA (a), mature resting cells is characterized by a ring shaped nucleolus (b) and terminal pre-apoptotic cell by a micronucleolus (c). Nucleoli – arrows. Thick black bars represent 10 μm.

the transfer of transcription and processing factors to the nucleolus. In contrary, some experimental observations indicated that such transfer is not essential for nucleolar biosynthetic activities since in some cells coiled bodies are absent (Olson et al. 2000).

On the other hand, only the sole fact of the nucleolar ribosomal RNA transcription and ribosomal assembly indicates the nucleolar direct participation in cell differentiation, maturation, aging and death as well as various cell functional activities because all these cell states depend on the cytoplasmic ribosomal translation machinery. Thus nucleoli appeared to be simple and convenient markers of mentioned cell activities. In past years, nucleoli, i.e., the nucleolar size and structural organization were used for the cell state evaluation (Smetana 2003).

NUCLEOLAR CLASSIFICATION

The nucleolar classification based on the nucleolar size and distribution of nucleolar components seemed to be very useful for such evaluation (Bernhard and Granboulan 1963, Busch and Smetana 1970, Smetana and Busch 1974, Schwarzacher and Wachtler 1983, Smetana 2003).

Large nucleoli with a relatively uniform RNA were usually present in proliferating cells (Fig. 6). Such nucleoli are very active in respect of RNA transcription. Smaller dominant ring shaped nucleoli with RNA present only in the nucleolar peripheral part (Fig. 6) are characteristic for resting cells, which, however, may return to the proliferation and cycling state again.

Ring shaped nucleoli are characterized by a reversible decrease of the RNA transcription. Micronucleoli (Fig. 6), the size of which is smaller than 1 μm, are mostly in cell terminal maturation or pre-apoptotic states (Smetana 2005). Such nucleoli

are characterized by the irreversible cessation of RNA transcription and may participate in the rearrangement of nuclear ribonucleoprotein structures during the apoptotic process (Biggiogera et al. 2004).

Micronucleoli must be distinguished from pronucleoli – prenucleolar bodies – at the end of the mitotic division. Pronucleoli are mostly in the proximity or at mitotic chromosomes and, in contrary to micronucleoli, they are characterized by the onset of the RNA transcription.

It should be also mentioned that some cell might possess several nucleolar types in one and the same nucleus. It is natural that in cells containing " active" large nucleoli, and other nucleolar types such as ring shaped nucleoli and micronucleoli in one nucleus, the former are functionally dominant. Simultaneously, "resting" ring shaped nucleoli are dominant in cells that in addition contain "inactive" micronucleoli. It seems to be interesting that cells exhibiting the nucleolar asynchrony, i.e. cells containing large nucleoli and ring shaped nucleoli in one and the same nucleus are less sensitive to the cytotoxic and cytostatic treatments (Smetana 2002, 2003).

At present, on the base of the present knowledge, the above reported classification of nucleoli should be slightly modified. To the class of large "active nucleoli" should be added that such nucleoli should contain small multiple fibrillar centers, which in nucleoli stained for RNA, appear as small unstained areas and after silver reactions as small silver stained particles (Fig. 3).

It seems to be likely that in fully active large nucleoli each fibrillar center possibly correspond to the single transcription unit (Ochs and Smetana 1989). In contrary, large nucleoli after inhibition of the RNA transcription are without fibrillar centers, i.e. they look very compact with decreased number or without silver stained particles (Smetana et al. 2008). Similar compact nucleolar appearance with reduced number or without fibrillar centers was also seen in the electron microscope (Smetana et al. 2004).

BIOMEDICAL CONTEXT

In addition, certain cytostatic drugs, antibiotics, infectious agents and UV or X radiation produce in large nucleoli nucleolar segregation. It seems to be likely that this phenomenon is produced by inhibition of the RNA transcription and DNA damage due to various mechanisms including the alteration of RNA polymerase system (Busch and Smetana 1970, Smetana and Busch 1974, Boulon et al. 2010). In addition to RNA transcription inhibitors such as cytostatic drug or antibiotics, the nucleolar segregation was produced by UV as well as X radiation and some infectious agents (Busch and Smetana 1970, Boulon et al. 2010).

Recent study suggested that the nucleolar segregation might represent a reaction to the cellular stress (Boulon et al. 2010). However, the nucleolar segregation was also observed in some maturing or neoplastic cells (Busch and Smetana 1970, Smetana and Busch 1974). On the other hand, the possibility cannot be eliminated that malignant cells exhibiting nucleolar segregation were noted in patients previously treated with the cytostatic therapy.

The phenomenon of the nucleolar segregation easily visible by electron microscopy is expressed by a distinct separation of main nucleolar components to defined areas containing only one of them. The formation of RNA containing nucleolar caps may be also observed in the light microscope using sensitive methods for RNA and DNA visualization.

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