PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 62 (2014), No 4
doi:10.3409/fb62_4.293

Age-Dependent Change in the Morphology of Nucleoli and Methylation of Genes of the Nucleolar Organizer Region in Japanese Quail (*Coturnix japonica*) Model (Temminck and Schlegel, 1849) (Galliformes: Aves)

Katarzyna ANDRASZEK, Magdalena GRYZIŃSKA, Ewa WÓJCIK, Sebastian KNAGA, and Elżbieta SMALEC

Accepted October 02, 2014

ANDRASZEK K., GRYZIŃSKA M., WÓJCIK E., KNAGA S., SMALEC E. 2014. Age-dependent change in the morphology of nucleoli and methylation of genes of the nucleolar organizer region in Japanese quail (*Coturnix japonica*) model (Temminck and Schlegel, 1849) (Galliformes: Aves). Folia Biologica (Kraków) **62**: 293-300.

Nucleoli are the product of the activity of nucleolar organizer regions (NOR) in certain chromosomes. Their main functions are the formation of ribosomal subunits from ribosomal protein molecules and the transcription of genes encoding rRNA. The aim of the study was to determine the shape of nucleoli and analyse methylation in the gene RN28S in the spermatocytes of male Japanese quail (*Coturnix japonica*) in two age groups. Nucleoli were analysed in cells of the first meiotic prophase. Their number and shape were determined and they were classified as regular, irregular or defragmented. In the cells of the young birds no defragmented nucleoli were observed, with regular and irregular nucleoli accounting for 97% and 3%, respectively. In the cells of older birds no regular nucleoli were observed, while irregular and defragmented nucleoli accounted for 37% and 67%, respectively. MSP (methylation-specific PCR) showed that the gene *RN28S* is methylated in both 15-week-old and 52-week-old quails. In recent years an association has been established between nucleolus morphology and cellular ageing processes.

Key words: Meiotic chromosomes, spermatogenesis, nucleoli, DNA methylation, epigenetics, MSP (methylation-specific PCR), *RN28S* gene.

Katarzyna ANDRASZEK, Ewa WÓJCIK, Elżbieta SMALEC, Institute of Bioengineering and Animal Breeding, Siedlce University of Natural Sciences and Humanities, Prusa 14, 08-110 Siedlce, Poland.
E-mai: andrasz@uph.edu.pl
Magdalena GRYZIŃSKA, Sebastian KNAGA, Department of Biological Basis of Animal Production, University of Life Sciences, Akademicka 13, 20-950 Lublin, Poland.
E-mail: magdalena.gryzinska@up.lublin.pl

For years animals have served man not only as companions and a source of food, but also as models in medical and genetic research. Laboratory animals have most often been mammals, but since the mapping of the genome of *Gallus domesticus* (Linnaeus, 1758) increasing attention has been devoted to birds as model organisms. The publication of the complete genome sequence of the chicken revealed syntenic regions between the human and avian genome, indicating that despite 300 million years of evolutionary separation of mammals and birds, many DNA sequences have remained conserved. For years the genome of *Gallus domesticus* was the only avian genome to be mapped, and it has been used as a model in comparative genetics for at least two reasons. First, the genome is relatively small, one-third of the size of the human genome, yet it has orthologues to most of the human genes (GREGORY 2002; ICGSC 2004; RUBIN *et al.* 2010).

Gallus domesticus is used as a model organism in biomedical and evolutionary research, comparative genomics, and epigenetic research. It is also the basic model in studies of embryology and development and in research on viruses and cancer. Another species of bird which is increasingly serving as a model species, replacing *Gallus domesticus*, is the Japanese quail (*Coturnix japonica*), for which the mitochondrial genome (NISHIBORI *et al.* 2002) and nuclear genome (FRÉSARD *et al.* 2012) have been sequenced. Quails are rapidly-maturing organisms with rapid turnover of generations. They can be used in cytogenetic, molecular and epigenetic research on ageing processes and

changes in cell structures taking place due to ageing. Moreover, like chickens they are excellent models for studies on age-dependent chromatin stability (WÓJCIK *et al.* 2012, 2013).

Nucleoli are the product of the activity of nucleolar organizer regions (NOR) in chromosomes. Their main function is to form the large and small ribosomal subunits from ribosomal molecules of specific proteins. Moreover, transcription of genes encoding rRNA takes place in the nucleoli (SHAW & JORDAN 1995; SCHEER & HOCK 1999; RAŠKA *et al.* 2004; HERNANDEZ-VERDUN 2006). The shape of nucleoli is an indicator of the transcriptional activity of rRNA genes (OLSON 2004). Nucleoli in young cells are regularly shaped, while in the ageing cell their shape becomes irregular (SHAW & JORDAN 1995; KŁYSZEJKO-STEFANOWICZ 2002). The shape of nucleoli is also affected by physiological or pathological states in the organism (OLSON *et al.* 2002; DERENZINI *et al.* 2005; HERNANDEZ-VERDUN 2006). Nucleoli are present in the nuclei of nearly all eukaryotic cells because they contain housekeeping genes, with the exception of sperm cells and mature erythrocytes in all vertebrates (KŁYSZEJKO-STEFANOWICZ 2002; RAŠKA *et al*. 2006). The mass of nucleoli is greater than that of the nucleoplasm in which they are suspended. Owing to their characteristic density, compact structure and low water content (10%), the nucleolus is an organelle that can easily be isolated. Following fragmentation of the cell nucleus, the nucleolus remains preserved in saline solution even after most of the structures of the nucleus have been destroyed, and it can be isolated after centrifugation. The isolated nucleolus is the same as in the nucleus of a live cell, and in some cases even retains its transcriptional activity (OLSON 2004; HERNANDEZ-VERDUN 2006; RAŠKA *et al.* 2006). The size of the nucleolus in vertebrates ranges from 1 to 5 μm. It varies depending on the cell's demand for ribosomes. Cells that do not carry out intensive protein synthesis, e.g. skeletal muscle cells, have small nucleoli, while liver cells, for example, which are characterized by a high level of protein synthesis, have larger nucleoli (SHAW & JORDAN 1995).

Using methods of molecular biology it is possible to analyse methylation of the entire genome and of individual genes. These methods are classified according to whether 5-methylcytosine is determined quantitatively or qualitatively. The MSP (methylation-specific PCR) technique used in the present study is a qualitative technique. It is based on the reaction of sodium hydrogen with nucleic acid (COLLEMAN & RIVENBARK 2006; HERMAN *et al.* 1996), deaminating cytosine to uracil in single-stranded DNA, whereas 5-methyl cytosine is unaffected (AZHIKINA & SVERDLOV 2005).

Modified DNA serves as a template in the MSP reaction. DNA amplification is carried out using two primer pairs designed to differentiate methylated and unmethylated bases in DNA. The first primer pair (methylated specific) recognizes 5-methylcytosine as cytosine and thus is complementary to and binds with the unconverted DNA fragment. The second primer pair (unmethylated specific) is complementary to the sequence in which cytosine has been converted into uracil. The advantages of this method are the short time required for the analysis, the possibility of obtaining results from small quantities of DNA, specificity, and sensitivity (methylation can be detected even when 0.1% of alleles are methylated) (GRYZIŃSKA *et al.* 2013a, 2013b, 2014).

Epigenetic mechanisms acting at the level of DNA methylation affect the morphology of nucleoli. Genes encoding rRNA occur in two forms. Their 'open' conformation is transcriptionally active, while their 'closed' conformation is transcriptionally refractive. Entire NOR regions or only certain genes may be silenced. This is directly linked to the progressing methylation process associated with the age of the organism (WEISENBERGER & SCHEER 1995; GRUMMT & PIKAARD 2003; RAŠKA *et al.* 2006).

The aim of the study was to determine the shape of nucleoli and analyse DNA methylation in the gene RNA28S in the spermatocytes of male Japanese quail (*Coturnix japonica*) belonging to two age groups, using MSP (methylation-specific PCR) and BSSCP (bisulphite single-strand conformation polymorphism).

Material and Methods

The study was conducted on male Japanese quails, *Coturnix japonica* (Temminck and Schlegel, 1849) in two age groups, 15 and 52 weeks. Each group was represented by 20 individuals. The birds came from flocks belonging to the Department of Biological Basis of Animal Production of the University of Life Sciences in Lublin. The material was collected in accordance with the 2nd Lublin Local Ethical Commission for Animal Experiments Resolution 8/11 of 15/03/2011.

Identification of nucleoli

Meiotic chromosomes were isolated using the POLLOCK & FECHHEIMER (1978) method. The nucleoli were identified by means of nucleolar organiser region silver staining in mitotic chromosomes (HOWELL & BLACK 1980). A 50% $AgNO_3$ solution and a colloidal gelatine solution were applied to 1-week-old preparations. The prepara-

tions were covered with a cover glass and incubated for 15-20 minutes at 60°C, in complete humidity. After the preparations turned brown in colour, the chemical reaction was interrupted and the preparations were rinsed several times with distilled water. The preparations were analysed with an OLYMPUS BX 50 microscope, using the Multiscan system. For each animal, 50 plates with chromosomes in the first stage of the meiotic prophase were analysed. The number and size of nucleoli in the cell were identified and the mean and standard error were calculated. The shape of the nucleoli at different ages was evaluated using the Mann-Whitney U test ($P \leq 0.05$).

Methylation analysis

DNA was isolated from the cell suspension used for isolation and identification of nucleoli with the Genomic Midi AX kit (A&A Biotechnology). Cytosine was converted to uracil with sodium hydrogen sulfate using an Imprint® DNA Modification Kit (Sigma-AldrichTM). The primers used for the reaction were designed so that one pair would bind at the site of methylation where methylcytosine remained as cytosine and the other pair at the point where unmethylated cytosine deaminated to uracil. The primers (Table 1) were designed using the software MethPrimer®, based on a sequence of the gene RN28S encoding 28S ribosomal RNA (*Gallus gallus*) (NCBI, ID 100861559).

The melting temperature for the primers was determined using the software Tm Calculator (Applied Biosystems). The reactions (25 μl total volume) contained 5 μl of DNA after conversion and 1.0 U Taq polymerase (Ampli Taq Gold 360 DNA Polymerase, Applied Biosystems) in the manufacturer's buffer, adjusted to a final concentration of 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 0.1 mM of each primer. A Thermal Cycler (MJ Research) was programmed for an initial incubation at 94°C for 5 min, 35 cycles each with denaturing at 94°C for 30 sec, annealing at 55°C/56°C for 30 sec, extension at 72°C for 30 sec and a final cycle at 72°C for 5 min.

Methylation analysis was performed using MSP (methylation-specific PCR) and BSSCP (bisulphite single-strand conformation polymorphism). In the PCR the amplification products were size-separated by electrophoresis in 1.8 % agarose gel and visualized by ultraviolet illumination after staining with ethidium bromide. In BSSCP, the amplification product (single-stranded DNA) was separated in a non-denaturing 8 % polyacrylamide gel. Bands were visualized using a DNA Silver Stain kit without access to light.

Results

Table 2 presents the results of the analysis of the shape of the nucleoli in two age groups of quails. The percentage of nucleoli of each shape varied depending on the age of the birds. A comparison of the shape of the nucleoli at different ages, evaluated using the Mann-Whitney U test, is presented in Table 3.

In the cells of 15-week-old quails, mainly nucleoli with a regular, oval shape were observed. Over 96% of the total 1128 nucleoli were regularly shaped. The average number of regular nucleoli in the 15-week-old quails was 54.5 (±3.65). Of the total 1128 nucleoli in this age group 38 irregular nucleoli were identified – on average 1.9 (±1.86). Irregular nucleoli accounted for slightly over 3% of all nucleoli, while no defragmented nucleoli were observed in this group. In the cells of the 52-week-old quails no regular nucleoli were observed. Irregular nucleoli accounted for over 37% (415 nucleoli) of all the 1110 nucleoli identified. The average number of irregular nucleoli in the 52-week-old quails was 20.75 (±3.86). In this age

Table 1

Description of primers for MSP

No	Primer	Sequence 5' → 3'	Product size	Tm	%GC
1	28RRNA_LM	TAAGCGATTTCGGAGAAGTC	91 bp	55.70	45
2	28RRNA_RM	CTCGAAACGAACCCGTT		55.50	53
3	28RRNA1_LU	TAAGTGATTTCGGAGAAGTCGG	94 bp	59.19	45
4	28RRNA_RU	TCTCTCAAAACAAACCCATT		53.24	35

Tm – melting temperature
28RRNA_LM - complementary primer for the leading strand at the methylation site;
28RRNA_RM - complementary primer for the delayed strand at the methylation site;
28RRNA1_LU - complementary primer for the leading strand at the non-methylated site;
28RRNA_RU - complementary primer for the delayed strand at the non-methylated site.

Table 2

Number and shape of nucleoli depending on the age of the quail

Quail	Age of quail (weeks)							
	15				52			
	regular	irregular	fragmented	total	regular	irregular	fragmented	total
1	51	4	0	55	0	21	34	55
2	54	0	0	54	0	23	31	54
3	47	5	0	52	0	19	35	54
4	53	1	0	54	0	23	32	55
5	58	4	0	62	0	26	31	57
6	63	0	0	63	0	24	37	61
7	58	0	0	58	0	18	40	58
8	55	2	0	57	0	19	38	57
9	55	4	0	59	0	26	34	60
10	52	4	0	56	0	29	29	58
11	54	0	0	54	0	18	33	51
12	53	0	0	53	0	17	35	52
13	54	1	0	55	0	17	38	55
14	52	5	0	57	0	13	44	57
15	59	2	0	61	0	24	35	59
16	55	0	0	55	0	21	33	54
17	56	1	0	57	0	19	33	52
18	59	0	0	59	0	22	30	52
19	52	2	0	54	0	18	38	56
20	50	3	0	53	0	18	35	53
Total	1090	38	0	1128	0	415	695	1110
$\bar{x}$	54.5	1.9	0	56.4	0	20.75	34.75	55.5
SD±	3.65	1.86	0.00	3.12	0.00	3.86	3.64	2.84
%	96.63	3.37	0	100	0	37.39	62.61	100

Table 3

Comparison of the shape of nucleoli at different ages (Mann-Whitney U test)

Shape	Z value	P
regular	5.46*	0.000
irregular	5.033*	0.000
fragmented	5.203*	0.000

* significant at $P \leq 0.05$

group defragmented nucleoli constituted the largest proportion, accounting for 62% – 695 nucleoli, on average 34.75 (±3.64). In the present study nucleoli of different shapes were identified in primary spermatocytes of quails of different ages. From 1 to 2 nucleoli were observed in the cell. The nucleoli were classified as regular (Fig. 1), irregular (Fig. 2), and defragmented (Fig. 3).

The MSP reaction showed that the gene *RN28S* is methylated in both 15-week-old and 52-week-old quails. In the electrophoresis gel (Fig. 4) only MSP products indicating the presence of methylated *RN28S* are visible in the case of both age groups. BSSCP confirmed the result obtained in MSP. The analysis showed that the 28S rRNA gene is methylated and silenced in both 15- and 52-week-old quails. Conclusions regarding differences in the methylation level of the gene RN28S were based on the intensity of the band in the electrophoresis gel (Fig. 4). The more intense band in the 15-week-old quails indicates a higher level of methylation.

The morphology of the nucleolus depends on the organism. The nucleoli have a regular shape in the

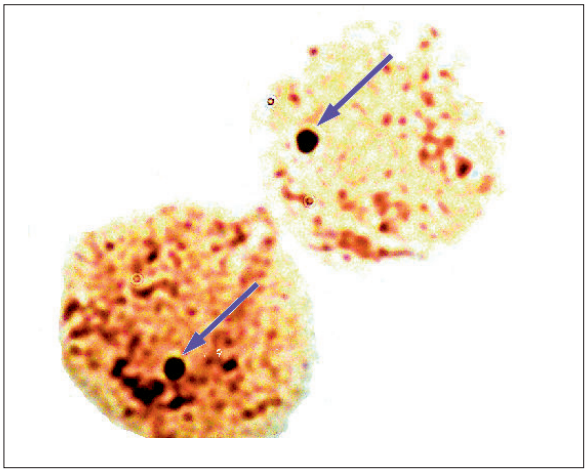

Fig. 1. Cell nuclei of first-order spermatocytes in a 15-week-old quail. Arrows indicate regularly shaped nucleoli.

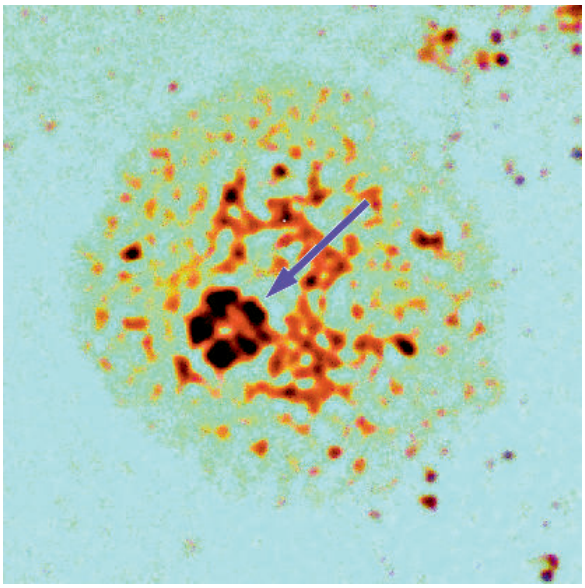

Fig. 2. Cell nucleus of a first-order spermatocyte in a 52-week-old quail. The arrow indicates an irregularly shaped nucleolus.

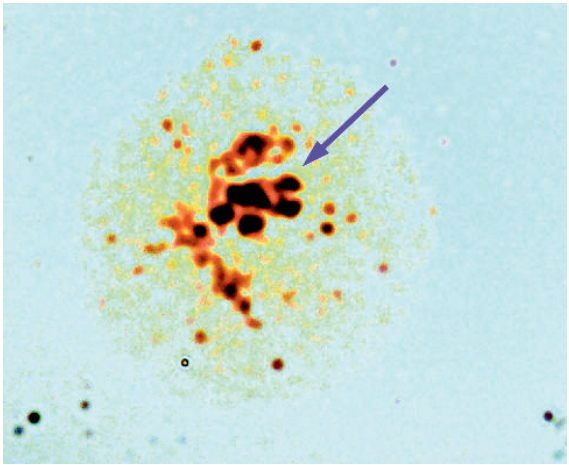

Fig. 3. Cell nucleus of a first-order spermatocyte in a 52-week-old quail. The arrow indicates a defragmented nucleolus.

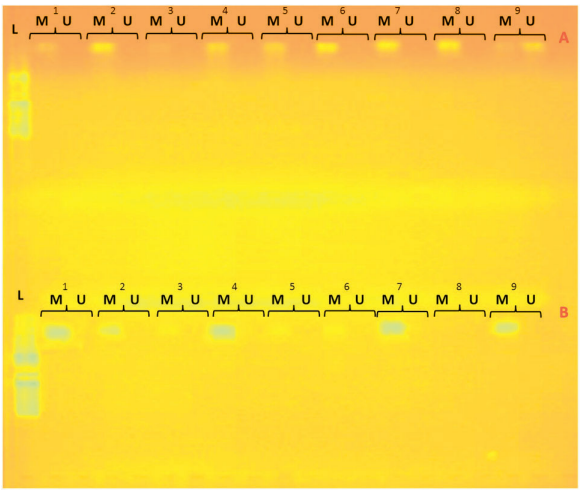

Fig. 4. Results of the MSP reaction for the RN28S gene in (A) 15- and (B) 52-week-old quails. L – Ladder (100 bp ladder); M – methylated cytosine; U – unmethylated cytosine; 1-9 – the number of the individual; top row (A) –15 week-old quails; bottom row (B) – 52 week-old quails

cells of young individuals. Ageing causes a change in the shape of the nucleolus; it takes on an irregular shape. The gene *RN28S* undergoes methylation in both young and old individuals. A higher level of methylation was observed in the older birds. Because RN genes occur in open and closed conformation, at this stage of research it cannot be definitively stated whether methylation of these genes is associated only with age or with the cell cycle as well. Analysis of methylation of all genes located in the nucleolar organizer region (NOR) is needed.

Discussion

A standard technique for isolating meiotic chromosomes (POLLOCK & FECHHEIMER 1978) in combination with a modified procedure for identifying nucleolar organizing regions (HOWELL & BLACK 1980) could be used successfully to isolate, observe and identify nucleoli in spermatocytes during the first meiotic prophase (ANDRASZEK & SMALEC 2007; ANDRASZEK *et al.* 2009; ANDRASZEK & SMALEC 2012; ANDRASZEK *et al.* 2012).

The number of nucleoli in a normal cell nucleus is equal to or less than the number of nucleolar organizer regions of mitotic chromosomes. Nucleoli lying close to each other may become fused together (RAŠKA *et al*. 2006), or satellite chromosomes may become associated with active NOR (ANDRASZEK *et al*. 2009). Moreover, genes located in different NOR-chromosomes are involved in

the formation of one nucleolus. Literature data show that at most four active NOR, associated with one pair of macrochromosomes and one pair of microchromosomes, are present in the quail karyotype (MCNALLY 2004). The maximum number of nucleoli identified in the present study was two, confirming that the number of nucleoli is equal to or less than the number of active NOR.

Variation in the number and size of nucleoli in the cell, both at the individual and species levels, is unquestionably influenced by changes in the activity of nucleolar organizing regions. Cytogenetic studies indicate that polymorphism of the number and size of NOR can occur within a species. This variability may be determined by breed or individuals. Furthermore, it may be influenced by the production purpose, age and physiological state of the animal (RAO & MOHANTY 2005; RAŠKA *et al.* 2006; ANDRASZEK *et al.* 2010).

In the present study methylation of the gene *RN28S* was analysed by MSP in quails belonging to two age groups. The gene was observed to have been methylated in both age groups. BSSCP confirmed the result obtained in MSP. The 28S rRNA gene in these developmental periods is silenced, indicating that gene methylation may be associated not only with age but with the cell cycle. Analysis of all genes located in the NOR is needed to confirm the relationship between methylation and lack of expression and an association with the cell cycle.

The relationship between age and increased methylation in the 18S and 28S rRNA genes has been studied in mice. The results showed that the methylation process of these genes began between the 6th and 8th month of life in the brain, liver and spleen of the individuals (SWISSHELM *et al.* 1990). Another study analysed the number of nucleolar organizing regions stained with silver depending on age in skin fibroblasts from people in two age groups. The results showed that the average number of Ag-NOR per cell decreased substantially with the age of the individual (THOMAS & MUKHERJEE 1996). In another study the quantitative method RT-PCR was used to analyse the expression of six housekeeping genes, including the 18S rRNA gene in the NOR, in blood samples belonging to 8 groups of healthy volunteers aged 35-74 years. Reduced expression of the 18S rRNA gene was observed in individuals aged 70-74 in comparison with the other groups, which was linked to hypermethylation progressing with age (ZAMPIERI *et al*. 2010). This result is consistent with a study that analysed housekeeping genes during brain maturation. A low level of expression of the 18S rRNA gene was also observed in the brain of adult mice in comparison with young individuals (BODA *et al.* 2009). In the case of rRNA genes, further analysis is needed to precisely determine the level of methylation of these genes depending on age. It is known that NOR activity is not identical for all NOR-chromosomes. The number of stained NOR in mitosis is believed to be dependent on the activity of transcriptional rDNA. It is assumed that stained NOR include those in which transcription will take place in the next cell cycle; not all nucleolar organizing regions are active at the time of staining and not all potential NOR are stained at the same time (OLSON 2004; RAŠKA *et al.* 2006). This selective activity of genes may be determined by epigenetic mechanisms.

In addition to its direct association with ribosome biogenesis, the nucleolus is also associated with or involved in other cell processes such as ageing, cancerogenesis, regulation of telomerase function and stabilization of the protein p53 (PEDERSON 1998; SANTORO & GRUMMT 2001; OLSON *et al.* 2002; GERBI *et al.* 2003; RAŠKA *et al.* 2006). There is also a direct relationship between the activity of nucleolar organizer regions and cell division (DERENZINI 1990; RAŠKA *et al.* 2006). It is currently uncertain whether these unconventional roles are the main functions of the nucleolus or adaptations in individual species or groups of species.

The morphology of the nucleolus has been one of the key factors used in cancer staging. The morphometric parameters of nucleoli are number, size and distance from the nuclear membrane (NAFE & SCHLOTE 2004; SMETANA *et al.* 2005, 2006; RAŠKA *et al.* 2006). Taking into account previous observations by MONTGOMERY (1895), biologists researching tumours quickly created a link between stained AgNOR and cell proliferation (DERENZINI 1990; RAŠKA *et al.* 2006). In cancer cells nucleoli are of the compact type. Nucleoli of this type are characterized by a dense granular component filling their interior. Their dimensions are not uniform, owing to several fibrous centres. Because rRNA transcription proceeds faster in them, the nucleoli are much larger than properly functioning nucleoli of normal structure (HORKÝ *et al.* 2002).

Numerous studies have been published concerning the promotion and suppression of forming tumours by mechanisms based on cytogenetic diagnosis of nucleoli. The c-Myc proteins (the product of the protooncogene c-*myc*) are localized in the nucleolus and control synthesis of rRNA (OSKARSSON & TRUMPP 2005; RAŠKA *et al.* 2006). It has also been demonstrated that c-Myc is capable of regulating the activity of all three polymerases in mammalian cells and coordinating ribosome synthesis and cell growth (ARABI *et al.* 2005). These observations indicate that c-Myc plays a key role in the development of tumour-promoting activity by regulating ribosome biogenesis.

The proteins pRb (retinoblastoma protein) and p53 play an important role in controlling the progress of the cell cycle and also ensure normal cell growth in daughter cells. These are onco-suppressor proteins, concentrated in the nucleolus (RYAN *et al.* 2001; TRERE *et al.* 2004). The gene encoding ARF/p16INK4a is the second most frequently occurring inactive gene of tumours in humans (RUAS & PETERS 1998). ARF is located near the nucleolus, where it interacts with p53 (KASHUBA *et al.* 2003). A direct functional link between the nucleolus and p53 has been established as well (RYAN *et al.* 2001; OLSON 2004; RUBBI & MILNER 2003). Most stress-inducing treatments that activate p53 also lead to the breakdown of the structure of the nucleolus.

A substantial proportion of somatic cells do not have active telomerase, which limits the number of cell division cycles, suggesting that this is a factor in ageing and cancerogenesis (MASER & DEPINHO 2002; KHURTS *et al*. 2004). Telomerase has been found to be present in the nucleolus. Its function is associated with regulation of nucleolus activity (TEIXEIRA *et al.* 2002). hTERT, i.e. reverse transcriptase of the catalytic subunit of human telomerase, is also located in the nucleolus (MITCHELL & COLLINS 2000; KHURTS *et al.* 2004). Evidence supporting a functional relationship between telomerase and nucleoli was provided by a study on human cell lines (WONG *et al.* 2002). In tumours and transformed cell lines hTERT was found to be inactive in the nucleolus (WONG *et al.* 2002). The nucleolar phosphoprotein nucleolin interacts with hTERT, and this interaction is also dependent on RNA telomerase. The interaction is probably involved in the dynamic localization of telomerase. Telomere binding factor TRF2 is associated with the nucleolus during the entire cell cycle (KHURTS *et al.* 2004; RAŠKA *et al*. 2006).

In recent years a link has been established between nucleolar morphology and cellular ageing processes. Research conducted on yeast cells has confirmed that damage to rRNA genes causes nucleoli to disintegrate, which is one of the symptoms of ageing in the organism.

A pilot study on the age-dependent structure of nucleoli in domestic horses confirmed that disintegration of nucleoli was associated with the age of the individuals studied (ANDRASZEK *et al.* 2010). The morphology of nucleoli is also influenced by epigenetic mechanisms acting at the level of DNA methylation.

Methylation is an extremely dynamic process that changes continually depending on the age of the organism. It is also characterized by specificity within particular tissues, organelles and species (VANYUSHIN 2005). BOCKLANDT *et al.* (2011) also found that the methylation level in individual genes substantially increased with the age of the organism. Cytosine methylation is a very good marker in research on age-dependent gene expression. Nucleoli can be successfully used in cytogenetic research on ageing processes and changes in cell structures taking place due to ageing in the organism.

References

ANDRASZEK K., SMALEC E. 2007. Number and size of nucleoli in the spermatocytes of European domestic goose (*Anser anser*). Arch. Geflügelk. **71**: 237-240.

ANDRASZEK K., SMALEC E. 2012. Structure of the nucleoli in domestic cattle spermatocytes. Folia Histochem. Cytobiol. **50**: 346-351.

ANDRASZEK K., HOROSZEWICZ E., SMALEC E. 2009. Nucleolar organizer regions, satellite associations and nucleoli of goat cells (*Capra hircus*). Arch. Tierz. **52**: 177-186.

ANDRASZEK K., DANIELEWICZ A., SMALEC E., KAPROŃ M. 2010. Identification of the nucleoli in domestic horse spermatocytes – preliminary investigations. Rocz. Nauk. PTZ **6**: 13-21.

ANDRASZEK K., GRYZIŃSKA M., KNAGA S., WÓJCIK E., SMALEC E. 2012. Number and size of nucleoli in the spermatocytes of chicken and Japanese quail. Folia Biol. (Kraków) **60**: 121-127.

ARABI A., WU S., RIDDERSTRALE K., BIERHO V.H., SHIUE C., FATYOL K., FAHLEN S., HYDBRING P., SODERBERG O., GRUMMT I., LARSON L.., WRIGHT A. P. 2005. C-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. Nat. Cell Biol. **7**: 1465-7392.

AZHIKINA T.L., SVERDLOV E.D. 2005. Study of tissue-specific CpG methylation of DNA in extended genomic loci. Biochemistry (Moscow) **70**: 596-603.

BOCKLANDT S., LIN W., SEHL M.E., SANCHEZ F.J., SINSHEIMER J.S., ORVATH S., VILAIN E. 2011. Epigenetic predictor of age. PLOS ONE **6**: 1-6.

BODA E., PINI A., HOXHA E. 2009. Selection of reference genes for quantitative Real-Time PCR studies in mouse brain. J. Mol. Neurosci. **37**: 238-253.

COLLEMAN W.B., RIVENBARK A.G. 2006. Quantitative DNA methylation analysis. J. Mol. Diagn. **8**: 152-156.

DERENZINI M., PASQUINELLI G., O'DONOHUE M.F., PLOTON D., THIRY M. 2005. Structural and functional organization of ribosomal genes within the mammalian cell nucleolus. J. Histochem. Cytochem. **54**: 131-145.

FRÉSARD L., LEROUX S., DEHAIS P., SERVIN B., GILBERT H., BOUCHEZ O., KLOPP C., CABAU C., VIGNOLES F., FEVE K., RICROS A., GOURICHON D., DIOT C., RICHARD S., LETERRIER C., BEAUMONT C,, VIGNAL A,, MINVIELLE F., PITEL F. 2012. Fine mapping of complex traits in non-model species: using next generation sequencing and advanced intercross lines in Japanese quail. BMC Genomics **13**, 551.

GERBI S.A., BOROVJAGIN A.V., LANGE T.S. 2003. The nucleolus: A site of ribonucleoprotein maturation. Curr. Opin. Cell Biol. 15: 318-325.

GREGORY T.R. 2002. A bird's-eye view of the C-value enigma: genome size, cell size, and metabolic rate in the class aves. Int. J. Org. Evol. **56**: 121-130.

GRUMMT I., PIKAARD C.S. 2003. Epigenetic silencing of RNA polymerase I transcription. Nature reviews. Mol. Cell Biol. **4**: 641-649.

GRYZIŃSKA M., ANDRASZEK K., JOCEK G. 2013a. DNA Methylation analysis of the gene *CDKN2B* in *Gallus gallus* (Chicken). Folia Biol. (Kraków) **61**: 165-171.

GRYZIŃSKA M., BŁASZCZAK E., STRACHECKA A., JEŻEWSKA-WITKOWSKA G. 2013b. Analysis of Age-Related Global DNA Methylation in Chicken. Biochem. Genet. **51**: 554-563.

GRYZIŃSKA M., ANDRASZEK K., JEŻEWSKA-WITKOWSKA G. 2014. Estimation of global content of 5-methylcytosine in DNA during allantoic and pulmonary respiration in the chicken embryo. Folia Biol. (Kraków) **62**: 97-101.

HERMAN J.G., GRAFF J.R., MYOHANEN S., NELKIN B.D., BAYLIN S.B. 1996. Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. PNAS **93**: 9821-9826.

HERNANDEZ-VERDUN D. 2006. Nucleolus: From structure to dynamics. Histochem. Cell Biol. **125**: 127-137.

HOWELL W.M., BLACK D.A. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer a 1-step method. Experentia **36**: 1014-1015.

HORKÝ M., KOTALA V., ANTON M., WESIERSKA-GADEK J. 2002. Nucleolus and Apoptosis. Ann. N. Y. Acad. Sci. **973**: 258-264.

ICGSC 2004. Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. Nature **432**: 695-716.

KASHUBA E., MATTSSON K., KLEIN G., SZEKELY L. 2003. p14ARF induces the relocation of HDM2 and p53 to extranucleolar sites that are targeted by PML bodies and proteasomes. Mol. Cancer **2,** 18.

KHURTS S., MASUTOMI K., DELGERMAA L., ARAI K., OISHI N., MIZUNO H., HAYASHI N., HAHN W.C., MURAKAMI S. 2004. Nucleolin interacts with telomerase. J. Biol. Chem. **279**: 51508-51515.

KŁYSZEJKO-STEFANOWICZ L. 2002. Cytobiochemia (third edition). PWN, Warsaw, Poland.

MASER R.S., DEPINHO R.A. 2002. Keeping telomerase in its place. Nat. Med. **8**: 934-936.

MCNALLY L.R. 2004. Chromosomal localization of a proinsulin transgene inserted with a transposon-based vector into japanese quail, Coturnix coturnix. PhD, Louisiana State University.

MITCHELL J.R., COLLINS K. 2000. Human telomerase activation requires two independent interactions between telomerase RNA and telomerase reverse transcriptase. Mol. Cell **6**: 361-371.

NAFE R., SCHLOTE W. 2004. Histomorphometry of brain tumours. Neuropathol. Appl. Neurobiol. **30**: 315-328.

NISHIBORI M., TSUDZUKI M., HAYASHI T., YAMAMOTO Y., YASUE H. 2002. Complete nucleotide sequence of the *Coturnix chinensis* (blue-breasted quail) mitochondrial genome and a phylogenetic analysis with related species. J. Hered. **93**: 439-344.

OLSON M.O., HINGORANI K., SZEBENI A. 2002. Conventional and nonconventional roles of the nucleolus. Int. Rev. Cytol. **219**: 199-266.

OLSON M.O. 2004. The nucleolus. Kluwer Academic Plenum Publisher, London.

OSKARSSON T., TRUMPP A. 2005. The Myc trilogy: Lord of RNA polymerases. Nat. Cell Biol. **7**: 215-217.

PEDERSON T. 1998. The plurifunctional nucleolus. Nucleic Acids Res. **26**: 3871-3876.

POLLOCK D.L., FECHHEIMER N.S. 1978. The chromosomes of cockerels (*Gallus domesticus*) during meiosis. Cytogenet. Cell Genet. **21**: 267-281.

RAO B.V., MOHANTY G.D. 2005. Nucleolar organising region evaluation using new NOR FISH probe. Indian J. Hum. Genet. **11**: 44-46.

RAŠKA I., KOBERNA K., MALINSK J., FIDLEROVA H., MASATA M. 2004. The nucleolus and transcription of ribosomal genes. Biol. Cell **96**: 579-94.

RAŠKA I., SHAW P.J., CMARKO D. 2006. New insights into nucleolar architecture and activity. Int. Rev. Cytol. **255**: 177-235.

RUAS M., PETERS G. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives., Biochim. Biophys. Acta 1378: F115-F177.

RUBBI C.P., MILNER J. 2003. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. EMBO J. **22**: 6068-6077.

RUBIN C.J., ZODY M.C., ERIKSSON J., MEADOWS J.R., SHERWOOD E., WEBSTER M.T., JIANG L., INGMAN M., SHARPE T., KA S., HALLBÖÖK F., BESNIER F., CARLBORG O., BED'HOM B., TIXIER-BOICHARD M., JENSEN P., SIEGEL P., LINDBLAD-TOH K., ANDERSSON L. 2010. Whole-genome resequencing reveals loci under selection during chicken domestication. Nature **464**: 587-591.

RYAN K.M., PHILIPS A.C., VOUSDEN K.H. 2001. Regulation and function of the p53 tumor suppressor protein. Curr. Opin. Cell Biol. **13**: 332-337.

SANTORO R., GRUMMT I. 2001. Molecular mechanisms mediating methylation dependent silencing of ribosomal gene transcription. Mol. Cell **8**: 719-725.

SCHEER U., HOCK R. 1999. Structure and function of the nucleolus. Curr. Opin. Cell Biol. **11**: 385-390.

SHAW P.J., JORDAN E.G. 1995. The nucleolus. Annu. Rev. Cell Dev. Biol. **11**: 93-121.

SMETANA K., KLAMOVA H., PLUSKALOVA M., STOCKBAUER P., HRKAL Z. 2006. To the intranucleolar translocation of AgNORs in leukemic early granulocytic and plasmacytic precursors. Histochem. Cell Biol. **125**: 165-170.

SMETANA K., KLAMOVA H., PLUSKALOVA M., STOCKBAUER P., JIRASKOVA I., HRKAL Z. 2005. Intranucleolar translocation of AgNORs in early granulocytic precursors in chronic myeloid leukaemia and K 562 cells. Folia Biol. (Praha) **51**: 89-92.

SWISSHELM K., DISTECHE C.M., THORVALDSEN J., NELSON A., SALK D. 1990. Age related increase in methylation of ribosomal genes and inactivation of chromosome specific rRNA gene clusters in mouse. Mutat. Res. **237**: 131-146.

TEIXEIRA M.T., FORSTEMANN K., GASSER S.M., LINGNER J. 2002. Intracellular tracking of yeast telomerase components. EMBO Rep. **3**: 652-659.

THOMAS S., MUKHERJEE A.B. 1996. A long itudeinal study of human age – related ribosomal RNA gene activity as detected by silver -stained NORs. Mech. Ageing Dev. **92**: 101-109.

TRERE D., CECCARELLI C., MONTANARO L., TOSTI E., DERENZINI M. 2004. Nucleolar size and activity are related to pRb and p53 status in human breast cancer. J. Histochem. Cytochem. **52**: 1601-1607.

VANYUSHIN B. F. 2005. Enzymatic DNA methylation is an epigenetic control for genetic functions of the cell. Biochemistry **70**: 488-499.

WEISENBERGER D., SCHEER U. 1995. A possible mechanism for the inhibition of ribosomal RNA gene transcription during mitosis. J. Cell Biol. **129**: 561-575.

WONG J. M., KUSDRA L., COLLINS K. 2002. Subnuclear shuttling of human telomerase induced by transformation and DNA damage. Nat. Cell Biol. **4**: 731-736.

WÓJCIK E., ANDRASZEK K., GRYZIŃSKA M., WITKOWSKI A., PAŁYSZKA M., SMALEC E. 2012. Sister chromatid exchange in Greenleg Partridge and Polbar hens covered by the genepool protection program for farm animals in Poland. Poult. Sci. **91**: 2424-2430.

WÓJCIK E., ANDRASZEK K., CISZEWSKa M., SMALEC E. 2013. Sister chromatid exchange as an index of chromosome instability in chondrodystrophic chickens (*Gallus domesticus*). Poultry Sci. **92**: 84-89.

ZAMPIERI M., CICCARONe F., GUASTAFIERRO T., BACALINI M. G., CALABRESE R., MORENO-VILLANUEVA M., REALE A., CHEVANNE M., BURKLE A., CAIAFA P. 2010.Validation of suitable internal control genes for expression studies in aging. Mech. Aging Dev. **131**: 89-95.