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Histochemistry for nucleic acid research: 60 years in the European Journal of Histochemistry

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ABSTRACT

Since the discovery of DNA structure in 1953, the deoxyribonucleic acid has always been playing a central role in biological research. As physical and ordered nucleotides sequence, it stands at the base of genes existence. Furthermore, beside this 2-dimensional sequence, DNA is characterized by a 3D structural and functional organization, which is of interest for the scientific community due to multiple levels of expression regulation, of interaction with other biomolecules, and much more. Analogously, the nucleic acid counterpart of DNA, RNA, represents a central issue in research, because of its fundamental role in gene expression and regulation, and for the DNA-RNA interplay. Because of their importance, DNA and RNA have always been mentioned and studied in several publications, and the European Journal of Histochemistry is no exception. Here, we review and discuss the papers published in the last 60 years of this Journal, focusing on its contribution in deepening the knowledge about this topic and analysing papers that reflect the interest this Journal always granted to the world of DNA and RNA.

Key words: Cell nucleus, nucleic acids, chromatin, RNA processing, electron microscopy, immunohistochemistry, flow cytometry.

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A first gaze on DNA: chromatin

Founded one year after Watson and Crick core publication, "Rivista di Istochimica Normale e Patologica" (as the European Journal of Histochemistry was originally named) tried to emphasize and give space to those discoveries, studies and experiments whose targets were to highlight the new main scientific topic: DNA. The same goal was maintained as the title of the Journal evolved: from the original Rivista to "Basic and Applied Histochemistry", from "European Journal of Basic and Applied Histochemistry" to the current "European Journal of Histochemistry", many articles were published trying to shed light on the fascinating mystery of DNA. This nucleic acid, localized in the cell nucleus, is involved in a wealth of basic biological mechanisms. Nuclei must be considered dynamic structures,[1,2] inside which DNA is known to interact with other biomolecules such as RNA, lipids and proteins.[3]

Physics and biochemistry regulate the chromatin compartmentalization in microenvironments that permit processes such as transcription and replication.[4] Many articles published in this Journal assessed the study of DNA and chromatin in cell nuclei with different techniques (discussed in detail in next paragraphs). Among them, histochemistry has been holding a pivotal role, being able to provide important data alongside precise localization at the microscopy level.[5,6] Features of DNA typical of specific diseases are studied in histopathology:[7,8] for example, an increased amount of condensed chromatin has been found in myonuclei of aged type-II fibres by Malatesta and colleagues.[9] Alongside data reflecting a given moment, chromatin condition has been analysed to study changes over time: Fraschini and Biggiogera studied chromatin organization during the sperm maturation processes in the mouse epididymis, showing the presence of basic knobby fibres, and in the vas deferens, where thicker fibres could be seen in addition to the knobby ones.[10,11] Other works have been exploring the basic unity of nucleosomal filaments, the first level of DNA compaction, with the aim to investigate the existence of putative further-order structures. Combination of different techniques, such as cryo-electron microscopy (cryo-EM), electron spectroscopic imaging (ESI) and Feulgen-like osmium ammine reaction for transmission electron microscopy (TEM) indicated that mammalian mitotic chromatin does not show the 30 nm fibres, hypothesized and for a long time widely accepted by the scientific community. These studies revealed that the first level of organization of chromatin is the 10 nm thick fibre and that, at the most, higher structural unities result from association of multiples of the 10 nm fibres.[12] Other analyses were focused on the general internal architecture of chromatin in relation to DNA replication: in 2004, Fakan defined the borders of condensed chromatin as the principal site of DNA synthesis. Furthermore, he suggested that the newly synthetized nucleic acid molecules do not root on those locations: on the contrary, they are rapidly moving towards condensed chromatin areas, displaying fast internalization course.[13] Transcription sites have been visualized, among the others, by Trentani and colleagues using TEM: by incorporation into living cells of bromouridine (BrU) as an RNA precursor, followed by further revelation using specific antibodies, it was possible to confirm the periphery of the condensed chromatin as the incorporation site, where RNA is synthetized. Moreover, chromatin organization has been demonstrated to be directly associated to the level of transcriptional activity. The cell metabolic state, evaluated on the base of RNA polymerase II activity, is in fact reflected on DNA condensation. For example, in resting rat hepatocytes that are characterized by a low transcriptional level a major portion of chromatin appears to be in the heterochromatic status; on the contrary, the same hepatocytes in a regenerating state, thus with higher transcriptional activity, show a dispersed form of chromatin. As advanced confirmation, by selective inhibiting RNA polymerase II, it is possible to compact the otherwise vast majority of euchromatin.[14] All the above aspects held an important role, providing evidence on the complicated, finely regulated and still partially known chromatin structural and functional organization.[11]

From chromatin to chromosomes

Differences due to the transcriptional activity have not only been found in the euchromatin-heterochromatin ratio, but an effect on chromosomes is displayed, too. It has been shown how chromosomes during interphase are characterized by high plasticity, since the relationship between chromatin and nuclear space devoid of chromatin is significantly variable and depends on multiple parameters, from the metabolic rate to cell cycle phases.[15] Cremer and Cremer highlighted that, after undergoing the decondensation that ensues the mitotic event, chromosomes in interphase nuclei still occupy specific regions defined as chromosomes territories; these nuclear areas are separated from the domains (known as interchromatin compartments) lacking DNA.[16] Together, chromosome territories and interchromatin compartments form an intricate three-dimensional network, containing lacunas and channels almost completely devoid of genetic material, alternated with dense DNA regions. In this context, chromosome territories (and, by logical consequence, chromosomes), are differently located according to various parameters. Gene-dense chromosome territories are preferentially found in central regions, while the gene-poor ones seem to mainly locate at the nuclear periphery; chromosomes territories of large chromosomes are often spotted at the equator of the nucleus, whereas the smallest are found close to the nuclear centre. Phenomena of chromosomes re-positioning after condensation-decondensation cycles, and chromatin remodelling are therefore clear examples of the dynamic topography of the chromatin domains. This may suggest a connection with gene regulation: for instance, epigenetic modifications of histones or DNA itself could affect spatial-temporal formation of condensed chromatin domains, thus influencing gene expression, cell differentiation and much more.[2]

Nevertheless, many questions regarding chromosomes still remain open. During years, the European Journal of Histochemistry contributed to the current knowledge about this topic. For example, in 1975 Chiarelli proposed a model for suprachromosomal organization, suggesting, among the various features, that a chromosome is the result of the condensation of a single chromatin filament.[17,18] In 1981, Marchi and colleagues analysed mosquito's mitotic and pre-mitotic interphase nuclei with the aim to study the chromosome arrangement, revealing that centromeric regions are found clustered together, often close to the nuclear envelope.[19] A decade later, Sumner took advantage of the knowledge coming from chromosome banding to define the nuclear localization of gene-rich and gene-poor chromosomal regions.[20] Nonetheless, as time passed, analyses reached even the level of nucleotide sequence in chromosomes, highlighting some important features. Among the others, the effect on chromosomes of the DNA cleavage by restriction enzymes has been studied. Endonuclease activity has been reported to often promote a reorganization of the centromeric and paracentromeric heterochromatic regions: this is also reflected in a diffused "open" chromatin condition: by consequence, after performing fluorescence *in situ* hybridization (FISH), a stronger signal is detected due to the easier interaction between chromosomal and probe DNAs.[21]

More recently, the Journal investigated topics such as chromosomal polymorphisms. The study of differences in highly repeti-

tive DNA allowed identifying unexplored polymorphisms in insects, mammals and humans. This could reveal significant pathways to approach clinical issues, for instance it may be applied to bone marrow transplantation, where chimerism is produced.[22,23]

Methodological contribution for studying DNA

Interest in providing new methods for visualizing and studying DNA is a solid presence through the history of this Journal. Indeed, travelling back to the very first volumes, dated 1954-1955, Novelli[24] and Levi[25] provided innovative procedures for nucleic acid staining at light microscopy. Actually, visualization of DNA is a gold standard to evaluate the cell status. Among several methods, the Feulgen reaction has been one of the most utilized to specifically stain DNA. Robert Feulgen and Heinrich Rossenbeck described this technique almost 100 years ago.[26] A chemical reaction between aldehydes and the Schiff reagent gives DNA a magenta colour visible at bright-field microscopy. Being the staining stoichiometric to the DNA content, a quantification is also possible. The European Journal of Histochemistry helped to elaborate on this technique, publishing supportive and questioning papers on this topic. For instance, thanks to the Feulgen reaction, Manfredi Romanini and De Stefano showed for the first time that it was possible to retrieve the quantity of metaphasic DNA from human lymphoblast.[27] Moreover, the Journal has published original works demonstrating some limits of the technique such as the hydrolysis time and the effect that histonic proteins could have on the quantification efficacy, as shown respectively by Porcelli[28] and by Bernocchi and De Stefano.[29]

An analogue of the Feulgen reaction for EM is the osmium ammine staining. Proposed in 1973 by Cogliati and Gautier, and then perfected by Olins and colleagues, this method utilizes an electron-dense osmium-ammine complex as a Schiff-like reagent visible under the electron beam, therefore ensuring higher resolution.[30,31] In this Journal, it has been shown for the first time how the utilization of this method can be essential for the visualization of chromatin in sperm cells, one of the most difficult chromatin organization to be observed with previously described methods.[11] Moreover, Vázquez-Nin and colleagues improved this method publishing the important technical modification regarding the substitution of gaseous SO_2 with sodium metabisulfite.[32] In 1975, Marinozzi and Derenzini[33] noticed, without a clear chemical explanation, that there was another electron-dense staining for DNA consisting in the use of the phosphotungstic acid (PTA). The same authors have reviewed recently in the Journal all the achievement on chromatin structure obtained *in situ* by ultrastructural cytochemistry.[12]

Fukuda and coworkers[34] observed that in cells of several cancers and pre-cancer conditions chromatin DNA is more prone to acid hydrolysis than in cells of the corresponding non-tumor tissues: these authors proposed as a marker of malignancy the "DNA-instability test", This assay is based on the immunohistochemical detection of single-stranded DNA after acid hydrolysis, and -contrary to the histochemical methods used to reveal the presence of DNA damage at specific genomic regions- it is able to reveal the presence of DNA instability in the entire nuclear DNA. In cancer tissues, the immunodetection of single-stranded DNA regions after denaturing acid hydrolysis makes it possible to detect cancer cell clones also in borderline malignancies or at an early stage of carcinogenesis.[35-44]

The DNA content quantification in the years has mainly been achieved thanks to the use of flow cytometry, recognised as a rapid and reliable technique for DNA quantification, since the 1970s.[46] In 1975, Prenna and colleagues described a different cytofluorometry strategy through the usage of pulsed and tunable lasers.[46] Later on, in the 1991, De Vita and colleagues utilized flow cytometry to quantify DNA from extracted nuclei from paraffin embedded breast cancer samples, thus highlighting the possibility to use archive samples in cancer research.[47] Finally, Crissman and Steinkamp, using four different intercalating DNA agents with different intensity, demonstrated that, with flow cytometry, it is possible to study chromatin changes upon the different cell cycle phases.[48] New approaches have also been developed to visualize DNA structures. For instance, Dávila-Rodríguez and colleagues exploited the features of the whole comparative genomic hybridization (W-CGH), a technique that identifies gains or losses of specific chromosomal region, when different genomes, labelled with hybridized fluorophores, are compared. The authors observed, comparing metaphase chromosomes, high level of polymorphism in chromosome 6, 5, 9, 10, 14, and 15 of healthy individuals.[49]

DNA and proteins, a close relationship

In this section we report various examples of papers focused on the study of one or few DNA-associated proteins. These studies highlight the importance of analysing the role of chromatin proteins to understand DNA nature and function inside nuclear organization.

Capitani and colleagues[50] analysed the interaction of a histone protein with DNA. They have already shown in 1976 how the lipid component has a relevant influence on this dynamic affecting chromatin organization, a topic that has recently been resumed with great interest. Specifically, they studied how phospholipids interaction with the histone F2b (now called H2B) influences its ability to associate with DNA, through thermal denaturation. Analysis of the denaturation profiles shows that sphingomyelin, phosphatidylserine, phosphatidyl-ethanolamine and bovine lecithin have a destabilizing effect on the DNA-F2b histone binding, in direct relationship with lipid concentration. Lipid interactions weaken the DNA-histone association, making the DNA molecule more susceptible to thermal denaturation.

Few years later, an article focused on non-histone chromatin proteins was published.[51] Although the existence of a heterogeneous series of proteins of a non-histone nature associated with DNA was already recognized, their function was largely unknown, although it was partly deduced that they could have a role in the regulation of gene function. The major difficulty in these studies was the ability to isolate proteins that associate with DNA, to measure their amount, and to identify the DNA-binding sequences. Some methods allowed to distinguish between DNA sequences deemed transcriptionally active compared to the inactive ones, because those actively transcribed are more exposed and sensitive to the action of nucleases or to mechanical shearing (in fact, the association of these DNA sequences with RNA polymerase II was often detected). In this article, the authors proposed another method for identifying transcriptionally active chromatin sequences, based on the visualization of nascent RNA fibrils. In 1993 a paper[52] underlines instead very clearly how important is the quantitative relationship between DNA and proteins in defining the morphological and functional properties of the cell and consequently of the whole tissue: using dual-parameter cytophotometry, it has been possible to quantify DNA (stained by Feulgen reaction) and protein contents. From this experiment emerged that hypertrophic myocardium shows polyploidy and an increase in protein content proportional to ploidy. Increased ploidy and protein quantity sustain ventricular hypertrophy and are responsible for heart dysfunction.

More recently, in 2006, a review[53] emphasized the role of the

proteins associated to the nuclear envelope, demonstrating that these proteins have not merely a structural function as a physical barrier preserving and confining the genome, but also deserve an active role in modulating chromatin organization. This concept is discussed in a series of articles that analyse the effects of mutations in these proteins, which give rise to genetic diseases called laminopathies. All of these diseases determine syndromic phenotype, which involve many tissues, because mutations in the nuclear lamina lead to alterations in the organization of chromatin which is common to all cells. Microscopical observation of these cells shows an abnormal heterochromatin distribution, which is no longer associated with the nuclear lamina, as it normally occurs: this feature could possibly be related to mutations in lamin A, which abnormally accumulates in the nucleus, leading to severe chromatin disorganization and pre-mature aging and/or lipodystrophy phenotype, due to the downstream alteration in the distribution and solubility properties of heterochromatin-associated proteins, such as HP1. A series of chromatin remodelling defects determines a cascade of epigenetic events, so that cells are unable to maintain the silenced chromatin state that allows/preserves terminal differentiation. This study revealed the importance of nuclear envelope protein such as lamin A in chromatin remodelling, distribution of heterochromatin and so in the proper regulation of gene silencing.

Studying nuclear environment and chromatin dynamics from the second half of the 20th century to the present day

We shift our attention from the importance of the role played by one or a few proteins analysed individually, as in the previous section, to the activity of protein complexes that work in concert to regulate chromatin structure and gene function.

In 1976, a method was published based on the incorporation of radioactively labelled nucleotides to study synthetic processes of DNA molecules, which neither concerns replication for the subsequent mitotic division, nor synthetic processes connected to repair mechanisms of DNA damage. This synthetic activity was designated by the name "metabolic DNA", to distinguish it from the aforementioned DNA process.[54] This study was carried out in plant tissues. In order to distinguish replicative synthesis from metabolic DNA, having both in common the incorporation of labelled nucleotides, they run the experiment in the collenchyma, which unlike other plant tissues such as phloem, xylem and cambium, is a stable terminally differentiated tissue and therefore not subject to replication and mitosis. Some evidence presented in this article also suggests that the incorporation of labelled nucleotides in collenchyma, after 24-hour treatment and 168-hour chase with water, would not be attributable to DNA repair processes. Another study published in 1984,[55] once again proposes the use of tritiated deoxyribonucleotides to detect DNA synthetic process unrelated to replication. Even in this case, the experiment was performed on stable tissue, specifically on cerebellar Purkinje cells. It was highlighted that the incorporation of labelled precursors is maximum in 12 to 30 days old rats, with a predominantly nucleolar localization. In this case, the authors suggest that the detected synthetic activity is attributable to DNA repair mechanisms or to the synthesis of nucleic acid surplus, even though no specific function for this latter was indicated.

Going on through the years, a work published in 1993[56] showed that in this period processes related to the DNA were no more studied as separate molecular mechanisms, but the awareness was gaining ground that specific 3D chromatin organization inside the nucleus allows coordination and integration of a myriad of different molecular reactions. In fact, although this article is focused on the repair of nicks generated in DNA during cell differentiation, it is well underlined how this process is integrated into a dynamic network of events, designed to allow acquisition of the morphological and functional properties typical of mature spermatozoa. In particular, the formation and repair of these DNA nicks are studied in relation to nuclear architecture dynamics. For this reason, sperm cells are a very good model as they undergo strong changes in DNA content, chromatin domains organization, and nuclear protein composition and quantities during differentiation from spermatids to spermatozoa.

All the studies conducted in the second half of the 20th century have led to the development of various techniques nowadays available to automatically and rapidly analyse specific DNA processes in many cells, opening the way to their applications even at the diagnostic level. For example, Cortés-Gutiérrez *et al*., in 2012 described the possibility of detecting DNA breakage in buccal cells by FISH.[57] This method allows the analysis of many cells singularly and rapidly, and to obtain quantitative data. Moreover, the breaking frequency could be detected in a specific DNA sequence or in the whole genome. All these advances have made it possible to transfer basic biology and scientific knowledge to the clinical setting.[23,58-60] This has also revolutionized our way of thinking and studying the nucleus as a microenvironment that houses an integrated network of chemical reactions, where fine regulation and probability, combined with the contribution of external stimuli, allow the realization of specific transcriptional programs and cell phenotypes.

RNA: an open chapter

So far, we focused on papers predominantly regarding DNA. Of course, alongside the plethora of investigations conducted on this topic and presented by the European Journal of Histochemistry, many other works have been carried out on RNA, either examining it as a stand-alone molecule or giving emphasis to the interplay between the two nucleic acids. For example, Ortiz and colleagues studied the distribution of both DNA and RNA during the synaptonemal complex formation, thus focusing on both the structural and transcriptional role of the two mentioned molecules.[61] On the other hand, in 2004, Pederson summarized techniques to study RNA and illustrated the branch of cytochemistry completely dedicated to this specific nucleic acid: fluorescent RNA cytochemistry, which provides potential to study the RNA dynamics in living cells. Briefly, this technique consists in the injection into cells of fluorescent RNA, obtained through the incorporation either of a fluorescently labelled ribonucleoside triphosphate or a modified uridine nucleotide post-transcriptionally coupled to a fluorescent reporter. In a nutshell, this technique gives the possibility to trace the RNA translocation inside a cell and, by coupling with fluorescence recovery after photobleaching (FRAP) or other approaches, it represents a valid instrument in gene expression research.[62]

Starting from this premise, in the next sections we direct our attention to the ribonucleic acid, in particular on papers describing both coding and non-coding RNA, RNA synthesis and processing, as well as its implication in several pathologies.

The nucleolus is a hub for the synthesis and transit of RNAs

The nucleolus is the nuclear domain where the synthesis of ribosomes takes place. Given this important role, it is the site of

intense RNA transcription and transit. The most quantitatively important transcriptional activity concerns the synthesis of ribosomal RNA, however, also some messenger RNAs are transcribed in the chromatin nucleolus-associated domains (NADs) and many non-coding RNAs pass through the nucleolus. Some of these are directly involved in the ribogenesis, while others play a role in different nucleolar functions. The organization and distribution of the nucleolar components, *i.e.*, the fibrillar centers (FCs), the dense fibrillar component (DFC), and the granular component (GC) depend on cell identity and functional status, but reflect the ribosome biogenesis, which is similar in the basic steps in animal and plant cells. FCs are most likely the anchoring sites for rDNA, and for the assembly of transcription complexes. Transcription of rRNA genes occurs in the transition zone between FCs and the DFC. In the DFC, not only transcription occurs but also some early and advanced steps of pre-rRNA processing; finally, the GC is the site of the later steps of pre-ribosomal processing.[63]

It is not surprising that the nucleolus has been recognized as a multifunctional domain that participates directly or indirectly in cell resting, proliferation, differentiation, maturation, and in programmed cell death. In fact, as the site of ribosome production and being the ribosomes necessary to translate all the proteins and enzymes involved in the different metabolic functions, the nucleolus greatly influences cell metabolism. Morphology and cytochemistry of nucleoli have been used for the evaluation of nucleolar biosynthetic activities in various cell states, under physiological and pathological conditions. Some nucleolar parameters have been used as markers for the diagnosis and prognosis of diseases and ageing.[64]

Both the number of nucleoli in a cell and the nucleolar area vary according to the cell type and the cell cycle stage. Increased nucleolar size is generally observed in cancer cells; therefore, several studies have focused on the possibility of using the size of the nucleolus as a diagnostic marker trying to establish standard criteria and conditions for its measurement. Berger[65] studied the nucleoli in mammalian circulating lymphocytes and insect circulating hemocytes, and found that smaller nucleoli correlated with lower proteosynthetic cellular activity in both these cells.

Smetana and collaborators also published many studies about the nucleolar size of white blood cells and its correlations with blood malignancies and ageing.[66-69] They studied human myeloblasts in the bone marrow of patients suffering from chronic myeloid leukemia, and measured nucleolar diameter using different cytochemical procedures for the visualization of RNA, DNA and AgNOR proteins, and discovered that nucleolar diameter depends on the procedures used for visualizing nucleoli. However, a close relationship was detected between the diameter of nucleoli and their number, where the larger number of nucleoli per cell corresponds to smaller nucleolar mean size. Moreover, they observed that the staining density of small and large nucleoli did not differ substantially after staining for RNA, thus they hypothesized that the nucleolar size is directly related to the total RNA content in myeloblasts.[66] Ageing in myeloblasts determines a significant decrease of the cells in S-G_2 phase of the cell cycle accompanied by a significant reduction of the mean diameter of nucleoli per cell. In contrast, treatment with the histone deacetylase inhibitor, Trichostatin A (TSA) produced an increase of cells in S-G_2 phase and also in the nucleolar size. In leukemic myeloblasts, nucleolar diameter might be used as a marker to estimate the occurrence of cells in the S-G_2 phase in smear preparations when the number of cells is very small.[67] Another study by Smethana *et al*. on cultured leukemic myeloblasts was performed to obtain information on nucleoli during apoptosis. The apoptosis was induced by TSA and nucleolar diameter and density were determined using computer-assisted measurement and densitometry in specimens stained for RNA. Apoptotic cells showed nucleolar mean diameter and nucleolar RNA density similar to the non-apoptotic myeloblasts, while the cytoplasmic RNA density in apoptotic cells was markedly reduced. They speculated that the transcribed RNA could remain "frozen" within the nucleolus, and its transport to the cytoplasm decreased or stopped, or RNA maybe degraded in the cytoplasm. Moreover, AgNORs staining revealed that nucleolar biosynthetic activity in apoptotic cells decreased or disappeared, indicating that large nucleoli intensely stained for RNA are not necessarily related to the high nucleolar biosynthetic activity and may be also present in apoptotic cells.[68]

The apoptotic process induced in the large or giant binucleate and multinucleate cells (LBMNCs, present in a small percentage in HL-60 cell culture) by photodynamic treatment (PDT) using 5-aminolaevulinic acid (ALA, as the precursor of the photosensitizer protoporphyrin IX), determines, also in this cell type, nucleolar changes as marked reduction or disappearance of silver-stained AgNORs. In addition, PDT also significantly reduced the number of nucleoli. These changes again seem to reflect the decrease or arrest of nucleolar biosynthetic activities.[69]

The nucleolus may also undergo disassembly either irreversibly in apoptosis or reversibly during mitosis, so that nucleolar proteins are redistributed. The three proteins, phosphorylated c-Myc, fibrillarin and Ki-67 showed independent behaviour as they relocated in distinct compartments during mitosis, while during apoptosis Ki-67 is cleaved and the other two are extruded into the cytoplasm with different kinetics. The separation of these nucleolar proteins continues in the cytoplasm and was still visible in the apoptotic blebs which contained different sets of nucleolar proteins. These observations confirmed that the apoptotic bodies may vary in size and may contain heterogeneous aggregates of nuclear proteins and/or nucleic acids.[70]

Interestingly the induction of apoptosis was also reported to determine the cellular re-localization of the entire nucleolus and other nuclear bodies. Short-term hypertonic (HT) stress induces apoptotic cell death in human EUE cells in culture. During this process, a rearrangement of nuclear ribonucleoprotein (RNP)-containing structures was detected, with the formation of Heterogeneous Ectopic RNP-Derived Structures (HERDS), which moved into the cytoplasm. Nucleolus-like bodies (NLBs), which morphologically resembled the nuclear functional nucleoli, were observed inside the cytoplasmic fragments blebbing-out at the cell surface. These NLBs still contain nucleolar proteins. This confirms that some RNP-containing structures of nuclear origin are extruded from the nucleus into the cytoplasm during apoptosis.[71]

Re-localization of proteins and RNAs in the nucleoli was also observed by ultrastructural immunocytochemistry in Hela cells during ageing. A significant amount of RNase A, was detected in roundish, electrondense foci within nucleoli of aged cells. These bodies also contained RNA, but lack ribosomal S3 proteins. Therefore, they could be storage sites or areas for RNA degradation.[72]

miRNA and lncRNA

The human genome is transcribed only for a minor part in protein-coding RNAs. The vast majority of RNAs have instead reduced or absent coding potential, and are categorised into two main groups: small and long RNAs. Small RNAs are divided into three main categories: microRNAs (miRNA), small nuclear RNAs, and piwi-interacting RNAs;[73] long non coding RNAs (lncRNAs) are instead 200 nucleotide-long fragments. miRNAs and lncRNAs are involved in several biological functions and associate to many diseases.

The physiological role of miRNAs is to inhibit gene expression by binding complementary sequence, thus subsequently inducing mRNA degradation.[74] Even in the nucleus, miRNAs can regulate various nuclear events such as transcription and RNA splicing.[75] lncRNAs act similarly on silencing even if, due to their length, the target sequences are larger portions of chromatin DNA. Generally, they can involve large chromatin remodelling, such as in the case of the X chromosome.[76]

The importance of miRNAs and lncRNAs has been widely reported in the literature due to their link with many biological conditions. In 2020, Tao and colleagues[77] reported the involvement of a miRNA, named miR-22, in the differentiation of pluripotent stem cells into alveolar epithelial cells type II (AECII). Overexpression of miR-22 induced pluripotent cells *in vitro* to enhance their differentiation into AECII.

Importantly, miRNAs are also linked to several diseases. Ouyang and colleagues reported that an interaction between a miRNA, named miR-126, and Golgi phosphoprotein 3 (GOLPH3, a protein related with pathways in gastric cancer) promotes GOLPH3 degradation, thereby reducing the insurgence of cancer.[78] Moreover, in 2019 another study demonstrated that one of the most abundant brain-enriched miRNAs, miR-132, is significantly reduced in the brains of Alzheimer's patients.[79]

Similarly, lncRNAs have been reported to play vital roles in regulating cellular pathways in various cancer types. Li and colleagues demonstrated that lncRNA GClnc1 is linked to ovarian cancer. Indeed, this latter is markedly higher in ovarian cancer samples than in the related normal tissues, due to its function on p53, one of the most important signalling pathway protein in tumours.[80]

The significance of these non-coding RNAs has been related also to other pathologies, such as osteoporosis. Li and colleagues reported in this journal that the expression of lncRNA named H19 was significantly upregulated in osteoporosis samples with respect to the controls. Furthermore, the knockdown of lncRNA H19 promotes the expression of pro-inflammatory mediators or inhibits cell apoptosis, suggesting this RNA as potential target for therapies.[81]

Impaired RNA processing: myotonic dystrophy as a "spliceopathy" case study

In the cell nucleus, gene primary transcripts undergo several maturation steps before generating mature mRNA ready to be exported to the cytoplasm. The vast majority of these events occurs on RNP-containing structures, whose location, organization and composition are crucial for pre-mRNA processing.[82]

Defects in this delicate process, such as in alternative splicing events, is known to contribute to pathogenesis of several genetic diseases. Those phenomena lead to the terms "toxic RNA" and "spliceopathy" being coined as a reference to accumulation of mutant RNAs with deleterious outcomes. Malatesta and colleagues analysed this topic in depth facing a major example of RNA-dominant disease, elaborating a series of papers about myotonic dystrophy (DM), a multisystem disorder characterized by multifactorial phenotype most probably due to alterations in post-transcriptional pre-mRNA processing.[83] Two forms of DM have been described, DM1 and DM2, both caused by nucleotidic expansion, respectively (CTG)n and (CCTG)n repeats. Both expanded transcripts are retained in the cell nucleus and accumulate in the form of focal aggregates called *foci* where splicing factors and RNP-containing complexes (snRNPs and hnRNPs) are sequestered. These RNPs are essential in early pre-mRNA processing, and usually accumulate in their functional sites such as perichromatin fibrils (PF) and interchromatin granules (IG): so, it is no surprising that their seizure in the *foci* causes hampering of the normal functionality of these components and compromise the regulation of alternative splicing.[84] Remarkably, DM shares common structural and functional alterations with another muscle disorder: sarcopenia. The sarcopenic process consists in a continuous loss of muscular mass determined by ageing and it is considered a risk factor in elderly. Likewise to DM, sarcopenia is characterized by ectopic accumulation of pre-mRNA processing factors apart from their usual sites represented by the RNP-containing structures.[9] The same delocalization of mRNA splicing and cleavage factors has also been found in multiple tissues and organs (*e.g.*, the liver and brain) of aged mammals, suggesting a common basic mechanism in age- and pathological-derived conditions rooted in the misregulation of the delicate mRNA production workflow.[85]

In conclusion, hampering pre-mRNA processing factor, either by their ectopic accumulation or by their delocalization being sequestered in specific sites, represents a massive accident for the whole machinery of post-transcriptional processing, leading to lower metabolic activity and impaired protein synthesis. This strongly highlights the importance this subtle equilibrium fulfils in cells and tissues. Moreover, the works we selected also indicate histochemistry and, in general, the possibility to detect the subnuclear distribution of these components as an important strategy in biomedical research, evaluating the presence and localization of RNA processing factors as univocal cytological markers to study pathological cell dysfunctions.[86]

Concluding remarks

Since it has been established, the European Journal of Histochemistry was aimed at publishing papers where histochemistry had been applied to investigate a variety of subjects in biology and biomedicine, providing the latest outcomes and giving insights in an assortment of topics. In this review, we reported examples of papers on DNA and RNA in which the histochemical approach, thanks to the application of refined techniques, proved to be pivotal for analysing the structural organization and function of these molecules in the nuclear microenvironment, and for elucidating their roles in cell proliferation, differentiation and death under normal and pathological conditions. All these findings not only enriched the knowledge in nucleic acid biology, but also were vanguard for practical applications that, as time passed, have been integrated and commonly used in different research fields. The same would likely be true for the most recent articles that are providing conceptual and technical foundations for suitably exploiting the experimental results in the clinical practice.

Histochemistry has the unique feature of providing evidence *in situ*, in tissues, cells or organelles, of the molecules' presence and function: in the years to come, this unique molecular approach will make histochemistry more and more crucial for studying biocomplexity in animals and plants. In this endeavour, the histochemical journals are called to play a major role to promote the development of novel technical improvements and applications, while being open forums where scientists in the biomedical field may present and discuss their original findings.

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