Вестник Российского университета дружбы народов. Серия: МЕДИЦИНА

2026 Том 30 № 3

Тема номера: КЛЕТОЧНАЯ БИОЛОГИЯ

DOI: 10.22363/2313-0245-2026-30-3
http://journals.rudn.ru/medicine

Научный журнал
Издается с 1997 г.

Издание зарегистрировано Федеральной службой по надзору в сфере связи, информационных технологий и массовых коммуникаций (Роскомнадзор)
Свидетельство о регистрации ПИ № ФС 77-61206 от 30.03.2015 г.
Учредитель: Федеральное государственное автономное образовательное учреждение высшего образования «Российский университет дружбы народов имени Патриса Лумумбы»

РЕДАКЦИОННАЯ КОЛЛЕГИЯ

RUDN JOURNAL OF MEDICINE

2026 Volume 30 No. 3

Theme of the issue: CELL BIOLOGY

DOI: 10.22363/2313-0245-2026-30-3
http://journals.rudn.ru/medicine

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Серия: МЕДИЦИНА

ISSN 2313-0261 (Online); 2313-0245 (Print)
Периодичность: 4 выпуска в год (ежеквартально).
Входит в перечень рецензируемых научных изданий ВАК РФ.
Языки: русский, английский.
Материалы журнала индексируются в БД: Белый лист РЦНИ, РИНЦ Российской научной электронной библиотеки, Scopus, Directory of Open Access Journals (DOAJ).

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Редакционная коллегия журнала приглашает к сотрудничеству ученых и научные коллективы по подготовке специальных тематических выпусков журнала: http://journals.rudn.ru/medicine/announcement/view/327

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Литературный редактор *К.В. Зенкин*
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117198, Москва, Россия, ул. Миклухо-Маклая, д. 8
e-mail: medj@rudn.ru

Адрес издательства:
115419, Москва, Россия, ул. Орджоникидзе, д. 3
Тел.: +7 (495) 955-07-16; e-mail: publishing@rudn.ru

Подписано в печать 24.08.2026. Выход в свет 05.09.2026. Формат 60×84/8
Бумага офсетная. Печать офсетная. Гарнитура «Tinos, Roboto».
Усл. печ. л. 10,8. Тираж 500 экз. Заказ № 1023. Цена свободная.

Федеральное государственное автономное образовательное учреждение высшего образования
«Российский университет дружбы народов имени Патриса Лумумбы» (РУДН)
117198, Москва, Россия, ул. Миклухо-Маклая, д. 6
Отпечатано в типографии ИПК РУДН
115419, Москва, Россия, ул. Орджоникидзе, д. 3
Тел.: +7 (495) 955-08-61; e-mail: publishing@rudn.ru

RUDN Journal of Medicine

ISSN 2313-0261 (Online); 2313-0245 (Print)
Publication frequency: 4 issues per year (quarterly).
Included in the list of peer-reviewed scientific publications of the higher attestation Commission of the Russian Federation.
Languages: Russian, English.
The journal articles are indexed by White List of RCSI, Russian Index of Science Citation, Scopus, Directory of Open Access Journals (DOAJ).

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Review editor *K.V. Zenkin*
Computer design: *M.V. Rogova*

Postal Address of the Editorial Board:
10 Miklukho-Maklaya St., 117198 Moscow, Russian Federation
e-mail: medj@rudn.ru

Address of the Publishing House
3 Ordzhonikidze St., 115419 Moscow, Russian Federation
Ph. +7 (495) 955-07-16; e-mail: publishing@rudn.ru

Printing run 500 copies. Open price.

Peoples' Friendship University of Russia Named After Patrice Lumumba (RUDN University)
6 Miklukho-Maklaya St., 117198 Moscow, Russian Federation

Printed at RUDN Publishing House:
3 Ordzhonikidze St., 115419 Moscow, Russian Federation
Ph. +7 (495) 955-08-61; e-mail: publishing@rudn.ru

СОДЕРЖАНИЕ

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

ТЕМА НОМЕРА: КЛЕТОЧНАЯ БИОЛОГИЯ
THEME OF THE ISSUE: CELL BIOLOGY

DOI 10.22363/2313-0245-2025-30-3-301-313
EDN JZDMMQ

REVIEW
ОБЗОР

Programmed neutrophil death in immunothrombosis

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Abstract. *Relevance.* Understanding the fundamental mechanisms of immunothrombosis is essential for developing effective treatments and prevention of thrombotic disorders. This review aims to summarize the current knowledge on the role of programmed neutrophil death in the molecular and cellular mechanisms underlying immunothrombosis, as well as to demonstrate how dysregulation of hemostasis initiated by neutrophil death is associated with various pathological conditions. Based on a search of the electronic scientific databases Google Scholar, PubMed (MEDLINE), Scopus, and Web of Science using keywords and their combinations, with the methodological quality assessed using the AMSTAR 2 software, the role of neutrophil death in the development of immunothrombosis is presented. The review describes the types of programmed neutrophil cell death, namely apoptosis, pyroptosis, necroptosis, ferroptosis, and neutrophil extracellular traps (NETs), along with their molecular triggers and mechanisms. A modern concept of immunothrombosis pathogenesis is presented, demonstrating the involvement of hemostatic proteins, platelets, and proinflammatory cells in its pathogenesis and taking into account the complex relationship between hemostasis and inflammation. Each type of programmed neutrophil death is shown to be involved in the development of immunothrombosis. In addition, the review presents data on therapeutic strategies employing immunometabolic regulators and NET inhibitors, which potentially can attenuate immunothrombotic activity without affecting hemostasis. Such treatment approaches offer advantages for managing patients with various thrombo-inflammatory conditions. *Conclusion.* Overall, the data demonstrate that neutrophil apoptosis has an antithrombotic effect, whereas other types of programmed cell death promote thrombus formation and vascular events. The latter differ in that they require specific triggers: a cytokine storm for pyroptosis, the release of death-associated molecular patterns (DAMPs) for necroptosis, and oxidative stress for ferroptosis. These types of cell death are unified by their capacity to initiate NET formation, thereby providing a trigger framework for the development of severe thrombotic complications.

Keywords: neutrophils, programmed cell death, immunothrombosis, apoptosis, pyroptosis, necroptosis, ferroptosis, neutrophil extracellular traps

Funding. This work was supported by the state contract “Structural and cellular-molecular mechanisms of age-related connective tissue remodeling in musculoskeletal diseases” No. 056-00055-24-00 dated January 14, 2024.

Author contributions. Plekhova N.G. — study concept and design, data analysis, manuscript editing; Mikhailov A.O. — data collection and processing and writing; Tsvetov N.V. — data collection and processing and writing; A.N. Voronova translation and writing. All authors made significant contributions to the conception, conduct of the study and preparation of the article, read and approved the final version before publication.

Conflicts of interest statement. The authors declares no conflict of interest.

Ethics approval — not applicable.

Acknowledgements — not applicable.

Consent for publication — not applicable.

Received 08.04.2026. Accepted 30.04.2026.

For citation: Plekhova NG, Mikhailov AO, Tsvetov NV, Voronova AN. Programmed neutrophil death in immunothrombosis. *RUDN Journal of Medicine*. 2026;30(3):301–313. doi: 10.22363/2313-0245-2026-30-3-301-313 EDN: JZDMMQ

Introduction

Thrombosis remains a serious challenge for medicine, manifesting in many diseases associated with high mortality across different demographic populations [1]. Based on an evolutionarily conserved link between the coagulation system and innate immunity, the process of thrombosis initiation involves cells and specific molecules of the host defense system [2, 3]. Under physiological conditions, this link facilitates the recognition of pathogens and damaged cells followed by their elimination, whereas, under dysregulated conditions, it manifests as thrombotic disorders in infectious and inflammatory pathologies. This process leads to increased blood coagulability and the progression of venous thromboembolism and arterial thrombosis, with high patient mortality rates, as observed, for example, in coronavirus disease COVID-19 [4, 5]. Inflammation involving immune cells strongly contributes to thrombosis development, but the precise mechanisms underlying this link remain incompletely understood.

The term “immunothrombosis” was first proposed in 2013 by Engelmann and Massberg to describe the interplay of innate immune cells, platelets, and blood coagulation factors in the local control of homeostasis aimed at immobilizing foreign pathogens or structures [6]. It is known that inflammation triggers coagulopathies and adverse thrombotic events, highlighting the importance of a balanced interaction between the immune and hemostatic systems [6, 7]. The causal relationship mediated by Toll-like receptors on leukocytes leading to platelet activation is reciprocal, whereby the immune system influences thrombus formation and vice versa [8, 9]. Pathogen-associated and damage-associated molecular patterns (PAMPs and DAMPs) have been shown to induce unregulated activation of blood coagulation factors, platelets, endothelial cells, and the complement system [7]. Together these processes resulting in surface expression of P-selectin on platelets and endothelium, which recruits leukocytes and leads to a thrombo-inflammatory state. According to many authors, neutrophils are the key

drivers of immunothrombosis, exhibiting a high capacity for tissue factor activity through aberrantly mediated signaling of the protease-activated receptor (PAR) coagulation factor [10, 11]. These cells are sensitive to exogenous stimuli, interact with various cell types, and express numerous pattern recognition receptors for damage- and pathogen-associated molecular patterns (DAMPs and PAMPs) on their surface [12]. Upon reaching the site of inflammation and/or injury, they are capable of forming network structures — neutrophil extracellular traps (NETs) — with the release of nuclear DNA and cytosolic granules into the extracellular space [13]. It is well established that during sterile inflammation, neutrophils can also form NETs when exposed to other immune cells, platelets, or activated endothelium. Moreover, excessive NET formation can lead to disseminated intravascular coagulation, serving as a scaffold for thrombus formation, and can also initiate tissue fibrosis with organ failure [3, 14, 15]. In this review, we discuss the various pathways through which programmed neutrophil death can be activated and how these processes may lead to thrombus formation. We also provide an assessment of these pathways with respect to targeted therapy aimed at developing approaches to reduce blood clot formation without increasing the risk of bleeding.

Programmed neutrophil cell death

Neutrophils constitute approximately 50–70 % of all circulating leukocytes, originate from hematopoietic stem cells in the bone marrow, and differentiate under the control of granulocyte colony-stimulating factor (G-CSF) and chemokines (CXCL1, CXCL2, CXCL5, and CXCL8) [16]. Structural features of these cells include a multilobed nucleus, specialized (primary, secondary, and tertiary) granules, and secretory vesicles containing antimicrobial factors. Upon recognition of microbial and/or inflammatory stimuli, they migrate to the site of inflammation through an adhesion/migration cascade regulated by cytokines [17, 18]. This process is initiated by receptor interaction with PAMP and DAMP molecules, followed by rolling, adhesion, and transendothelial migration of the cells. In peripheral tissues, neutrophils are attracted by chemoattractants (CXCL8, IL-1β, leukotriene B4 and others), triggering effector functions including phagocytosis, production of reactive oxygen species (ROS), degranulation, vesicle secretion, and cell death [18]. The speed with which these cells migrate and perform antimicrobial functions, including the production of proteases and microbicidal peptides along with the generation of substantial amounts of ROS — underpins their ability to modulate various immune responses. [16]. Under homeostatic conditions, mature neutrophils constitute a short-lived cell population with a lifespan of up to one day; upon activation, for instance at an inflammatory site, their life cycle can be prolonged [18]. Following tissue inflammation, the release of lipoxins and resolvins affects neutrophil apoptosis, promoting efferocytosis by macrophages, reducing IL-23 levels, and suppressing hematopoietic progenitor activity in the bone marrow [19]. Neutrophils may re-enter the bloodstream and, via reverse migration, return to the bone marrow [18]. In addition to apoptosis, these cells may undergo alternative forms of regulated cell death, including necroptosis, pyroptosis, and NETosis (NET formation) [17]. Each of these alternative mechanisms of neutrophil cell death has important implications for inflammatory responses and the initiation of immunothrombosis.

Neutrophil apoptosis. This evolutionarily conserved type of regulated cell death plays a predominant role in maintaining organismal homeostasis and occurs in response to extrinsic and intrinsic inducers (Figure 1). Activation of the proapoptotic caspase cascade with subsequent cleavage of their substrates induces structural changes in cells, including reduction in cell area, plasma membrane blebbing, chromatin condensation, nuclear DNA fragmentation, and formation of apoptotic bodies [17] (Figure 1). The intrinsic signaling pathway of neutrophil apoptosis is modulated by B-cell lymphoma 2 (Bcl-2) family molecules and is activated upon involvement of various cytosolic factors, including DNA damage, hypoxia, and metabolic stress [19]. Proapoptotic Bcl-2 family proteins, the Bcl-2-associated X protein (Bax) and/or its antagonist Bak, promote outer mitochondrial

membrane permeabilization, leading to the release of cytochrome C and other enzymes into the cytosol [20]. Their release into the cytoplasm triggers the assembly of the apoptosome, which activates caspase-9, which in turn initiates effector caspases 3 and 7 [19] (Figure 1). The extrinsic pathway of apoptosis is induced by ligands binding to death receptors such as CD95/Fas, tumor necrosis factor receptor 1 (TNFR1), and the TNF-related apoptosis-inducing ligand (TRAIL) cytokine (Figure 1).

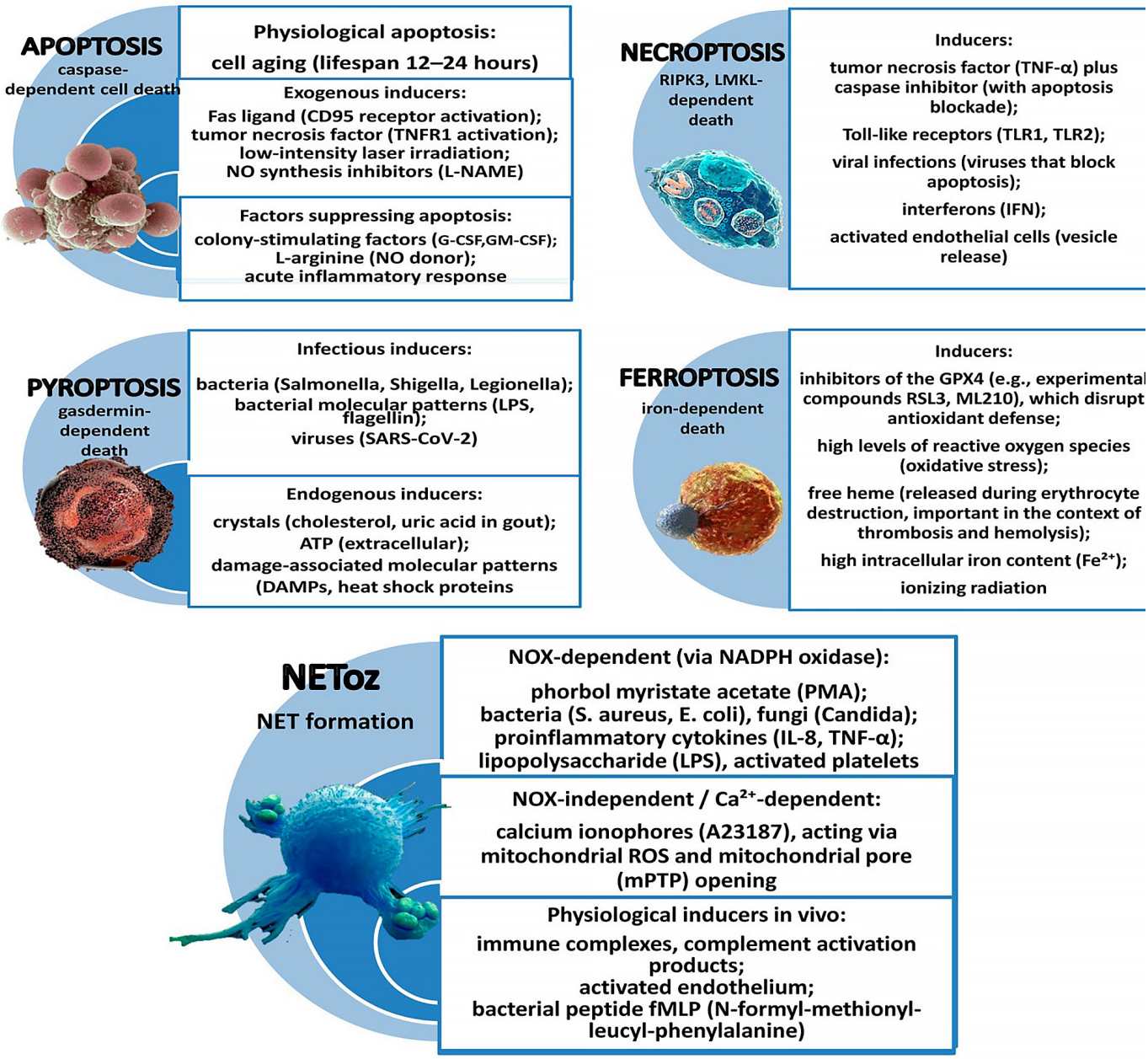

Figure 1. Exogenous and endogenous signals triggering the processes of programmed neutrophil death

Their activation triggers the assembly of the death-inducing signaling complex.

(DISC) with caspases 8 and 10, which promotes the cleavage of procaspases 3 and 7 [19, 20]. The kinetics of CD95/Fas-induced apoptosis can be modulated through the expression levels of pro- and antiapoptotic proteins [20]. Cathepsins are released from azurophilic granules of neutrophils, which cleave and activate caspases 8 and 3, and it has been reported that defective neutrophil apoptosis contributes to the development of chronic inflammatory diseases [21].

Neutrophil pyroptosis. Unlike apoptosis, this type of cell death is proinflammatory, as it is accompanied by plasma membrane rupture and release ofintracellular contents [22]. The key mediator of this mechanism is gasdermin D (GSDMD), which, upon activation, is cleaved by caspases, and its N-terminal fragment inserts into the cell membrane, forming pores. This alters osmotic pressure, increases the cytoplasmic volume of the cell, and leads to its destruction with the release of the proinflammatory cytokines IL-1β and IL-18 [17, 23, 24]. The role of caspase-1-dependent gasdermin D cleavage in neutrophils has long been known, and recent transcriptomic and immunoblotting data indicate that elastase from their cytosolic granules cleaves gasdermin D more efficiently than caspases do [25]. In aging neutrophils, activated protease alone is sufficient to induce pyroptosis, whereas upon bacterial stimulation, the NLRC4 inflammasome is formed, leading to IL-1β-dependent cleavage of gasdermin D by caspase-1 (Figure 1) [26].

Neutrophil necroptosis. Neutrophil necroptosis is independent of apoptotic signaling and is induced by the synergistic activity of receptor-interacting protein kinase-3 (RIPK3, RIP3) and the effector protein mixed lineage kinase domain-like (MLKL) (Figure 1) [27]. The molecular basis of neutrophil necroptosis is currently insufficiently understood; nevertheless, its key role in the development of many cardiovascular diseases and other pathologies has been demonstrated [28]. Necroptosis involves the strictly regulated intracellular assembly of the necrosome, which induces structural alterations in cells resembling those seen in necrosis. Degradation of organelle and granule membranes induced by reactive oxygen species (ROS) triggers endosomal and autophagosomal fusion, leading to the formation of extensive cytoplasmic vacuoles and an increase in cell volume [28]. The nuclear envelope is disrupted, chromatin undergoes condensation, and the plasma membrane becomes permeable, inducing the release of intracellular DAMPs, which exert a proinflammatory effect. This process is induced by ligation of TNFR1 and TRAIL receptors as well as adhesion molecules (CD44, CD11b, CD18, and CD15) on neutrophils (Figure 1) [17, 27, 28]. Unlike apoptosis, necroptosis is triggered upon caspase-8 inactivation, without DNA fragmentation, and is accompanied by a robust immune response driven by DAMPs [18]. Characterization of the necroptosis signaling pathway holds significant promise for the management of inflammation resulting from neutrophil dysfunction [8, 16].

Neutrophil ferroptosis. Neutrophil ferroptosis is a distinct type of programmed cell death characterized by the accumulation and peroxidation of phospholipids containing polyunsaturated fatty acids in the plasma membrane (Figure 1). This process is dependent on intracellular iron levels and is genetically and biochemically distinct from apoptosis, necrosis, and autophagy [29]. Ferroptosis manifests as disruption of plasma membrane integrity, swelling of the cytoplasm and mitochondria, and moderate chromatin condensation [30]. Neutrophils undergoing ferroptosis exhibit mitochondrial dysfunction with increased synthesis of ROS and lipid peroxidation products in the absence of morphological signs [31]. Ferroptosis is regulated by three main systemic pathways: (1) inactivation of the cysteine-glutamate antiporter, leading to inhibition of the antioxidant (REDOX) mechanism; (2) scavenging of peroxyl radicals and lipid oxidation involving the NADPH enzyme system with the suppressor protein FSP1 and coenzyme Q10; and (3) CoA ligase 4- and lipoxygenase-mediated activation of the system that esterifies polyunsaturated fatty acids into phospholipids [31]. Thus, during ferroptosis in neutrophils, redox reactions and iron and lipid transformations occur simultaneously [28, 30].

Neutrophil extracellular traps (NETs, NETosis). This is a lytic form of proinflammatory regulated cell death that is induced by an NADPH oxidase-mediated oxidative burst [32, 33]. NET formation is initiated by various signaling pathways, such as those stimulating

necroptosis, alterations in calcium ion levels, kinase phosphorylation, release of proteases from granules, and caspase-1 activation (Figure 1) [34]. Pyroptosis and NETosis are triggered by different molecules, but both types are mediated by nuclear histone H3-induced chromatin relaxation and DNA decondensation [18]. The formation of the web-like NET structure involves PAMP or DAMP receptors, which trigger a cascade of signaling pathways, culminating in changes in cell morphology with chromatin decondensation and translocation of DNA associated with cytosolic granular proteins [33]. This process depends on the activity of histone-cleaving serine proteases and procaspases. Furthermore, peptidyl arginine deiminase 4 (PAD4) promotes histone citrullination, which facilitates the interaction with negatively charged nuclear DNA, leading to its decondensation [1, 10, 34]. After DNA binds to granule components in the neutrophil cytoplasm, the subcortical actin network breaks down, and the decorated DNA, enclosed by the plasma membrane, is released into the extracellular space. In addition to the well-established role of NETs in trapping pathogens, their significance has been demonstrated in sepsis, autoimmunity, coagulation, and cancer [10, 13, 32, 33]. It has also been reported that NETs regulate the expression of the inflammatory cytokines IL-6 and pro-IL-1β in macrophages and participate in the development of vascular occlusion and thrombosis [1, 14].

Mechanisms of immunothrombosis and neutrophils

The contemporary concept of immunothrombosis pathogenesis, taking into account the complex interplay between hemostasis and inflammation, demonstrates the involvement of hemostatic proteins, platelets, and proinflammatory cells [7]. Coagulation factor III (tissue thromboplastin) is considered a marker of inflammation; it induces the biogenesis of Weibel-Palade bodies containing P-selectin in endothelial cells. This protein acts as a chemoattractant for leukocytes and can bind to platelets either directly or indirectly [7]. It consists of apoprotein III and a phospholipid complex and is expressed on the plasma membrane of fibroblasts, pericytes, vascular smooth muscle cells of subendothelial tissue, and activated monocytes [3, 7, 35]. Tissue thromboplastin interacts with coagulation factor VII, leading to its conversion to VIIa, which forms a complex with phospholipids and Ca^{2+} ions and activates the central component of the coagulation cascade, coagulation factor X [36]. In addition to this pathway, coagulation can also be triggered by contact of prekallikrein and its cofactor kininogen with activated factors XI and XII [37]. Thrombin and other proteases of the coagulation cascade, including factor Xa and VIIa, not only induce fibrin generation but also regulate cell functions, including hemostatic and inflammatory processes [7]. They can bind to G protein-coupled receptors (PARs), which are expressed on platelets (PAR1), thereby initiating platelet activation. Endothelial cells demonstrate the presence of all four members of the PAR receptor family and can be stimulated by activated protein C, as well as by plasmin, factors Xa and VIIa, and thrombin [37, 38].

Signaling through PAR receptors influences long-term mobilization of hematopoietic stem cell populations, the degree of adhesion marker, proinflammatory cytokine expression, and nitric oxide production [39]. These receptors are also expressed on leukocytes and influence proinflammatory and proapoptotic reactions. It has been shown that not only tissue thromboplastin synthesis by monocytes or endothelial cells exerts a procoagulant effect, but also neutrophil extracellular DNA can initiate coagulation through the contact pathway [11]. The fibrinolytic system plays a crucial role in immunomodulation by regulating the balance between pro- and anti-inflammatory processes through fibrin removal. Fibrin acts as a scaffold for immune cells and promotes their recruitment and activation. Endothelial and epithelial cells, as well as monocytes/macrophages, release tissue-type and urokinase-type plasminogen activators (uPA, tPA), thereby influencing the conversion of the main fibrinolytic enzyme plasminogen into plasmin [3, 7]. The acute-phase inflammatory mediator plasminogen activator inhibitor-1 (PAI-1) promotes bacterial elimination, enhances neutrophil migration, and regulates interferon-gamma (IFN-γ) synthesis in these cells, whereas plasminogen activator inhibitor-2 (PAI-2) attenuates the proteolytic activity of neutrophils [38, 39]. Thrombin-activatable carboxypeptidase (TAFI)

removes plasmin binding sites, which does not halt the process of plasmin binding to fibrin. As a result, numerous degradation products, including D-dimer and fibrinopeptide B, are released, which also possess immunomodulatory and chemotactic functions toward neutrophils [37].

It is also necessary to clarify the role of P-selectin (CD62P) interaction between activated platelets and the leukocyte glycoprotein receptor, for which monocytes exhibit the highest affinity, followed by granulocytes and lymphocytes [1, 40]. The initial attachment of platelets to leukocytes is stabilized by the involvement of other receptors and leads to targeted release of soluble mediators from the latter and activation of immune and inflammatory responses [6]. In addition to direct interaction with neutrophils, platelets release extracellular vesicles and soluble mediators, which modulate neutrophil functions [8, 11]. Platelet factor 4 (PF4/CXCL4) affects CD62P expression and IL-8 releasing by the endothelium, thereby influencing neutrophil extravasation [11]. Moreover, platelets stimulate cytokine production and the oxidative burst in neutrophils, which promotes NET formation [41]. The anticoagulant role of neutrophils consists of their ability to phagocytose platelets with membranes selectively modified by annexin A1 determinants of phosphatidylserine, thereby leading to active thrombus resolution [42]. Furthermore, these cells accelerate proplatelet maturation by eliminating defective cells [42].

Role of apoptosis in the context of immunothrombosis

Neutrophil apoptosis is a mechanism that limits and halts immunothrombosis (Figure 2). Its occurrence prevents cells from transitioning into necrosis or NETosis, which can activate platelets and subsequent thrombus formation [43]. This is supported by data demonstrating that suppression of apoptosis leads to an accumulation of "aged" and hyperactive neutrophils in tissues, which promote thrombus formation through the release of ROS and enzymes [44]. Such negative effects of apoptosis arrest, induced by cytokines and growth factors, have been noted in respiratory distress syndrome and multiorgan failure, where uncontrolled immunothrombosis plays a significant role [45]. Conversely, accelerated massive neutrophil apoptosis in sepsis patients with AIDS promotes immunosuppression, leading to neutropenia and impaired cell function, thereby increasing the risk of infections [43]. In turn, impaired clearance of neutrophil apoptotic bodies may lead to secondary necrosis or may serve as a source of autoantigens [19]. Thus, neutrophil apoptosis is a natural limiter of immunothrombosis, ensuring the removal of spent cells and preventing their transformation into triggers of thrombus formation. However, both its timely initiation and termination are critically important.

Pyroptosis, necroptosis, and ferroptosis of neutrophils in immunothrombosis

Unlike apoptosis, neutrophil pyroptosis is accompanied by plasma membrane rupture and the release of vacuolar and lysosomal contents, which recruits other immune cells and amplifies inflammation. The link between these processes is direct and interdependent, with gasdermin D playing a key role [2, 23]. When neutrophils activated, which cleaves gasdermin D, leading to subsequent pore formation in the membrane [23, 24].

Even before neutrophil death, this protein promotes their maturation and activation, increasing integrin expression on the membrane, which allows them to firmly adhere to the vessel wall [25]. This process activates platelets and triggers the coagulation cascade (factor XII, among others), leading to the formation of a fibrin thrombus, to which pathogens and immune cells adhere [2]. For instance, in a mouse model of thrombotic microangiopathy induced by cholesterol crystals, the absence or blockade of gasdermin D reduces immunothrombosis and kidney injury [17]. It has been reported that dysregulation of immunothrombosis involving neutrophil pyroptosis and massive NET formation leads to disseminated intravascular coagulation, lung injury, and multiorgan failure in COVID-19 viral infection [5, 41, 44]. Thus, during pyroptosis, gasdermin D activates neutrophils, which adhere to the vessel wall and begin to produce inflammatory molecules, creating a thrombus formation zone that recruits additional cells.

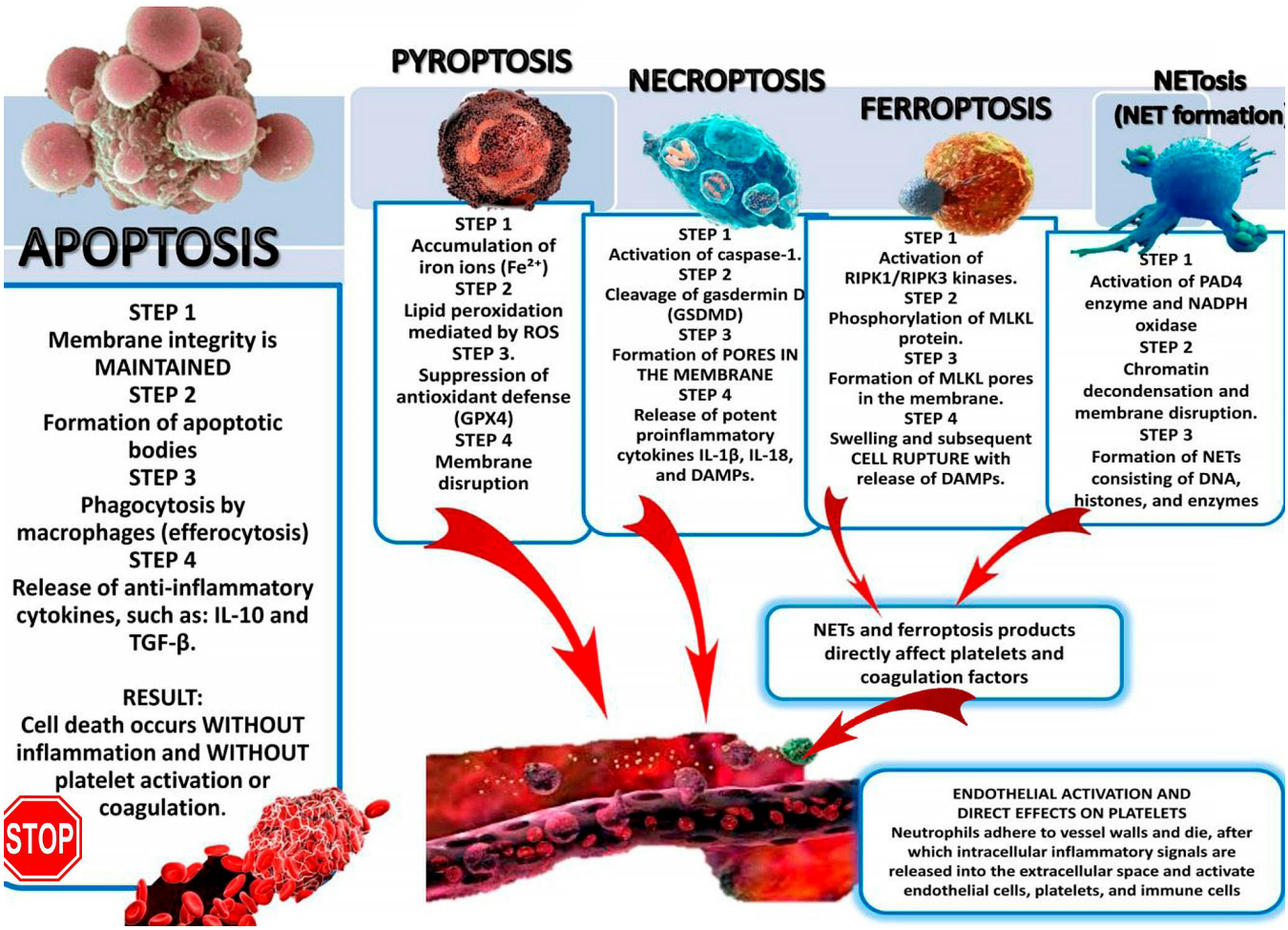

Figure 2. The influence of types of programmed neutrophil death on immunothrombosis

Two alternative neutrophil death programs, necroptosis and ferroptosis, also actively promote the development of immunothrombosis (Figure 2) [8]. Necroptosis is triggered by the activation of RIPK1, RIPK3 kinases and the MLKL protein, which form pores in membranes, leading to cell rupture with the release of DAMPs, whereas the main driving force of ferroptosis are iron ions, which stimulate membrane lipid peroxidation (Figure 1) [27, 29]. For the induction of immunothrombosis, the key link for both processes is the activation of the formation of NETs, which directly activate platelets and bind to coagulation factors [45–47]. During necroptosis, the release of intracellular DAMP molecules enhances inflammation and creates a prothrombotic environment, and inhibition of its key enzyme, RIPK1 kinase, abort this process and the formation of NETs [47]. In sepsis and acute respiratory distress syndrome, necroptosis of endothelial cells and neutrophils has been shown to contribute to hypercoagulability and microthrombosis in pulmonary vessels [45, 47]. Ferroptosis also stimulates the formation of NETs, which directly activate platelets expressing P-selectin on their surface, leading to thromboembolism [46]. This type of cell death is considered a key player in the pathogenesis of immunothrombosis in COVID-19, and it has been suggested that targeted therapy aimed at suppressing it may improve patient outcomes [5]. Understanding

the role of the above-mentioned death mechanisms is critical for the treatment of diseases in which immunothrombosis becomes dysregulated. Inhibition of key molecules, such as RIPK1 in necroptosis, may block the pathological immunothrombosis cascade, whereas for ferroptosis, iron-chelating agents or inhibitors of lipid oxidation are under investigation [45, 46].

Thus, pyroptosis, necroptosis, and ferroptosis represent three distinct yet functionally overlapping programs of neutrophil death that serve as critical drivers and amplifiers of immunothrombosis. Unlike apoptosis, which limits this process, the other types of cell death are united by their proinflammatory and prothrombotic potential, which is realized through several key mechanisms (Figure 2). A central event linking all three types of cell death to immunothrombosis is their ability to trigger or enhance NET formation. Despite this common outcome, each pathway makes a unique contribution to pathogenesis. The main feature of pyroptosis is not merely cell death but the accompanying massive release of potent proinflammatory cytokines (IL-1β, IL-18). This creates a systemic milieu that activates the endothelium and platelets, making them more sensitive to prothrombotic signals [48]. In necroptosis, which is often triggered when apoptosis is blocked by pathogens, DAMPs serve as powerful stimulators of inflammation and provoke thrombotic complications in sepsis and severe lung injury. As a metabolic catastrophe, ferroptosis is unique in its ability not only to affect neutrophils but also to directly activate platelets through lipid peroxidation mechanisms. This death pathway acts as a link between metabolic imbalance, platelet dysfunction, and neutrophil hyperactivity. Hyperactivation of pyroptosis, necroptosis, and ferroptosis transforms the protective response of immunothrombosis — aimed at isolating pathogens in the bloodstream — into a pathological factor. The presented data justify the promise of searching for therapeutic agents capable of selectively inhibiting these neutrophil death pathways. Blockade of key molecules (e.g., gasdermin D in pyroptosis, RIPK3 in necroptosis, and iron ions in ferroptosis) may represent a strategy to restrain pathological immunothrombosis while preserving its physiological protective functions [2, 18, 42, 45]. Of particular interest is a combined targeting, as these pathways may duplicate and amplify one another.

Role of neutrophil extracellular traps in the pathogenesis of immunothrombosis

Neutrophils contribute to the capture and degradation of pathogens through the formation of NETs, accompanied by the release of decondensed chromatin and antibacterial proteins. Initially, these structures were discovered in patients with stroke, where they provided a scaffold within blood vessels for recruiting erythrocytes, platelets, and leukocytes, as well as binding plasma proteins [28, 42]. It has also been demonstrated that in sepsis, inflammation accelerates neutrophil recruitment and activation, leading to NET formation, which initiates connective tissue remodeling and contributes to the development of venous thrombosis in the lungs [8, 10, 32]. In COVID-19 viral infection, conversion of Hageman factor (factor XII) to XIIa promotes immunothrombosis by influencing IL-6 and complement synthesis, with subsequent activation of NET formation [4].

Procoagulant proteins, including von Willebrand factor, factors XI and XII have been found on the insoluble reticular DNA structure containing histones and bactericidal enzymes [36]. Moreover, while histones with immobilized coagulation factors activate platelets, negatively charged DNA triggers activation of the coagulation cascade through XIIa-induced thrombin generation [49]. In contrast, elastase inactivates anticoagulant mechanisms by cleaving thrombomodulin and tissue factor pathway inhibitor [6]. Predisposition to NET formation is primarily exhibited by aging neutrophil subpopulations with high phagocytic capacity [40]. In addition to local effects through the release of tissue thromboplastin-containing vesicles, NETs have a systemic, long-term effect on microthrombus formation and vascular occlusion, inducing immunosuppression [50].

Indeed, during inflammation, the functions of NETs include not only the capture and elimination of pathogens, which enhances the microbicidal effect of neutrophils, but also prothrombotic and procoagulant stimulation [49]. This type of cell death is often recognized as an important therapeutic

target. For example, in sepsis, the effect of the TLR4 receptor blocker and the TNF-a synthesis inhibitor 2-acetamidopyranoside on reducing the level of circulating adhesion receptor ICAM-1, NET formation, and thrombus development has been demonstrated [51]. Statins and angiotensin receptor blockers possess similar effects, as they inhibit TLR4 signaling pathway activity, thereby reducing the release of proinflammatory cytokines [52]. One of the stimulators of NET formation is peptidylarginine deiminase 4 (PAD4); its inhibition reduces vascular damage and endothelial dysfunction [53, 54]. Treatment with low doses of heparin (250 U/kg) reduces NET formation and synthesis of proinflammatory factors, whereas increasing its concentration, conversely, induces NET formation and the production of antibodies against platelet factor 4, leading to thrombosis activation [54, 55]. These findings indicate the need for precise heparin dosing in clinical practice or the use of heparin alternatives.

Conclusion

Unlike apoptosis, which has an anticoagulant effect, the molecular mechanisms of neutrophil death namely pyroptosis, necroptosis, ferroptosis, and NETosis turn these cells into powerful prothrombotic drivers. These types of programmed cell death are united by their capacity to trigger NET formation and differ in their specific mechanisms: a cytokine storm in pyroptosis, DAMP release in necroptosis, and direct platelet activation via oxidative stress in ferroptosis. Collectively, the programs of neutrophil cell death other than apoptosis form a trigger basis for the development of severe thrombotic complications in many critical conditions of the organism.

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Запрограммированная гибель нейтрофилов при иммунотромбозе

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Аннотация. Понимание основных механизмов иммунотромбоза необходимо для разработки эффективных методов лечения и профилактики тромботических нарушений. Цель обзора – изложить существующие знания о роли запрограммированной гибели нейтрофилов в молекулярных и клеточных механизмах развития иммунотромбоза, а также представить, каким образом дизрегуляция гемостаза, инициированная смертью этих клеток, связана с различными патологическими состояниями. На основании поиска работ в электронных научных базах данных Google Scholar, PubMed (MEDLINE), Scopus и Web of Science по ключевым словам и их сочетаниям с использованием программы AMSTAR 2 представлена роль гибели нейтрофилов в развитии иммунотромбозов. Изложены данные о типах запрограммированной гибели нейтрофилов, а именно апоптозе, пироптозе, некроптозе, ферроптозе и нейтрофильных внеклеточных ловушках (НВЛ), их молекулярных триггерах и механизмах. Представлена современная концепция патогенеза иммунотромбоза, которая демонстрирует вовлеченность в его патогенез гемостатических белков, тромбоцитов, провоспалительных клеток и учитывает сложную связь между гемостазом и воспалением. По каждому из типов запрограммированной гибели нейтрофилов показано участие в развитии иммунотромбоза. Наряду с этим, изложены сведения о терапевтических стратегиях с использованием иммунометаболических регуляторов и ингибиторов НВЛ, которые потенциально могут ослабить иммунотромботическую активность, не влияя на гемостаз. Такие методы лечения имеют преимущества для лечения пациентов при многих тромбовоспалительных состояниях. *Выводы*. В целом, продемонстрированные данные указывают, что апоптоз нейтрофилов обладает противотромботическим эффектом, тогда как остальные типы запрограммированной гибели способствуют развитию тромбов и сосудистых катастроф. К отличиям последних относятся специфические триггеры, а именно, для пироптоза цитокиновый шторм, для некроптоза выброс молекулярных паттернов смерти и для ферроптоза окислительный стресс. Объединяет указанные типы гибели способность запускать НВЛ, результатом чего является формирование триггерной основы развития тяжелых тромботических осложнений.

Ключевые слова: нейтрофилы, запрограммированная гибель клеток, иммунотромбоз, апоптоз, пироптоз, некроптоз, ферроптоз, нейтрофильные внеклеточные ловушки

Информация о финансировании. Работа выполнена при финансовой поддержке государственного задания «Структурные и клеточно-молекулярные механизмы возрастного ремоделирования соединительной ткани при заболеваниях опорно-двигательного аппарата» № 056-00055-24-00 от 14.01.2024 г.

Вклад авторов. Плехова Н.Г. — концепция и дизайн исследования, анализ полученных данных, редактирование рукописи; Михайлов А.О., Цветов Н.В. — сбор и обработка материалов; Воронова А.Н. — написание и перевод текста. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

Информация о конфликте интересов. Авторы заявляют об отсутствии конфликтов интересов.

Этическое утверждение — неприменимо.

Благодарности — неприменимо.

Информированное согласие на публикацию — неприменимо.

Поступила 08.04.2026. Принята 30.04.2026.

Для цитирования: *Plekhova N.G., Mikhailov A.O., Tsvetov N.V., Voronova A.N.* Programmed neutrophil death in immunothrombosis // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 301–313. doi: 10.22363/2313-0245-2026-30-3-301-313 EDN: JZDMMQ

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

DOI 10.22363/2313-0245-2025-30-3-314–327
EDN KBUNAB

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
ORIGINAL RESEARCH

Comparative characteristics of the molecular biological portrait of the head and neck squamous cell carcinoma tumor and peritumoral tissue

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Abstract: *Relevance*. Head and neck squamous cell cancer (HNSCC) accounts for about 890,000 new cases of malignant neoplasms (approximately 4.5% of all diagnosed cancer cases worldwide) and 450,000 deaths per year. Even with radical antitumor treatment, more than 40–50% of patients develop relapse, 20–30% of them develop distant metastases, and the five-year survival rate in advanced cases is 25%. Patients with recurrent HNSCC have an unfavorable clinical prognosis with a median overall survival of about 12 months. A high recurrence rate indicates the possible existence of a “source” of tumor cells, which may be located near the primary tumor, namely in the peritumoral tissue, which necessitates its study. The aim of the study was to compare the structure and expression of genes in the tumor and peritumoral tissues of HNSCC. *Materials and methods*. Biopsy material of tumor and peritumoral (located at a distance of 2 cm from the tumor border) tissues was obtained from 35 patients with HNSCC. Biopsies were examined by histological method, then the levels of mRNA expression of *EGFR, PIK3CA, PTEN, YAP, SLC26A6, ARIH2, UBE2Z, PITX1* genes were determined by real-time polymerase chain reaction in samples of tumor tissue and peritumoral region. The RNA Solo kit was used for RNA isolation, and the MMLV RT Kit (Evrogen, Russia) was used for reverse transcription. The amplification reaction with real-time detection was performed on a Real-Time DTprime amplifier (DNA Technology, Russia). *Results and discussion*. All tumor samples, regardless of anatomical localization, exhibited characteristic morphological features of squamous cell carcinoma. The peritumoral specimens represented visually unchanged tissue with

preserved epithelial architecture. The expression of *EGFR* and *PIK3CA* genes was higher in tumor tissue samples, while *PITX1* expression was higher in peritumoral tissue samples. *Conclusion*. Increased expression of *EGFR* and *PIK3CA* genes in tumor tissue confirms the activation of signaling pathways associated with tumor cell proliferation, survival, and disease progression. In contrast, the higher expression of the putative tumor suppressor gene *PITX1* in peritumoral tissue suggests the preservation of regulatory mechanisms limiting malignant transformation outside the tumor focus.

Keywords: squamous cell carcinoma of the head and neck, peritumoral tissue, tumor tissue, gene expression

Funding. This work was supported by the tripartite agreement of the Russian Science Foundation № 24-24-00296 dated 29.12.2023 on the project "The effect of proton therapy on the molecular portrait of the microenvironment of head and neck tumors" of the competition 2023 "Conducting basic scientific research and search scientific research by small individual scientific groups" between RSCF, Lokhonina A.V. and RUDN.

Author contributions. Jumaniyazova E.D. — the concept and design of the study, analysis of the data obtained, writing the text; Arutyunyan I.V. — the concept and design of the study, processing of the material and conducting the experiment; Soboleva A.G. — processing of the material and conducting the experiment, analysis of the data obtained; Gulieva M.A. — writing the text; Vishnyakova P.A. — text editing, analysis of the data obtained; Lokhonina A.V. — responsible for document management; Makarov A.V. — text editing; Gordon K.B. — collection of clinical material. All authors made a substantial contribution to the study and writing the manuscript, read and approved the final version before publication.

Conflicts of interest statement. The authors declare that there are no obvious or potential conflicts of interest related to the publication of this article.

Ethics approval. The study was approved by the Independent Ethics Committee (Protocol No. 634 dated 11/17/2021, Protocol No. 684 dated 03/02/2022).

Acknowledgements — not applicable.

Consent for publication. All patients signed an informed consent to participate in the study.

Received 10.06.2025. Accepted 01.07.2025.

For citation: *Jumaniyazova ED, Arutyunyan IV, Soboleva AG, Gulieva MA, Vishnyakova PA, Lokhonina AV, Makarov AV, Gordon KB.* Comparative characteristics of the molecular biological portrait of the head and neck squamous cell carcinoma tumor and peritumoral tissue. *RUDN Journal of Medicine.* 2026;30(3):314–327. doi: 10.22363/2313-0245-2026-30-3-314-327 EDN: KBUNAB

Introduction

The most common morphological form of tumors of the head and neck is squamous cell carcinoma. According to data published by GLOBOCAN (2020), head and neck squamous cell carcinoma (HNSCC) ranks sixth in prevalence among other types of malignant neoplasms in the world. According to current data, HNSCC accounts for an estimated 890 000 incident cases of malignant neoplasms per annum, corresponding to approximately 4.5% of the global incidence of diagnosed cancers, and approximately 450 000 cancer-related deaths annually [1]. This type of cancer is more common in adults over 50 years of age, with a male-dominated gender ratio (the ratio of men to women is approximately 2:1) [2]. To date, the increase in the incidence of acute respiratory viral infections among young able-bodied people has become socially significant. The main generally recognized etiological factors are tobacco smoking, alcohol consumption, infection with human papillomavirus (HPV) or Epstein-Barr virus (EBV), as well as betel chewing, common among the peoples of Southeast Asia. Clinically, HNSCC is characterized by late diagnosis, aggressive course, locally widespread

growth pattern, and frequent relapses [3]. The lack of screening leads to late diagnosis, complex anatomical localization complicates the radical surgical stage of treatment, and the proximity of critical structures leads to complications during RT. In the early stages of HNSCC (stages I and II), surgical treatment and RT are highly effective, with one- and two-year survival rates of 88.7 and 79.8%, respectively [4]. However, more than half of the patients (approximately 2/3 of the patients) already have a locally advanced form of the disease (stages III and IV) at the time of diagnosis, which is a prognostically unfavorable factor. Even with radical antitumor treatment, more than 40–50% of patients develop relapse, 20–30% of them develop distant metastases [5, 6], and the overall five-year survival rate is 25%. Patients with recurrent HNSCC have an unfavorable clinical prognosis with a median overall survival of about 12 months [7]. It was shown that in the most unfavorable cohort of patients (with a low–grade tumor negative for HPV type 16), the recurrence rate in the first six months after treatment is 50.6%, within 1 year — 72.5%, within 2 years — 88.6%. Such statistical data indicate the possible existence of a source of preserved or, conversely, induced by the therapeutic effect of tumor cells, which may be located near the localization of the primary tumor, namely, in the peritumoral tissue, which necessitates its study.

The aim of our study was to compare the structure and expression of genes in the tumor and peritumoral tissues of squamous cell carcinoma of the head and neck.

Based on a literature review, genes were selected that play a key role in tumor progression, including long-known regulators of tumor progression, as well as genes whose role in tumor growth has become known relatively recently.

Materials and methods

Characteristics of the biomaterial

The biopsy material of the tumor and peritumoral tissue (located at a distance of 2 cm from the tumor border) of the patients was obtained from the A. Tsyb Medical Radiological Research Center (MRSC). During the study, biomaterials from 35 patients obtained during the surgical stage of treatment were analyzed, information about patients is presented in the Table 1.

Biomaterial was obtained from patients meeting the following criteria:

1. Men and women over the age of 18.

2. Morphologically or radiologically verified diagnosis of squamous cell carcinoma in the head and neck (C00-C14, C30-C33).

3. General condition on the Karnovsky scale of at least 70 points.

4. Informed consent to participate in the study.

Table 1

General characteristics of patients

Parameters	Patients n, %
Total quantity	35 (100%)
Gender	
Men Women	22 (62.8%) 13 (37.1%)
Age (years)	
Median Range	57.3 19—83
Grade of malignancy	
G1 *G2* *G3*	25 (71.4.4%) 8 (22.8.8%) 2 (5.7.7%)

Ending tabl. 1

Parameters	Patients n, %
Keratinization	
Keratinizing	17 (48.57%)
Non-keratinizing	18 (51.4%)
T stage	
1	3 (8.57%)
2	15 (42.8%)
3	10 (28.5%)
4	7 (20%)
N stage	
0	9 (25.7%)
1	18 (51.4%)
2	8 (22.8%)
M stage	
0	22 (62.8%)
1	13 (37.2%)
Localization	
Oral cavity	15 (42.8%)
Tongue	8 (22.8%)
Larynx	10 (28.5%)
Maxillary sinus	2 (5.7%)

Primary processing of the received material

The material was thoroughly washed from possible contaminating agents in a phosphate-salt buffer containing 0.02% EDTA (Versin solution), then in a phosphate-salt buffer containing 1× antibiotic-antimycotic, then in a phosphate-salt buffer or Hanks solution without additives. In each solution, the tissue was washed 3 times. Then the material was divided into 2 unequal parts: the fragment was preserved in RNAlater solution for subsequent PCR-RT and stored at –80˚C until the study, the rest of the tissue was used for morphological examination.

Morphological examination

To verify the diagnosis of squamous cell carcinoma, a histological examination of the obtained tissue samples (tumor and peritumoral) was performed. Unfixed tissue samples were transferred to a foil mold filled with Tissue-Tek O.C.T. Compound (Sakura Finetek), frozen at –80 °C until the mounting medium completely solidified, then stored at –20 °C until use. Cryosections with a thickness of 5 microns were made using a Leica CM1200 cryotome using Super Frost Gold (Menzel) glasses and dried at room temperature for 1 hour. For routine histological examination, cryosections were stained with hematoxylin and eosin, dehydrated in standard wiring, and enclosed in a mounting medium (Biovitrum). The survey was carried out using a Leica DM 4000 B direct microscope.

PCR-RT

The levels of mRNA expression of *EGFR, PIK3CA, PTEN, YAP, SLC26A6, ARIH2, UBE2Z,* and *PITX1* genes were determined using real-time polymerase chain reaction in samples of tumor tissue and the peritumoral region (Table 2). The RNA Solo kit was used for RNA isolation, and the MMLV RT Kit (Evrogen, Russia) was used for reverse transcription. The amplification reaction with real-time detection was performed on a Real-Time DTprime amplifier (DNA Technology, Russia). The relative mRNA concentration of these genes was calculated by direct data comparison using

the formula: [A]0/[B]0 = E DC(T), where [A]0 is the initial concentration of the gene's mRNA in the PCR mixture, [B]0 is the initial concentration of *GAPDH* mRNA in the PCR mixture, E is the reaction efficiency (assumed to be 1.98), DC(T) is the difference between the threshold cycles of *GAPDH* and the desired gene.

Table 2

Sequences of used primers

Gene	Sequence	
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA
EGFR	Forward	CCCCCTGACTCCGTCCAGTA
	Reverse	CCCAACTGCGTGAGCTTGTT
PIK3CA	Forward	TTCCGGGGGATTGTAGGCTC
	Reverse	TTCCGGGGGATTGTAGGCTC
YAP	Forward	CAGCAACTCCAACCAGCAGC
	Reverse	CAGCAACTCCAACCAGCAGC
SLC26A6	Forward	AGACAGCCAGAGATGCTGCC
	Reverse	GTAGGTGACCACGAAGCCGA
UBE2Z	Forward	CGGCACGAGACCATCAGAGT
	Reverse	TGGTAGTCAAAGTGGCCCCG
PTEN	Forward	CCCAGTCAGAGGCGCTATGT
	Reverse	CCCAGTCAGAGGCGCTATGT
TIMP1	Forward	CCTTCCAGGTGTTTCCCTGTT
	Reverse	CCTTCCAGGTGTTTCCCTGTT
TIMP2	Forward	GACCCACAAGGAGATTGGGG
	Reverse	GACCCACAAGGAGATTGGGG
PITX1	Forward	AACCGCTACCCCGACATGAG
	Reverse	CTGCACTAGGCCGCTGAACT

Statistical methods

The statistical analysis of the obtained data was carried out in the Statistica 8.0 program, using the Shapiro – Wilk test to assess the normality of the distribution, the Mann – Whitney criteria and the t-test for pairwise comparison. The differences were considered statistically significant at a significance level of $p<0.05$.

Results and discussion

In all the presented samples of tumor tissue, extensive invasion of tumor cells into their own plate of the mucous membrane was noted, and the integrity of the basement membrane was disrupted (Figure 1, 2). Histological preparations stained with hematoxylin and eosin revealed a pattern characteristic of squamous cell carcinoma: abundant invasive growth of tumor cells into the underlying tissues in the form of rounded clusters, strands or individual cells. Normal architectonics were preserved in the peritumoral tissue samples: the multilayer epithelium is separated from its own plate of the mucous membrane by a preserved basement membrane (Figure 1, 2).

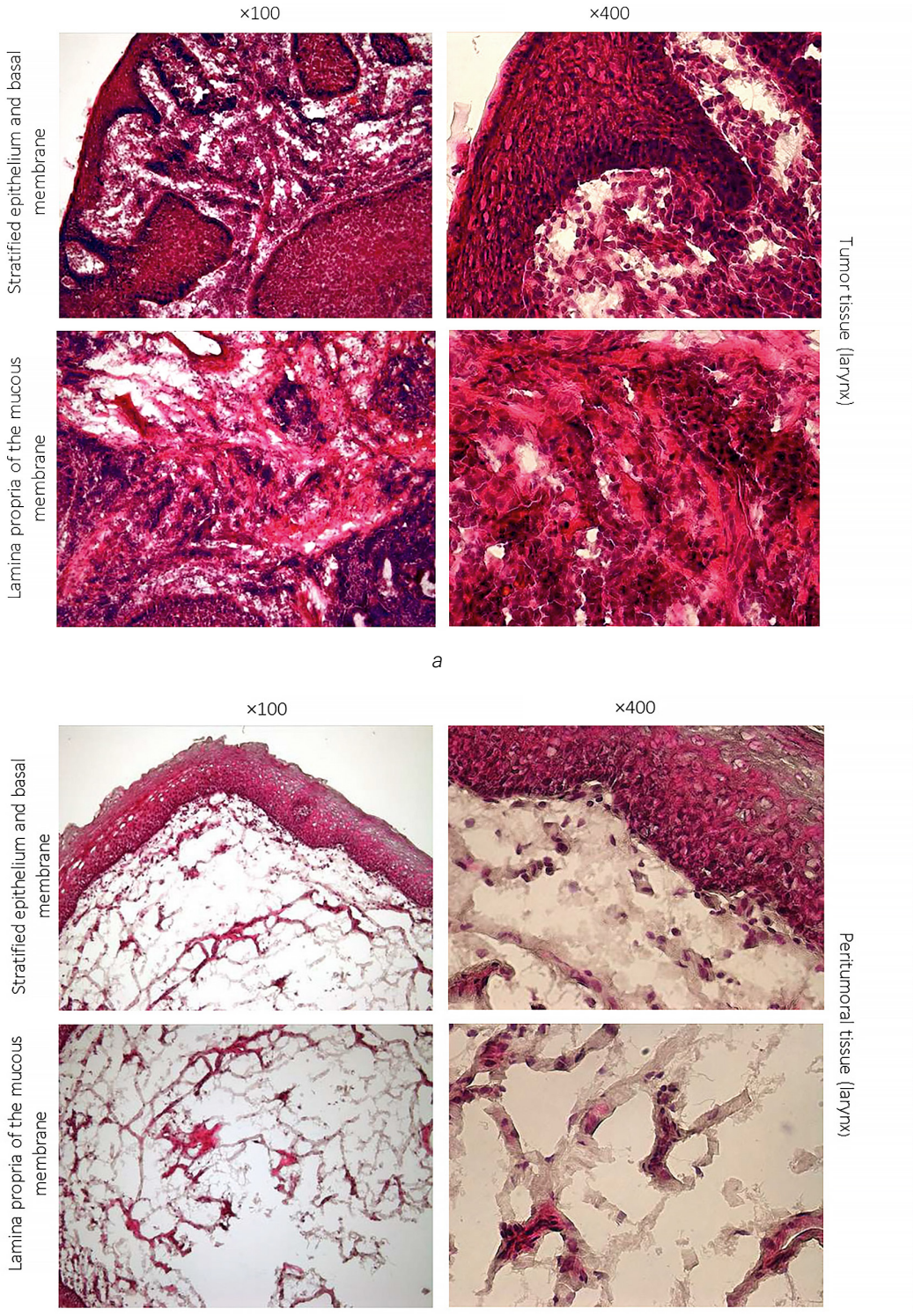
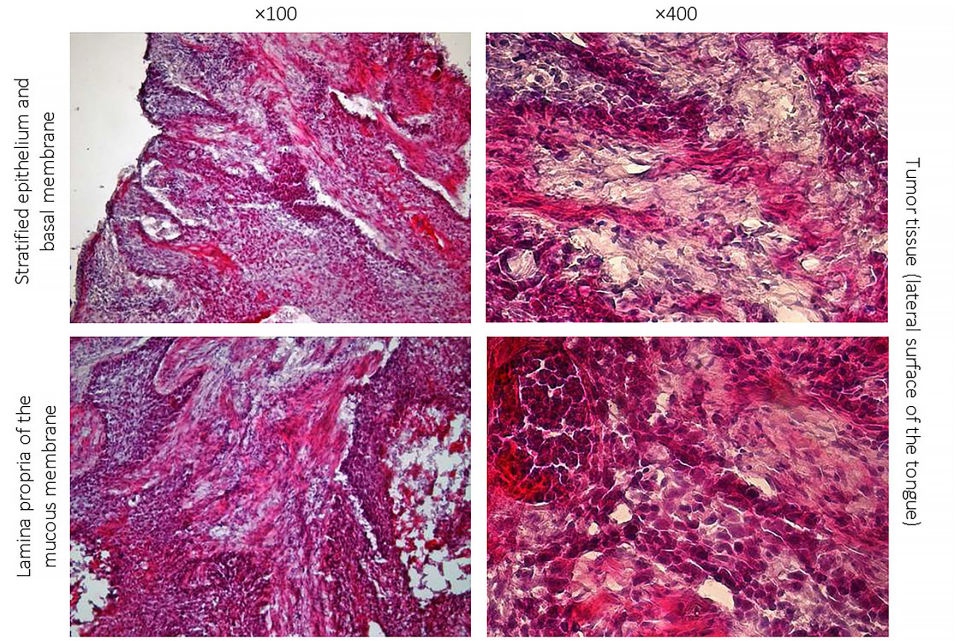
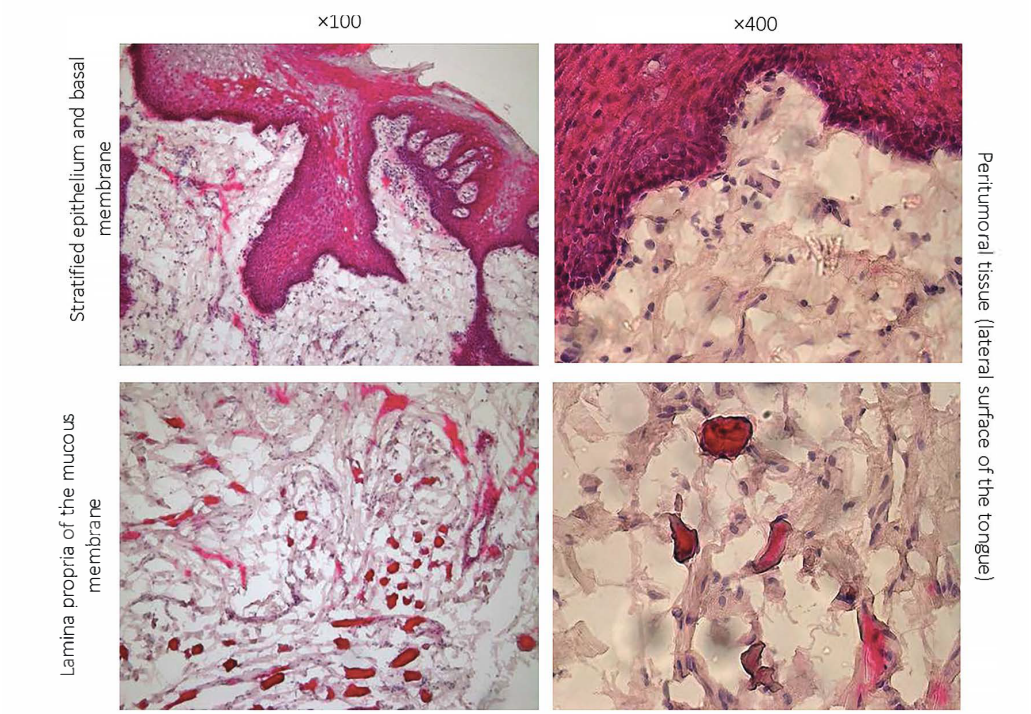


Figure 1. Squamous cell carcinoma of the larynx: tumor (*a*) and peritumoral (*b*) tissues. Hematoxylin and eosin staining. Light-field microscopy. The length of the scale segment is 100 microns and 50 microns



a



b

Figure 2. Squamous cell carcinoma of the tongue: tumor (*a*) and peritumoral (*b*) tissues. Hematoxylin and eosin staining. Light-field microscopy. The length of the scale segment is 100 microns and 50 microns

The levels of mRNA expression of the *PTEN*, *TIMP1*, *TIMP2*, *YAP*, *PIK3CA*, *EGFR*, *ARIH2*, *SLC26A6*, *UBE2Z*, and *PITX1* genes were evaluated using real-time polymerase chain reaction in tumor tissue and peritumoral tissue samples.

PTEN is a tumor suppressor gene that regulates the cell cycle and apoptosis by modulating the PI3K-PKB/Akt signaling pathway. Approximately 30% of cases of HNSCC have decreased *PTEN* expression. Moreover, the lack of *PTEN* expression often leads to the development of aggressive tumors and is associated with low disease-free and overall survival of patients [8, 9].

YAP is a transcriptional coactivator of genes involved in growth and tumor formation. Increased *YAP* expression is associated with an unfavorable prognosis of HNSCC and resistance to chemo and radiotherapy [10] (Figure 3).

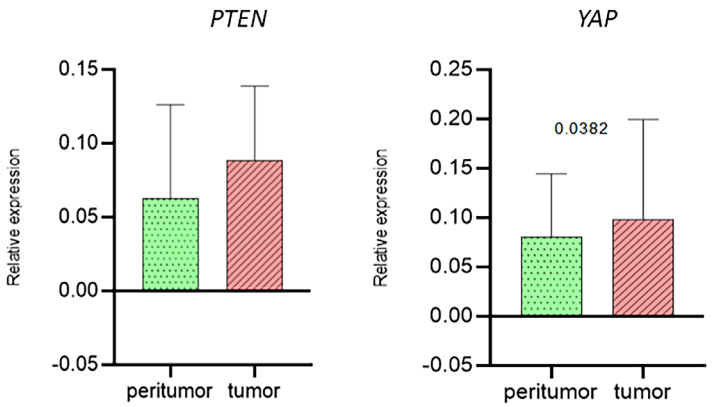


Figure 3. The level of relative expression of the *PTEN, YAP* genes in tumor and peritumoral tissues

Tissue metalloproteinase inhibitors (TIMPs) block the activity of matrix metalloproteinases, which leads to suppression of tumor progression [11], but their role in HNSCC is controversial. There was no significant difference in the expression of these genes in the samples of tumor and peritumoral tissues studied by us (Figure 4).

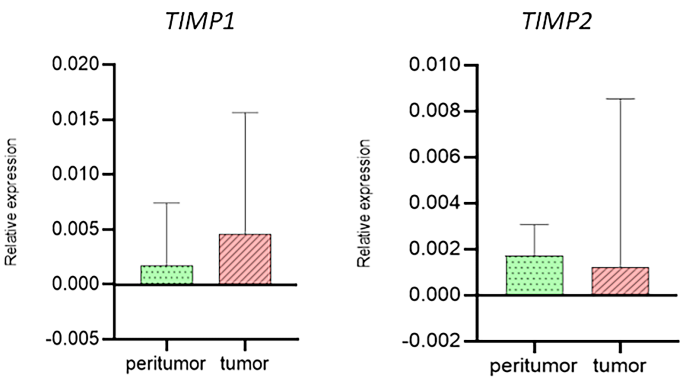


Figure 4. The level of relative expression of the *TIMP1* and *TIMP2* genes in tumor and peritumoral tissues

PIK3CA is one of the most frequently mutating oncogenes in PRGS: mutations were detected in 13.7% of cases (TCGA, Firehose Legacy). *PIK3CA* encodes p110a, a Class 1A PI3K catalytic subunit. With abnormal activation, PI3K stimulates several downstream signaling cascades, which leads to uncontrolled proliferation, survival, and migration of tumor cells [12].

We found that the expression level of the epidermal growth factor receptor (EGFR) is significantly higher in tumor tissue than in peritumoral tissue (Figure 5). *EGFR* is overexpressed in more than 90% of cases of HNSCC, and its increased expression correlates with an unfavorable outcome in patients [13]. Binding of ligands to EGFR leads to a conformational change in the receptor, followed by autoactivation of tyrosine kinase from the intracellular domain of the receptor. This process activates the intracellular signaling pathway, which leads to inhibition of apoptosis, activation of cell proliferation and angiogenesis, as well as to an increase in the potential for metastatic spread [14].

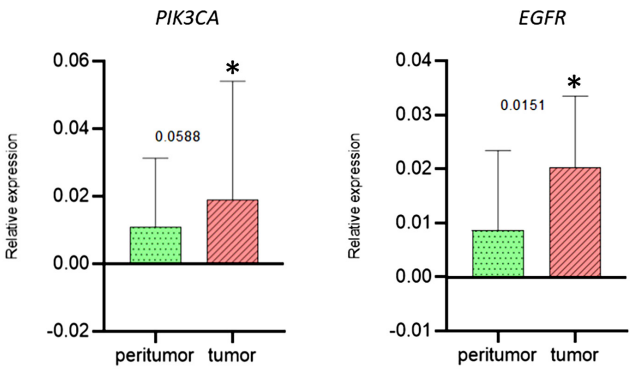


Figure 5. The level of relative expression of *PIK3CA* and *EGFR* genes in tumor and peritumoral tissues

Note: * — $p < 0.05$

The *ARIH2* gene is expressed everywhere, demonstrating the highest levels of expression in granulocytes [15]. Ubiquitin-protein ligase E3, encoded by the *ARIH2* gene, catalyzes the ubiquitination of target proteins and plays a key role in posttranslational modifications in various cellular processes. *ARIH2* is involved in DNA repair under genotoxic stress [16].

The family of solute carrier proteins 26 (SLC26) includes multifunctional transporters of substrates, including oxalate, sulfate, and chloride, and plays an important role in the physiology and pathophysiology of the kidneys [17]. One of the members of this family, SLC26A6, is of particular interest because it has some non-canonical properties. For example, it has recently been demonstrated that *SLC26A6* acts as an oncogene in hepatocellular carcinoma [18] and lung cancer [19]. In the studied samples, the expression levels of the *ARIH2* and *SLC26A6* genes were approximately the same in the tumor and peritumoral tissues (Figure 6).

Ubiquitination is one of the types of protein modification that modulates many cellular signaling processes, including DNA repair, transcription, cell membrane receptor recycling, intracellular transport, endocytosis, angiogenesis, and inflammatory signaling [20]. Ubiquitination occurs as a result of the joint work of three types of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), respectively (Upregulation of ubiquitin-conjugating enzyme E2Z is associated with human hepatocellular carcinoma). More and more studies show that most of the enzymes involved in ubiquitination play a significant role in the development of tumors [21]. Paired homeodomain transcription factor 1 (PITX1), also known as pituitary homeobox 1 (Ptx1), refers to highly conserved homeobox genes that encode sequence-specific transcription factors (SSTFS) that are involved in osteogenesis [22]. It is assumed that *PITX1* can act as a tumor suppressor gene and a potential biomarker for predicting the chemoresistance of tumor stem cells of HNSCC [23]. In our study, a decrease in the expression of this gene was noted in the samples of the tumor tissue of the HNSCC, compared with the tissue of the peritumoral region (Figure 7).

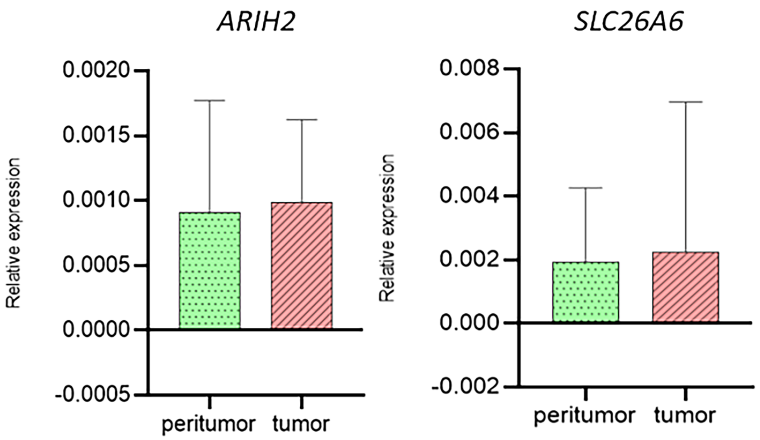


Figure 6. The level of relative expression of the *ARIH2, SLC26A6* genes in tumor and peritumoral tissues

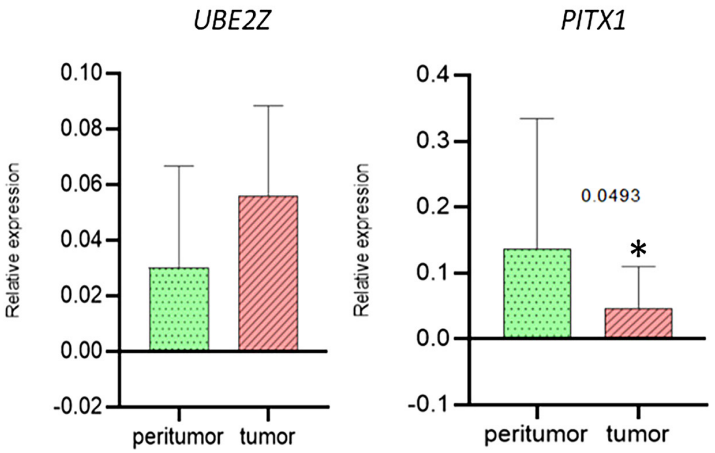


Figure 7. The level of relative expression of the *UBE2Z and PITX1* genes in tumor and peritumoral tissues

Note: * — $p < 0.05$

Conclusion

Increased expression of *EGFR* and *PIK3CA* genes in tumor tissue confirms the activation of signaling pathways associated with tumor cell proliferation, survival, and progression. In contrast, the higher expression of the putative tumor suppressor gene *PITX1* in peritumoral tissue suggests the preservation of regulatory mechanisms limiting malignant transformation outside the tumor focus.

The obtained results emphasize the importance of studying not only the tumor itself but also its surrounding peritumoral microenvironment, which may contribute to disease progression and recurrence. The identified molecular markers can be considered promising candidates for further studies aimed at improving prognostic assessment, identifying patients at high risk of relapse, and developing personalized therapeutic strategies for *HNSCC*.

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Сравнительная характеристика молекулярно-биологического портрета опухолевой и перитуморальной тканей плоскоклеточного рака головы и шеи

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Аннотация: *Актуальность*. На долю плоскоклеточного рака головы и шеи (ПРГШ) приходится около 890 000 новых случаев злокачественных новообразований (примерно 4,5 % всех диагностированных случаев в мире) и 450 000 смертей в год. Даже при проведении радикального противоопухолевого лечения у более чем 40–50 % пациентов развивается рецидив, у 20–30 % из них появляются отдаленные метастазы, а общая пятилетняя выживаемость составляет примерно 25%. Пациенты с рецидивами ПРГШ имеют неблагоприятный клинический прогноз с медианой общей выживаемости около 12 месяцев. Высокая частота местных рецидивов свидетельствует о возможном существовании «источника» опухолевых клеток, который может быть локализован недалеко от первичной опухоли, а именно — в перитуморальной ткани, что обуславливает актуальность ее изучения. *Цель исследования* — сравнительная оценка строения и экспрессии генов в опухолевой и перитуморальной тканях ПРГШ. *Материалы и методы*. Биопсийный материал опухолевой и перитуморальной (расположенной на расстоянии 2 см от границы опухоли) тканей получен от 35 пациентов с ПРГШ. Образцы биоптатов последовательно исследовали: на первом этапе — гистологическим методом, на втором — методом полимеразной цепной реакции; в образцах опухолевой ткани и перитуморальной области определяли уровни экспрессии мРНК генов *EGFR, PIK3CA, PTEN, YAP, SLC26A6, ARIH2, UBE2Z, PITX1*. Для выделения РНК использовали набор RNA Solo, а для обратной транскрипции — MMLV RT Kit (все — «Евроген», Россия). Реакцию амплификации с детектированием в режиме реального времени проводили на Real-Time амплификаторе DTprime («ДНК-Технология», Россия). *Результаты и обсуждение*. Все образцы опухоли, вне зависимости от анатомической локализации, имели характерную для плоскоклеточного рака картину. Образцы перитуморальной зоны представляли собой визуально неизмененную ткань с сохранной архитектоникой эпителия. Экспрессия генов *EGFR, PIK3CA* была выше в образцах опухолевой ткани, в то время как экспрессия *PITX1* была выше в образцах перитуморальной ткани. *Выводы*. Повышенная экспрессия генов *EGFR* и *PIK3CA* в опухолевой ткани указывает на активацию сигнальных путей, связанных с пролиферацией, выживанием клеток и опухолевой прогрессией. Напротив, более высокая экспрессия предполагаемого гена-супрессора опухолей *PITX1* в перитуморальной ткани указывает на сохранение регуляторных механизмов, ограничивающих злокачественную трансформацию за пределами опухолевого очага.

Ключевые слова: плоскоклеточный рак головы и шеи, перитуморальная ткань, опухолевая ткань, экспрессия генов.

Информация о финансировании. Работа выполнена при поддержке трёхстороннего соглашения Российского научного фонда № 24-24-00296 от 29.12.2023 по проекту «Влияние протонной терапии на молекулярный портрет микроокружения опухолей головы и шеи» конкурса 2023 года «Проведение фундаментальных научных исследований и поисковых научных исследований малыми индивидуальными научными коллективами» между РНФ, Лохониной А.В. и РУДН.

Вклад авторов. Джуманиязова Э.Д. — концепция и дизайн исследования, анализ полученных данных, написание текста; Арутюнян И.В. — концепция и дизайн исследования, обработка материала и проведение эксперимента; Соболева А.Г. — обработка материала и проведение эксперимента, анализ полученных данных; Гулиева М.А. — написание текста; Вишнякова П.А. — редактирование текста, анализ полученных данных; Лохонина А.В. — ответственная за документооборот; Макаров А.В. — редактирование текста; Гордон К.Б. — сбор клинического материала. Все авторы внесли существенный вклад в проведение исследования и подготовку рукописи, прочитали и одобрили окончательную версию перед публикацией.

Информация о конфликте интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией данной статьи.

Этическое утверждение. Исследование одобрено независимым комитетом по этике (протокол № 634 от 17.11.2021, протокол № 684 от 02.03.2022).

Благодарности — неприменимо.

Информированное согласие на публикацию. Все пациенты подписали информированное согласие на участие в исследовании.

Поступила 10.06.2025. Принята 01.07.2025.

Для цитирования: *Jumaniyazova E.D., Arutyunyan I.V., Soboleva A.G., Gulieva M.A., Vishnyakova P.A., Lokhonina A.V., Makarov A.V., Gordon K.B.* Comparative characteristics of the molecular biological portrait of tumor and peritumoral tissue of the head and neck squamous cell carcinoma // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 314–327. doi: 10.22363/2313-0245-2026-30-3-314-327 EDN: KBUNAB

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

DOI 10.22363/2313-0245-2025-30-3-328-338
EDN KMPBFW

REVIEW
ОБЗОР

Typing of adaptive immunity cells: markers of populations and their functional significance

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Abstract. *Relevance.* Single-cell sequencing technologies (scRNA-seq) provide unique opportunities for studying the heterogeneity of immune populations, enabling the analysis of expression profiles, ligand-receptor interactions, and differentiation trajectories at the single-cell level. However, the quality of cell typing significantly depends on the accuracy of data annotation. Marker ambiguity, variability in their expression depending on physiological context, limitations of automated tools such as CellTypist, Azimuth, and SingleR, and the incompleteness of databases like PanglaoDB and CellMarker, including insufficient information on the functional roles of markers, substantially hinder the identification of cell populations and require additional efforts to achieve meaningful results. *Aim.* To systematize markers of T- and B-cells of the adaptive immune response to facilitate scRNA-seq data annotation. *Materials and Methods.* An analysis of 30 scientific publications from PubMed, Scopus, and Web of Science (2008–2024) and marker data from PanglaoDB and CellMarker was conducted. *Results and discussion.* Key and additional T-cell (*CD3D, CD3E, CD3G, CD4, CD8A, CD8B, FOXP3, GZMA, TBX21*) and B-cell (*CD19, MS4A1, CD27, IGHM, IGHD*) markers, their roles in immune processes, including activation, cytotoxicity, and regulation, were examined. Subpopulations such as Th1 cells (T-bet, IFN-γ), follicular helper cells (CXCR5, BCL-6), and regulatory B-cells (CD24, IL-10) and their functions in health and disease were described. *Conclusion.* The systematization of T- and B-cell markers was developed to improve the quality of single-cell sequencing data annotation and is applicable for enhanced typing of cell functional states in health and pathological conditions, including infectious, autoimmune, and oncological diseases. This opens opportunities for a deeper understanding of immune processes and the development of immunotherapy approaches, such as CAR-T therapy. However, the limited specificity of markers and the lack of standardized annotation algorithms highlight the need for further research to refine typing methods.

Keywords: T-cells, B-cells, adaptive immunity, single-cell sequencing, immune markers, typing, immunotherapy

Funding. This project was supported by the Russian Science Foundation [grant no. 22–75–10128].

Author Contributions: Fedorov A.A. — literature collection and analysis, table preparation, manuscript writing; Fedorenko A.A. — database analysis, manuscript writing; Patysheva M.R. — conceptualization, data analysis, editing; Gerashchenko T.S. — manuscript review and editing.

Conflict of interest statement. The authors declare no conflict of interest.

Ethical approval — not applicable.

Acknowledgments — not applicable.

Consent for publication — not applicable.

Received 04.07.2025. Accepted 01.08.2025.

For citation: Fedorov AA, Fedorenko AA, Patysheva MR, Gerashchenko TS. Typing of adaptive immunity cells: markers of populations and their functional significance. *RUDN Journal of Medicine*. 2026;30(3):328–338. doi: 10.22363/2313-0245-2026-30-3-328-338 EDN: KMPBFW

Introduction

Deciphering immune cell composition and function is key to understanding both normal immunity and its disruption in disease [1]. Modern sequencing technologies, such as single-cell RNA sequencing (scRNA-seq), provide unprecedented opportunities to investigate immune populations by enabling the analysis of gene expression profiles, ligand–receptor interactions, and differentiation trajectories at single-cell resolution [2]. In the field of cancer research, the analysis of immune cell types through scRNA-seq holds great importance, as the makeup and functional traits of the tumor microenvironment (TME) affect molecular diagnostics, the creation of targeted treatments, and the prognosis of the disease. For instance, identifying T-cell subpopulations with exhaustion markers (PD-1$^+$, LAG-3$^+$) in the TME indicates immunosuppression, making immune checkpoint inhibitors, such as anti-PD-1 antibodies, a preferred therapeutic strategy to restore immune function. High infiltration of CD8+ cytotoxic T cells is associated with favorable prognosis in breast cancer and other malignancies, whereas a predominance of regulatory T cells (FOXP3$^+$, CD25$^+$) may necessitate combined approaches, such as CTLA-4 inhibitors or CAR-T cell therapies, to overcome immunosuppression [1, 3]. Identifying markers such as CXCR5$^+$ and BCL-6$^+$ for follicular helper T cells aids in assessing humoral immune activity and selecting patients likely to respond to immunotherapy [3, 4].

The advancement of single-cell sequencing technologies has led to the development of numerous computational tools, including automated annotation methods such as CellTypist, Azimuth, and SingleR, which aim to streamline and standardize this process [5–7]. While these tools are widely used for basic immune cell population typing, they have limitations in performing detailed subpopulation analysis. They often lack information on expression variability across different physiological states and do not provide insights into the origin or functional roles of specific markers. As a result, accurately distinguishing clusters into biologically meaningful cell populations can be challenging. To achieve higher-resolution annotations, manual reclustering is frequently required, typically guided by expert knowledge and supported by curated cell marker databases such as PanglaoDB, CellMarker, and MonacoImmuneData [8–10]. However, these resources often lack detailed information on the functional roles and expression contexts of markers, limiting the depth and accuracy of annotations and necessitating additional manual literature searches. Emerging tools such as CellMeSH, which utilize indexed scientific literature for probabilistic cell type annotation, offer a promising solution to these challenges but have not yet become part of standard practice [11].

Given the limitations of current automated annotation methods and the incomplete characterization of immune cell markers in existing databases, this review aims to systematize key markers of T and B lymphocytes within the adaptive immune system, taking into account their developmental origin,

functional roles, and transcriptional context. As central mediators of adaptive immunity, T and B lymphocytes play critical roles in shaping the antitumor immune response, thereby influencing treatment outcomes and disease prognosis. Markers of innate immune cells will be discussed in a forthcoming publication. This review serves as a complementary resource for the accurate annotation and interpretation of single-cell RNA sequencing (scRNA-seq) data, supporting more reliable identification of immune cell populations.

T cells

T cells are central components of the adaptive immune system, providing protection against pathogens and maintaining immune homeostasis. They originate in the bone marrow from a common lymphoid progenitor (CLP) and undergo maturation in the thymus through processes of positive and negative selection, ultimately giving rise to functionally competent $CD4^+$ and $CD8^+$ T cells (Figure) [3].

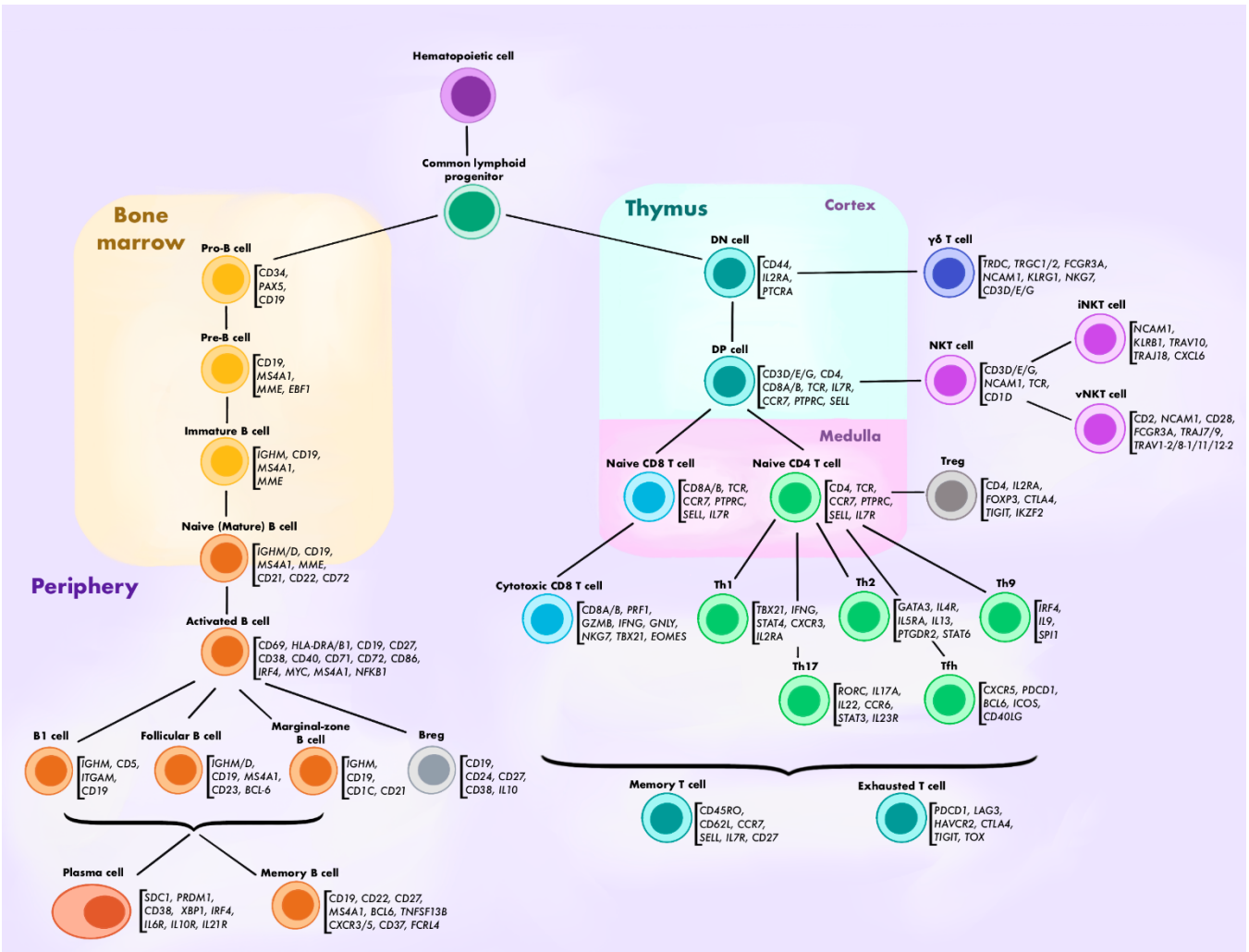

Figure. Markers of adaptive immune cell populations. The figure shows a scheme of adaptive immune cell differentiation, with key markers indicated at various stages. Colors represent the maturation sites of different cell populations

T-cell development in the thymus

T cell differentiation begins with the migration of hematopoietic progenitor cells from the bone marrow to the thymus, where they undergo maturation and selection. In the thymus, thymocytes undergo development through three main stages: double-negative (DN), double-positive (DP), and single-positive (SP).

At the double-negative stage (DN, DN1-DN4), thymocytes lack expression of *CD4* and *CD8A*/*CD8B* coreceptors but exhibit variable levels of *CD44* and *IL2RA*. A critical milestone is the DN3 stage, where the proto-TCR (*PTCRA*) begins to be expressed, enabling progression to the double-positive stage [3, 12].

During the double-positive (DP) stage, T cells co-express *CD4* and *CD8A/CD8B* and undergo selection based on the specificity of their T cell receptor (TCR). Depending on the outcome, cells differentiate into $CD4^+$ or $CD8^+$ lineages. At this stage, molecules such as *CCR7* and *PTPRC* are expressed, playing key roles in cell migration and functional maturation [13].

At the single-positive stage (SP), T lymphocytes acquire either a $CD4^+$ or $CD8^+$ naive T-cell phenotype. The differentiation trajectory is determined by TCR interactions with MHC class I or II molecules. The cytokine microenvironment and activation of transcription factors, such as *Runx3* for $CD8^+$ and *GATA-3* for $CD4^+$, are pivotal in this process [14]. Following the SP stage, NKT cells may emerge, serving regulatory functions at the interface of innate and adaptive immunity.

Peripheral activation and memory cell formation

Following their maturation in the thymus, naïve T cells circulate to peripheral lymphoid tissues, where antigen presentation triggers their activation and differentiation into effector T cells. Some activated T cells directly eliminate pathogens or infected cells, while others differentiate into memory T cells, including central memory, effector memory, and tissue-resident memory subsets. These memory cells persist in lymphoid organs and tissues, ensuring a faster and more effective response upon re-exposure to antigens. During memory formation, both $CD4^+$ and $CD8^+$ T cells may express similar markers, such as *SELL*, *CCR7*, and *PTPRC* [13].

T-cell activation and specialization are orchestrated by networks of transcription factors (e.g., *TBX21*, *GATA3*, *FOXP3*), epigenetic mechanisms (e.g., DNA methylation, histone modification), and metabolic shifts, such as transitions between glycolysis and oxidative phosphorylation [14].

$CD4^+$ T-helper cells differentiate into various subpopulations under the influence of specific cytokines and signaling pathways: Tfh, Th1, Th2, Th9, Th17, and Tregs. Follicular helper T cells (Tfh), expressing *CXCR5*, *PDCD1*, and *BCL6*, regulate B-cell maturation and germinal center formation [3, 15]. Th1 cells, characterized by *TBX21* expression and IFN-γ production, are critical for defense against intracellular pathogens by activating macrophages [3]. Th2 cells express *GATA3* and produce IL4, IL5, and IL13, promoting humoral immunity and stimulating B cells to produce antibodies [3]. Th9 cells, associated with *IRF4*, *SPI1*, and *IL9*, contribute to defense against helminths and are involved in allergic responses [14]. Th17 cells, expressing *RORC* and producing IL17A and IL22, protect mucosal surfaces against fungi and bacteria [3]. Regulatory T cells (Tregs), expressing *CD4*, *IL2RA*, and *FOXP3*, suppress excessive immune responses and prevent autoimmune reactions by secreting IL-10 and TGF-β [3, 16, 17].

$CD8^+$ cytotoxic T cells are key in eliminating virus-infected and transformed cells through the production of cytotoxic proteins (granzymes, perforin) and antiviral cytokines, such as IFN-γ. Upon activation, they form pools of effector and memory cells, regulated by transcription factors *TBX21* and *EOMES* [3].

Additional subpopulations include γδ T cells, which express *TRDC*, *TRGC1*, *TRGC2* and are capable of rapid antigen recognition independent of MHC presentation. [18, 19]. These cells are divided into subsets such as $V\delta1^+$ (tissue surveillance) and $V\delta9V\delta2^+$ (mucosal protection). NKT cells combine features of NK and T cells, expressing *CD3D*, *CD3E*, *CD3G* and NK markers (*NCAM1*) [20]. They recognize lipid antigens presented by *CD1D* and differentiate into functional subsets: NKT1 (*TBX21*, *IFNG*), NKT2

(*GATA3*, *IL-4*), and NKT17 (*RORC*, *IL-17*) [21]. Two types of NKT cells are distinguished: invariant NKT (iNKT) and variable NKT (vNKT). iNKT cells express an invariant TCR composed of the *TRAV10*, *TRAJ18* α-chain and co-express markers such as *CD3D*, *CD3E*, *CD3G*, *NCAM1*, *KLRB1*, and *CXCR6*. They recognize lipid antigens via *CD1D* and swiftly secrete diverse cytokines such as IFN-γ, IL-4, and IL-17, bridging innate and adaptive immunity [20,22]. vNKT cells have more diverse TCRs (e.g., *TRAV12–2*, *TRAJ9*, *TRAV1–2*, *TRAJ7*, *TRAV8–1*, *TRAV11*) and express *CD3D*, *CD3E*, *CD3G*, *NCAM1*, *FCGR3A*, *CD2*, and *CD28*. These cells produce proinflammatory mediators such as TNF-α and IL-2, playing a significant role in infection control and tumor immune surveillance [20].

T-cell functional activity is defined by various markers. Cytotoxicity is characterized by the expression of granzymes (*GZMA*, *GZMB*), *PRF1*, *FASLG*, and *TNFSF10*, particularly in $CD8^+$ and γδ T cells [23]. Cytokine secretion, such as IFN-γ, TNF-α, IL-2, and IL-17, varies by subpopulation, with Th1 cells producing IFN-γ and Th2 cells producing IL-4 and IL-5 [1]. T-cell activation and proliferation are accompanied by the expression of *IL2RA* and *MKI67*, reflecting cell division in response to antigenic stimulation. Apoptosis is regulated by molecules such as *FAS*, *TNFRSF10A*, *TNFRSF10B*, and *CASP*, preventing excessive inflammation [3]. In conditions of chronic antigenic stimulation, such as viral infections or tumors, T cells may exhibit exhaustion, marked by the expression of inhibitory receptors like PD-1, CTLA-4, TIM-3, and LAG-3 [24]. A list of T-cell subpopulation markers and their functional significance is provided in Table 1.

Table 1

Markers and functional significance of T-cell subpopulations

Subpopulation/Stage	Main markers	Additional markers	Functional significance of markers	References
Double negative cells (DN1–DN4)	*CD44, IL2RA* (CD25), *PTCRA* (pre-TCRα)	–	Absence of CD4 and CD8 indicates early development; CD44 and CD25 regulate migration and proliferation on DN1–DN4 substages; PTCRA on DN3 initiates transition to DP stage	[3, 12]
Double positive cells (DP	*CD4, CD8A, CD8B, TCR, CCR7, PTPRC* (CD45RA), *SELL* (CD62L), *CD3D, CD3E, CD3G, IL7R* (CD127)	–	Simultaneous expression of CD4 and CD8 reflects TCR specificity testing; CD3D/E/G form TCR complex; CCR7 supports migration	[13]
Single positive cells (SP), naive T-cells	*CD4, CD8A/CD8B, TCR, CCR7, PTPRC* (CD45RA), *SELL* (CD62L), *IL7R* (CD127)	CD27, CD28	Differentiation into CD4+ or CD8+ upon interaction with MHC–II or MHC–I; CCR7 and CD62L ensure migration to lymphoid organs	[14]
$CD4^+$ Tfh (follicular helper)	*CXCR5, PDCD1* (PD-1), *BCL6, ICOS, CD40LG* (CD40L)	MAF, IL21R	Support B-cell maturation and germinal center formation; ensure antibody class switching	[3 ,15]
$CD4^+$ Th1	*TBX21* (T-bet), *IFNG* (IFN-γ), *STAT4, CXCR3, IL2RA* (CD25)	IL18R1, IL18RAP, CXCR6	Activate macrophages; form antiviral immunity and immunity against intracellular infections	[3]
$CD4^+$ Th2	*GATA3, IL13, PTGDR2* (CRTH2), *STAT6, IL4R, IL5RA*	*IL10, IL1RL1*(ST2)	Support humoral immunity; stimulate antibody production (IgE)	[3]
$CD4^+$ Th9	*IRF4, SPI1* (PU.1), *IL9*	*STAT5, STAT6, TGFBR1* (TGF-βR)	Protection against helminths; participation in allergic reactions	[3,14]

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Subpopulation/Stage	Main markers	Additional markers	Functional significance of markers	References
CD4⁺ Th17	*RORC* (RORγt), *IL17A* (IL-17), *IL22, CCR6, STAT3, IL23R*	IL21, CCL20	Protection of mucosal surfaces from fungi and bacteria; regulation of neutrophil recruitment and activation	[3]
Treg (regulatory T-cells)	*CD4, IL2RA* (CD25), *FOXP3, CTLA4, TIGIT, IKZF2* (Helios)	*CD127, IL10, TGFB1* (TGF-β)	Suppression of autoimmune reactions; maintenance of tolerance to autoantigens (via IL-10, TGF-β)	[3, 16]
CD8+ cytotoxic (effector T-cells)	*CD8A, CD8B, PRF1* (Perforin), *GZMB* (Granzyme B), *IFNG* (IFN-γ), *GNLY, NKG7, TBX21* (T-bet), *EOMES*	GZMK, KLRG1	Destruction of infected and tumor cells via perforin and granzymes; participation in antiviral immunity (via IFN-γ synthesis)	[3, 23]
γδ T-cells	*TRDC, TRGC1, TRGC2, FCGR3A* (CD16), *NCAM1* (CD56), *CD3D, CD3E, CD3G, KLRG1, NKG7*	IL17A, CCR5	Rapid antigen response without MHC presentation; tissue surveillance (Vδ1+) and mucosal protection (Vδ9Vδ2+)	[18, 19]
NKT-cells	*CD3D, CD3E, CD3G, NCAM1* (CD56), *TCR, CD1D*		Combine functional properties of T- and NK-cells; secretion of cytokines (IFN-γ, IL-4, IL-17) in response to lipid antigens	[20, 21]
iNKT (invariant)	*CD3D, CD3E, CD3G, NCAM1* (CD56), *TRAV10, TRAJ18* (TCR Vα24-Jα18), *KLRB1* (CD161), *CXCR6, NCAM1*	*IFNG* (IFN-γ), *IL4, IL17A* (IL-17)	Immunoregulation, rapid cytokine response, antitumor activity.	[20, 22]
vNKT (variable)	*TRAV12–2, TRAJ9, TRAV1–2, TRAJ7, TRAV8–1, TRAV11* (TCR Vα3.2-Jα9, Vα1-Jα7, Vα8, Vα11), *FCGR3A* (CD16), *CD3D, CD3E, CD3G, NCAM1* (CD56), *CD2, CD28*	TNF, IL2	Antiviral and antitumor activity, immune response modulation.	[20]
Memory T-cells	*CD45RO, CD62L, CCR7, SELL* (CD62L), *IL7R* (CD127), *CD27*	KLRG1, CX3CR1	Ensure enhanced and rapid secondary immune response; divided into central (TCM), effector (TEM), and resident (TRM) cell	[3]
Exhausted T-cells	*PDCD1* (PD-1), *CTLA4, HAVCR2* (TIM-3), *LAG3, TIGIT, TOX*	*EOMES, CD244* (2B4)	Indicate T-cell dysfunction under chronic antigenic stimulation (tumors, viral infections); loss of effective proliferation and cytotoxic response	[3, 24]

Notes: Main markers are associated with the specific subpopulation, functionally relevant, and used for cell identification in flow cytometry and scRNA-seq. Additional markers are less specific, support biological interpretation, may vary depending on the cell state, and are validated by the CellMarker 2.0 and PanglaoDB databases.

B cells

B cells are key components of adaptive immunity, responsible for humoral responses through antibody secretion and antigen presentation. Derived from CLPs they mature in the bone marrow through immunoglobulin (Ig) gene rearrangement, forming unique B-cell receptors (BCR). Their heterogeneity is reflected in diverse functional subtypes, differentiation stages, and activation statuses, identifiable by distinct surface and transcriptional markers [25, 26].

B-cell development in the bone marrow

B-cell development is divided into early (pro-B, pre-B) and late (immature, mature) stages. In the bone marrow, pro-B cells, characterized by *CD34*, *CD19*, and the transcription factor *PAX5*, initiate heavy-chain immunoglobulin gene rearrangement under the influence of EBF1 and RAG1/2 enzymes [25,27]. Successful heavy-chain rearrangement leads to pre-B cells, which express C*D19*, *MS4A1*, *MME,* and components of the pre-B cell receptor (pre-BCR) including *IGHM* (μ chain), *VPREB1* and *IGLL1* (λ-like chain). The pre-BCR comprises an immunoglobulin μ heavy chain and a surrogate light chain formed by VPREB1 and λ5 proteins. Interactions between these components activate signaling pathways via adapter molecules Igα and Igβ, promoting pre-B cell proliferation and initiating light-chain gene rearrangement [25, 28]. During B cell development, cells expressing *IGHM*, *CD19*, *MS4A1*, *MME*, and *CD22*, undergo negative selection to eliminate autoreactive clones, a process mediated by BCR recognition of autoantigens and signaling through *CD79A* [25, 28]. Mature B cells, co-expressing IgM and IgD, complete maturation and migrate to peripheral lymphoid organs, such as lymph nodes and the spleen, guided by chemokines like CXCR4 [25, 27]. Mature B cells remain naïve until antigen encounter and are generally defined by the absence of CD27 and the presence of IGHD expression [25].

Peripheral activation and memory cell formation

In peripheral lymphoid organs, mature B cells are activated upon antigen binding to the BCR, triggering signaling cascades via *CD79A*/*CD79B* and activation of transcription factors such as *NFKB1* and *IRF4*. T-dependent activation requires co-stimulation from T-helper cells through CD40-CD40L interactions and cytokine secretion (e.g., IL-4, IL-21, BAFF), activating STAT5 and STAT6 pathways to promote proliferation and differentiation. This process leads to germinal center formation, where B cells expressing *BCL6* undergo somatic hypermutation and class-switch recombination, producing high-affinity antibodies of IgG, IgA, or IgE isotypes. T-independent activation, induced by polyvalent antigens like bacterial polysaccharides, relies on signals through CD21 and Toll-like receptors, activating NF-κB. Activated B cells express *CD69* and *HLA-DRA*/*HLA-DRB1*, reflecting their proliferative and antigen-presenting status [25, 28].

Upon activation, B cells differentiate into plasma cells expressing *SDC1* and *PRDM1*, which secrete antibodies (IgM, IgG, or IgA) to neutralize pathogens. Others become memory B cells, marked by *CD27* and *BCL6*, ensuring faster and stronger immune responses during subsequent encounters with the same antigen [25, 29].

B cells demonstrate considerable functional and phenotypic diversity, resulting in the formation of specialized subpopulations with unique immunological functions (Figure 1). Follicular B cells, expressing *FCER2* and *BCL6*, localize in lymph nodes and participate in T-dependent immune responses, forming germinal centers. Marginal zone B cells, predominantly in the spleen, express high levels of *CR2*, *IGHM* and *CD1c*, providing rapid T-independent responses to blood-borne pathogens. B1 cells, located in serous cavities, produce natural antibodies (IgM^{hi}) and express *CD5*, playing a key role in early immune responses to infections [25, 27].

Regulatory B cells (Bregs) are critical for maintaining immune tolerance, suppressing excessive Th1/Th17 responses through the secretion of IL-10, TGF-β, and IL-35. They express *CD19*, *CD24*, *CD38*, and *CD27* and are activated by CD40L, CpG, and IL-21 signals [26, 30, 31].

B-cell functional activity encompasses three main roles. First, antibody production is carried out by plasma cells expressing *SDC1* and *PRDM1*, which secrete IgM, IgG, or IgA antibodies to neutralize pathogens. Second, antigen presentation is mediated by activated B cells expressing *HLA-DRA*/*HLA-DRB1* and *CD69*, which efficiently interact with T-helper cells to enhance adaptive immune responses. Third, immunosuppression is driven by Bregs and transitional B cells through IL-10, TGF-β, and IL-35 secretion, as well as PD-L1 expression, suppressing effector T cells and preventing autoimmune reactions. These functions are regulated by transcription factors (*PAX5*, *BCL6*, *PRDM1*, *IRF4*, *NFKB1*) and epigenetic modifications, such as DNA methylation [25, 29]. A list of B-cell subpopulation markers and their functional significance is provided in Table 2.

Table 2

Markers and functional significance of B-cell subpopulations

Subpopulation/Stage	Main markers	Additional markers	Functional significance of markers	References
Pro-B cells	CD34, CD19, PAX5	*DNTT* (TdT), *RAG1, RAG2*	Initiate heavy chain immunoglobulin rearrangement for BCR formation	[25, 27]
Pre-B cells	*CD19, MS4A1* (CD20), *MME* (CD10), *EBF1*	*CD79A* (Igα), *CD79B* (Igβ)	Complete light chain rearrangement, forming a functional BCR	[25, 27, 28]
Immature B-cells	*IGHM* (IgM), *CD19, MS4A1* (CD20), *MME* (CD10)	*CD22, CD79A* (Igα)	Undergo negative selection to eliminate autoreactive clones	[25, 27, 28]
Naive (Mature) B-cells	*IGHM* (IgM), *IGHD* (IgD), *CD19, CD21, CD22, CD72, PAX5, TCL1A, IL21R, TNFRSF13C* (BAFFR)	*PTPRC* (CD45), *CD79A* (Igα), *CD79B* (Igβ), *MS4A1* (CD20)	Maintain antigen-independent status, readiness for activation in peripheral lymphoid organs	[25, 27, 28]
Follicular B-cells	*CD19, MS4A1* (CD20), *CD23, IGHM* (IgM), *IGHD* (IgD)	BCL6	Participate in T-dependent responses, form germinal centers	[25, 27]
Marginal zone B-cells	*CD19, CD21, IGHM* (IgM), *CD1C*	*MS4A1* (CD20)	Provide T-independent response to pathogens circulating in peripheral blood	[25, 27, 29]
B1-cells	*CD19, CD5, ITGAM* (CD11b), *IGHM* (IgM)	CD43	Produce natural antibodies, participate in early immune response	[25, 27]
Memory B-cells	*CD19, CD27, MS4A1* (CD20), *BCL6, CD22, TNFSF13B* (CD30L), *CD37, CD39, CD44, CD73, CD80, CD86, CXCR3, CXCR5, FCRL4*	*CD72, CD79B* (Igβ), *CD82, FAS* (CD95), *EBI2, ITGAX* (CD11c), *PDCD1* (PD-1), *CD274* (PD-L1), *TBX21* (T-bet), *ZEB2*	Secondary immune response, reaction to repeated antigen	[25, 27, 29]
Plasma cells	*SDC1* (CD138), *PRDM1* (BLIMP-1), *XBP1, IRF4, CD38, IL6R, IL10R, IL21R*	*BHLHE40, CD19, CD27, CD45RO, ITGA4* (CD49d), *CD69, CD71, FAS* (CD95)	Produce antibodies after terminal differentiation	[25, 27, 29]
Regulatory (Breg)	CD19, CD24, CD38, CD27, IL10	*CD274* (PD-L1), *TGFB1* (TGF-β), *CD5*	Suppress inflammatory reactions	[26, 29, 31]
Activated B-cells	*CD19, CD69, CD86, NFKB1, MS4A1* (CD20), *CD21, CD22, CD23, CD27, CD38, CD39, CD40, CD71, CD72, HLA-DRA, HLA-DRB1* (HLA-DR), *IRF4, MYC*	AICDA, BACH2	Ensure proliferation and antigen presentation	[25, 28]

Notes: Main markers are associated with the specific subpopulation, functionally relevant, and used for cell identification in flow cytometry and scRNA-seq. Additional markers are less specific, support biological interpretation, may vary depending on the cell state, and are validated by the CellMarker 2.0 and PanglaoDB databases.

Conclusion

The rapid development of scRNA-seq has enabled deeper insights into cellular heterogeneity and immune cell function. Yet, the value of these insights' hinges on precise and reliable cell type annotation. This review provides a systematic overview of markers for adaptive immune cells, ranging from early progenitors to functionally specialized subpopulations. These marker panels can significantly improve the accuracy of automated annotation by reducing the rate of false positives and false negatives. Such standardization has important practical implications in oncoimmunology, facilitating a better understanding of disease pathogenesis, more accurate prediction of responses to immunotherapy, and the identification of novel therapeutic targets. Nevertheless, a comprehensive understanding of the immune system requires the analysis of both adaptive and innate immune compartments. The systematization of markers for innate immune cells will be the focus of future work.

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Типирование клеток адаптивного иммунитета: маркеры популяций и их функциональное значение

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Аннотация. *Актуальность.* Технологии секвенирования единичных клеток (scRNA-seq) открывают уникальные возможности для изучения гетерогенности иммунных популяций, позволяя анализировать экспрессионные профили, лиганд-рецепторные взаимодействия и траектории дифференцировки на уровне отдельных клеток. Однако качество типирования клеток существенно зависит от точности аннотации данных. Неоднозначность маркеров, вариабельность их экспрессии в зависимости от физиологического контекста, ограничения автоматических инструментов, таких как CellTypist, Azimuth и SingleR, а также неполнота баз данных PanglaoDB и CellMarker, включая недостаток сведений о функциональной роли маркеров, значительно затрудняют идентификацию клеточных популяций и требуют дополнительных усилий для достижения значимых результатов. *Цель.* Систематизировать маркеры Т- и В-клеток адаптивного иммунного ответа для упрощения аннотации данных scRNA-seq. *Материалы и методы.* Проведен анализ более 30 научных публикаций из баз PubMed, Scopus и Web of Science за 2008–2024 годы, а также данных маркеров из баз PanglaoDB и CellMarker. *Результаты и обсуждение.* Рассмотрены ключевые и дополнительные маркеры Т-клеток (*CD3D, CD3E, CD3G, CD4, CD8A, CD8B, FOXP3, GZMA, TBX21*) и В-клеток (*CD19, MS4A1, CD27, IGHM, IGHD*), их роль в иммунных процессах, включая активацию, цитотоксичность и регуляцию. Описаны субпопуляции, такие как Th1-клетки (T-bet, IFN-γ), фолликулярные хелперы (CXCR5, BCL-6), регуляторные В-клетки (CD24, IL-10), и их функции в норме и патологии. *Выводы.* Систематизация маркеров Т- и В-клеток разработана для улучшения качества аннотации данных секвенирования единичных клеток и применима для расширенного типирования функциональных состояний клеток в норме и при патологии (инфекционные, аутоиммунные и онкологические заболевания). Это открывает возможности для углубленного понимания иммунных процессов и разработки подходов к иммунотерапии, включая CAR-T-терапию. Однако ограниченная специфичность маркеров и отсутствие стандартизированных алгоритмов аннотации подчеркивают необходимость дальнейших исследований для совершенствования методов типирования.

Ключевые слова: Т-клетки, В-клетки, адаптивный иммунитет, секвенирование единичных клеток, иммунные маркеры, типирование

Информация о финансировании. Данный проект был поддержан Российским Научным Фондом [грант № 22-75-10128]. Вклад авторов: Федоров А.А. — сбор и анализ литературы, подготовка таблиц, написание текста; Федоренко А.А. — анализ баз данных, написание текста; Патышева М.Р. — концепция, анализ данных, редактирование; Геращенко Т.С. — рецензирование и редактирование рукописи. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

Информация о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение — неприменимо.

Благодарности — неприменимо.

Информированное согласие на публикацию — неприменимо.

Поступила 04.07.2025. Принята 01.08.2025.

Для цитирования. *Fedorov A.A., Fedorenko A.A., Patysheva M.R., Gerashchenko T.S.* Typing of adaptive immunity cells: markers of populations and their functional significance // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 328–338. doi: 10.22363/2313-0245-2026-30-3-328-338 EDN: KMPBFW

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

DOI 10.22363/2313-0245-2025-30-3-339-355
EDN KOYSOO

ORIGINAL RESEARCH
ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ

Study of the neuroprotective effect of conditioned medium from human iPSCs-derived glial progenitor cells in a model of glutamate excitotoxicity

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Abstract. *Relevance*. In modern biomedicine, therapeutic strategies for neurological disorders are increasingly focused on the development of multitarget therapeutic approaches. Among the promising avenues, preparations based on conditioned media stand out. In the present work, for the first time, a genome-wide transcriptomic analysis of differential gene expression was performed in primary cortical neuron cultures incubated with conditioned medium from human glial progenitor cells under conditions of induced glutamate excitotoxicity. This study allows us to infer potential molecular mechanisms underlying the observed protective effect. *Materials and methods*. Conditioned medium was collected from human glial progenitor cell cultures and subjected to purification and concentration by tangential ultrafiltration. Excitotoxic glutamate exposure was modeled in primary cortical neurons from newborn (P0) rats by adding sodium glutamate to a final concentration of 100 μM. Cell viability was assessed using the MTT assay. Transcriptomic libraries were sequenced on the NextSeq 1000 platform (Illumina, USA); read quality control was performed with the FastQC tool. The R package edgeR was used for differential gene expression analysis (FDR < 0.05). *Results and discussion*. The conditioned medium was found to exert a dose-dependent neuroprotective effect: at a preparation concentration of 45 μg/mL, cell viability recovered to the level of the intact control. According to transcriptomic profiling data, the addition of conditioned medium against a background of glutamate stress was accompanied by upregulation of 173 genes and downregulation of 479 genes. The identified genes were grouped into functional categories with statistical significance. Among the most represented biological processes associated with activated genes were regeneration, cell–cell adhesion, response to oxidative stress, inhibition of apoptosis, and stimulation of the TGF-beta signaling pathway. Downregulated

genes were predominantly linked to calcium transport, glutamatergic signal transmission, and regulation of neurite outgrowth and branching. The results obtained may indicate that components of the conditioned medium trigger antioxidant defense and repair cascades in neurons while simultaneously limiting excessive activation of glutamate receptors and calcium ion influx. *Conclusion.* The conditioned medium exhibits neuroprotective properties under conditions of glutamate-induced excitotoxicity. Presumably, this effect is based on the simultaneous induction of genes promoting survival and suppression of genes mediating glutamatergic neurotransmission. The totality of the data obtained confirms the promise of further development of therapeutic agents based on conditioned media from human glial progenitor cells for the treatment of neurological disorders.

Keywords: conditioned medium, glutamate excitotoxicity, transcriptome analysis, glial progenitor cells, iPSCs

Funding. The work is supported by the Ministry of Education and Science of the Russian Federation, budget classification code: 075 0110 47 2 U8 70440 621. Scientific topic code: FSSF-2025-0004.

Authors contributions. Shedenkova M.O. — experiment design, data analysis and writing the text; Guryanova A.A. — data analysis; Sudina A.K. — writing the text and text translation; Maksimov Y.M. — text translation; Guguchin E.P. — data analysis; Karpulevich E.A. — data analysis; Fakhudinov T.K. — study idea and design; Goldshtein D.V. — study idea and design; Salikhova D.I. — scientific editing of the text and study idea and design. All authors made a significant contribution to the development of the concept and manuscript writing, read and approved the final version before publication.

Conflict of interest statement. The authors declare no conflicts of interest.

Ethics approval. Animal experiments are conducted in accordance with these principles and regulations, the Foundation's preferred low standards (ESF) and the Declaration on Humane Animal Nutrition, as well as in accordance with the Order of the Ministry of Health and Social Development of Russia dated August 23, 2010, No. 708n "On approval of laboratory practice". Animal care, breeding and experimental procedures are carried out in accordance with the requirements of the Ethics Committee of the A.P. Avtsyn Research Institute of Human Morphology of the B.V. Petrovsky Russian Scientific Center of Surgery. Protocol No. 38 (14) dated May 31, 2022. Informed consent was obtained from all patients participating in the treatment when taking skin biopsies from donors. The protocol was approved by this Institutional Institutional Review Board of the Medical Genetics Research Center (Protocol No. 2019-2/3 dated October 13, 2020).

Acknowledgments — not applicable.

Consent for publication — Voluntary written consent was obtained from the patient for the investigation and publication of relevant medical information according to WMA Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects, 2013.

Received 13.04.2026. Accepted 12.05.2026

For citation: Shedenkova M, Gurianova A, Sudina A, Guguchin E, Karpulevich E, Fatkhudinov T, Goldshtein D, Salikhova D. Study of the neuroprotective effect of conditioned medium from human iPSCs-derived glial progenitor cells in a model of glutamate excitotoxicity. *RUDN Journal of Medicine.* 2026;28(3):339–355. doi: 10.22363/2313-0245-2026-30-3-339-355 EDN: KOYSOO

Introduction

Glutamate is a key excitatory neurotransmitter of the central and peripheral nervous system, involved in neurotransmission and synaptic plasticity, which determines its fundamental role in the physiology of nervous tissue and cognitive functions. The signaling function of glutamate is realized through binding to ionotropic and metabotropic receptors, which trigger cascades of intracellular reactions controlling axonal growth, dendritic branching and dendritic spine formation, mechanisms of neuroplasticity, as well as the mitochondrial pathway of apoptosis [1]. Maintaining the homeostatic concentration of glutamate in the synaptic cleft is a necessary condition for stable

neuronal functioning. An increase in the amount of glutamate in the extracellular space (excitotoxicity) results in overstimulation of the receptors of this neurotransmitter, leading to the opening of voltage-gated calcium channels and an avalanche-like influx of Ca^{2+} into the cell [2]. An excess of free Ca^{2+} disrupts the homeostasis of this ion and initially overloads the cytoplasmic buffer system, followed by the mitochondrial system, which functions via the calcium uniporter [3]. The consequence is depolarization of the inner mitochondrial membrane, leading to an increase in the number of molecules associated with oxidative stress, such as reactive oxygen species, free radicals, or hydrogen peroxide [4]. This disrupts the function of the electron transport chain, ATP production, and triggers endoplasmic reticulum (ER) stress, contributing to the accumulation of misfolded proteins within the cell and destabilizing the redox balance [5]. Furthermore, mitochondrial dysfunction leads to a decrease in NAD^+ levels, which is necessary for the proper function of the DNA repair system [6]. All of these impairments lead to the activation of intracellular cascades of caspase proteins responsible for programmed neuronal cell death. Cell death via apoptosis leads to extensive damage to nervous tissue, often culminating in necrosis, and is also accompanied by cognitive dysfunctions. Dysregulation of the glutamatergic system exerts a profound negative impact on neuronal physiology and acts as a key pathogenetic factor in a range of neurological disorders, such as dementia, stroke, traumatic brain injury, and amyotrophic lateral sclerosis [3, 7]. Given the multifactorial nature of the excitotoxic action of glutamate, the current strategy for pharmacotherapy of neurodegenerative diseases is increasingly shifting towards the use of drugs with a multimodal mechanism of action, allowing for the simultaneous modulation of various components of the pathological process.

Conditioned media (CM) are currently considered as novel multicomponent agents that target several pathological pathways simultaneously. Using a model of glutamate excitotoxicity, the neuroprotective properties of CM derived from mesenchymal stem cells, neural stem cells, oligodendrocytes, and astrocytes have already been demonstrated [8–10]. Owing to their diverse composition, which includes extracellular vesicles, microRNAs, proteins, peptides, and metabolites, CM exhibit multitargeted effects aimed at stabilizing oxidative stress, inhibiting apoptosis, and reducing ER stress. Previously, our laboratory demonstrated the therapeutic properties of conditioned media from glial derivatives of human induced pluripotent stem cells (GPCs) in a stroke model and a neuritogenesis model [11–14].

The aim of this study: evaluation of the neuroprotective effect of CM derived from human glial progenitor cells and analysis of the main biological pathways and signaling cascades contributing to neuroprotection in a glutamate excitotoxicity model using transcriptomic analysis.

Materials and methods

Preparation of conditioned media from glial progenitor cells (CM-GPC)

The cellular material for obtaining the conditioned media from glial progenitor cells preparation (CM-GPC) consisted of glial progenitor cells previously derived from induced pluripotent stem cells of a healthy donor according to the protocol described in [15]. GPCs were cultured in a media of the following composition: 1% N2, 1 mM L-glutamine, 50 U/mL penicillin/streptomycin (PanEco, Russia), 1% fetal bovine serum (Gibco, USA), supplemented with growth factors: 10 ng/mL FGF-2, 20 ng/mL EGF, and 20 ng/mL CNTF (Peprotech, USA). Upon reaching 80–90% confluence, the cells were washed three times with Hanks' solution (PanEco, Russia) to remove residual serum and transferred to a media devoid of serum, N2, and growth factors for 16 hours. After incubation, the conditioned media was collected and then centrifuged for 30 minutes at 10,000 × g to remove apoptotic bodies and debris. Subsequently, the supernatant was collected and subjected to sequential vacuum filtration through membrane filters with pore diameters of 0.45 μm and 0.22 μm (Millipore, Germany), after which it was concentrated by tangential ultrafiltration using a Labscale TFF system (Millipore,

Germany) with a Pellicon XL 3 kDa cartridge to a final protein concentration of 250 μg/mL. The finished CM preparations were stored at –80 °C. Protein concentration was measured using a ready-to-use Quick Start™ Bradford Protein Assay kit (Biorad, USA).

Establishment of a Neuroglial Culture and Induction of Glutamate Excitotoxicity In Vitro

Modeling of glutamate excitotoxicity *in vitro* was performed on primary neuroglial cultures of the cerebral cortex isolated from newborn rat pups (P0) [15]. Cells were cultured in Neurobasal medium (Gibco, USA) supplemented with 1 mM L-glutamine, 50 U/mL penicillin-streptomycin (PanEco, Russia), and B27 supplement (Gibco, USA). On day 10 after plating, CM-GPC preparations were added to the cultures at final concentrations of 5, 15, and 45 μg/mL. An equivalent volume of serum-free DMEM/F12 medium was used as a negative control to exclude any neuroprotective effect of the vehicle medium itself. On the following day (11 DIV), glutamate excitotoxicity was induced according to a previously described protocol [15]. The experimental protocol involved the use of three types of solutions:

(1) a normalized buffer (140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 20 mM HEPES) for washing the cultures;

(2) a glutamate solution (100 μM glutamate, 10 mM glycine, 2 mM $CaCl_2$ in normalized buffer);

(3) a calcium-free solution (normalized buffer with 2 mM $MgCl_2$ and without $CaCl_2$ addition).

The modeling procedure was as follows: after removing the growth medium and washing with solution (1), solution (1) was left in the control wells, while the experimental wells received solution (2), followed by incubation for 1 hour. After incubation, the cells were washed twice with solution (3) and the previously collected native culture medium was returned to the wells.

Assessment of Cell Viability by a Biochemical Method (MTT Assay)

Assessment of cellular metabolic activity was performed using the MTT assay. For this purpose, the reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Merck, Germany) was preliminarily dissolved in dimethyl sulfoxide (DMSO, ThermoFisher, USA) and added to the culture medium to a final concentration of 0.1 mg/mL. The samples were incubated for 1 hour in a CO_2 incubator at 37 °C. Following incubation, the supernatant was removed, and the formed formazan crystals were dissolved in DMSO. The absorbance of the resulting solution was measured using a ClarioStar plate spectrophotometer (BMG Labtech, Germany) at 520 nm and 690 nm. The final optical density values were expressed as a percentage relative to the control (intact cultures were taken as 100%).

Statistical analysis

Each experiment was repeated at least four times using cultures derived from unrelated cells. Data processing was performed using GraphPad Prism 10 software (GraphPad Software, USA). Comparisons between groups were conducted using one-way analysis of variance (one-way ANOVA) followed by Dunnett's post hoc test for multiple comparisons correction. The Shapiro–Wilk test was used to assess the normality of distribution. Final values are expressed as mean ± standard deviation (Mean ± SD).

Transcriptomic Analysis

Isolation and Purification of Total mRNA

To identify intracellular signaling pathways modulated by the CM-GPC preparation under conditions of glutamate excitotoxicity, total RNA sequencing was performed. CM-GPC was added to the cultures 24 hours prior to damage induction, and glutamate stress was induced the following day according to the procedure described above. Four hours after completion of the glutamate incubation, total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The obtained samples were stored and transported at –80 °C.

Residual genomic DNA was removed from the RNA preparations using the Turbo DNA-Free Kit (Thermo Fisher Scientific, USA) in a reaction volume of 50 μL. Subsequent nucleic acid purification was performed using Agencourt RNAClean XP reagent (Beckman Coulter, USA), strictly following the

manufacturer's instructions. RNA concentration was measured fluorometrically using the Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific, USA). The integrity and quality of the isolated RNA were assessed on an Agilent bioanalyzer using Agilent RNA 6000 Pico Chips (Agilent Technologies, USA).

Preparation of Transcriptomic Libraries and RNA Sequencing

Procedures for library preparation and bioinformatic analysis were performed according to approaches previously validated in our studies [16]. Starting aliquots of 250 ng of total RNA were used for transcriptomic library synthesis. Libraries were prepared using a poly(A)-mRNA enrichment module on magnetic particles (NEBNext) and the KAPA RNA Hyper Kit (Roche, Switzerland) according to the manufacturer's instructions. Intermediate RNA purification was performed using RNA Clean XP, and final purification of the completed libraries was carried out using Agencourt AMPure XP magnetic particles (Beckman Coulter, USA). Fragment size distribution and quality parameters of the libraries were determined using the Agilent High Sensitivity DNA Kit (Agilent Technologies); quantification was performed fluorometrically using the Quant-iT DNA Assay Kit, High Sensitivity (Thermo Fisher Scientific). The resulting libraries were pooled in equimolar ratios to a final pool concentration of 750 pM. Sequencing was performed on a NextSeq 1000 platform (Illumina) using a NextSeq 1000/2000 P2 Reagents Kit (200 cycles) v3 with the addition of 2% PhiX control.

Bioinformatic Processing of Sequencing Results

Initial read quality assessment was performed using the FastQC utility [17]. Low-quality regions and technical adapter sequences were removed using Trimmomatic [18]; additional read processing was conducted using the Cutadapt utility with the parameter — nextseq-trim 20. Quantification of gene expression levels was performed using Salmon (mapping-based mode) [19]. The complete transcript set for the R. norvegicus genome assembly rn6 from the ENSEMBL database version 106 was used as the reference transcriptome. During reference transcriptome indexing, the genomic nucleotide sequence was included as a decoy sequence to prevent erroneous read mapping. Aggregation of expression values from the individual transcript level to the gene level was performed using the R package tximport [20]. Statistical analysis of differential gene expression between compared groups was performed using the R package edgeR with the glmLRT test [21]. The threshold for statistical significance was set at FDR < 0.05.

Results and discussion

Assessment of the Neuroprotective Effect of CM-GPC

Twenty-four hours after exposure to 100 μM glutamate on primary rat cortical neuron cultures, a significant decrease in the number of viable cells was recorded. Their viability relative to the intact control (taken as 100%) decreased to 61.7 ± 12% (Figure 1). In this case, pre-incubation of cultured neurons for 24 hours with CM-GPC at concentrations of 5, 15, and 45 μg/mL led to a significant increase in the number of viable neurons to 86.2 ± 15% ($p = 0.0005$) for 15 μg/mL and to control values upon addition of 45 μg/mL ($p < 0.0001$). Furthermore, upon addition of DMEM/F12 medium alone as a negative control (since glial progenitor cells were incubated in DMEM/F12 during conditioned medium preparation), no difference was observed compared to the group incubated with glutamate only (Figure 1).

Transcriptomic analysis of neuroglial cultures in a model of glutamate excitotoxicity

Analysis of differential gene expression upon addition of conditioned medium to the cultures against the background of glutamate exposure

The addition of conditioned medium led to a statistically significant change in the expression of 652 genes: of these, 173 exhibited upregulated transcription in the group treated with conditioned medium from glial progenitor cells (CM-GPC), whereas 479 genes, conversely, showed downregulated expression ($p < 0.05$, FC > 1.5; FC < –1.5).

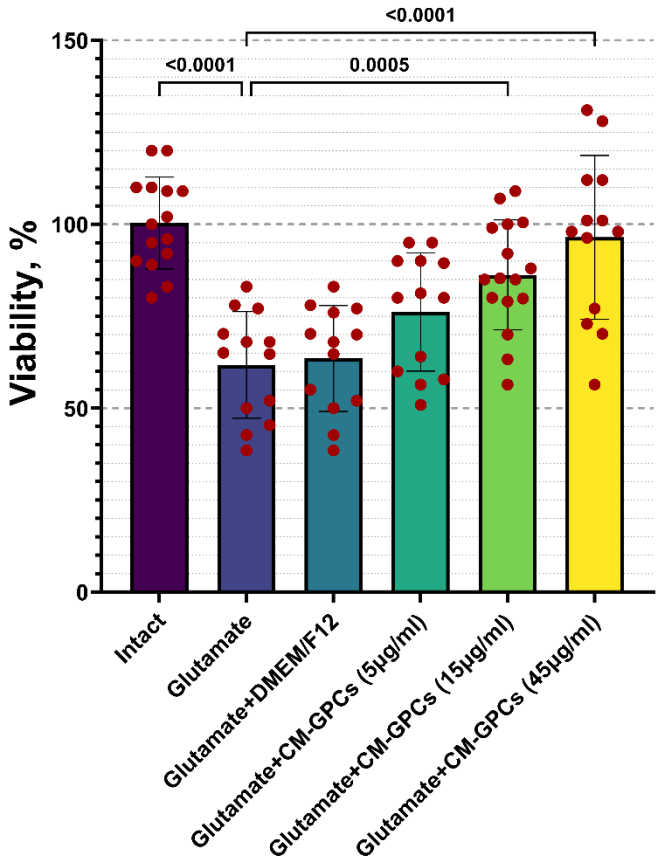


Figure 1. Determination of the viability of cortical neurons using the MTT-test

Note: Intact — intact group, Glutamate — addition of glutamate (100 μM), Glutamate+DMEM/F12 — addition of DMEM/F12 24 hours before glutamate, Glutamate+CM-GPCs (5, 15, 45 μg/ml) — addition of CM-GPCs (5, 15, 45 μg/ml) 24 hours before glutamate. Data were analyzed using one-way ANOVA with Dunnett's post-hoc test. Data are presented as means and standard deviations.

Functional annotation of differentially expressed genes using the Gene Ontology and KEGG databases revealed their significant enrichment in categories related to regeneration, wound healing, negative regulation of apoptosis, response to oxidative stress, and response to growth factors. Specifically, the upregulated genes were grouped into the following functional clusters: positive regulation of cell adhesion (24 genes, including *Fbln2* and *Fstl3*, which encode proteins involved in brain extracellular matrix remodeling during reparative processes; *Lgals1* and *Fn1* — the genes encoding galectin-1 and fibronectin, which are essential for the growth of damaged axons; *Efemp2*, whose product ensures the structural organization of the extracellular matrix [22–25]); wound healing (16 genes, including *Nog* and *Igf1*, which control neuronal differentiation and survival; *Csrp1* — a regulator of the actin cytoskeleton; *Timp1* — a metalloproteinase inhibitor that activates anti-apoptotic genes of the Bcl-2 family; *Ccn1*, which stabilizes synapses during brain development and under neurotoxic conditions [26–30]); regeneration (12 genes: *Spp1*, required for mitochondrial function and activated upon neuronal injury; *Ptpru*, whose phosphatase dephosphorylates β-catenin, thereby affecting intercellular adhesion and stabilizing focal contacts; *Bace2* — an α-secretase that reduces β-amyloid production and its associated toxicity [31, 32]); gliogenesis (11 genes: *Olig1* — a transcription factor of oligodendrocytes that promotes astrocyte survival and axonal growth; *Metrn*, which acts as a neurotrophic factor, stimulating

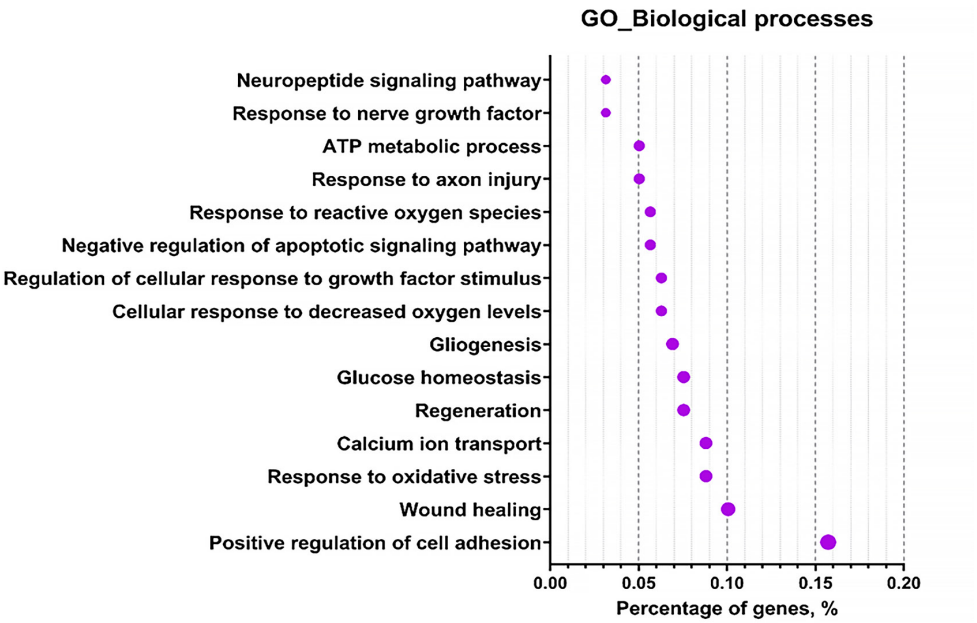
the proliferation of neural precursors and neuroblast migration; *Apcdd1* — a potent inhibitor of the Wnt/β-catenin and BMP signaling pathways, which play a key role in neuronal development and differentiation [33–35]); and response to axon injury (8 genes: *Spp1*, the same osteopontin whose expression is upregulated in motoneurons upon axonal damage and stimulates regeneration; *Tspo* — the gene encoding the 18 kDa mitochondrial translocator protein, which is involved in neurosteroidogenesis [36, 37]). The increased expression of these gene groups indicates that CM-GPC initiates programs aimed at maintaining intercellular adhesion and restoring damaged processes, which is critically important for preserving the integrity of neuronal networks and the overall viability of nerve cells.

The presence of the CM-GPC preparation also contributed to an increase in the expression of genes associated with the response to oxidative stress, the regulation of cellular respiration, and apoptosis. These genes were reliably classified into the following categories: Response to oxidative stress (14 genes: *Gpx3* — the gene encoding glutathione peroxidase 3, one of the key antioxidants; *G6pd* — the gene for glucose-6-phosphate dehydrogenase, which plays a crucial role in neurons by ensuring NADPH production; *Txn1* — the gene for thioredoxin 1, another antioxidant in nerve cells; *Atox1* (Antioxidant 1) encodes a chaperone protein that plays a critical role in copper transport in neurons to maintain homeostasis and protect against oxidative damage [38–41]); Calcium ion transport (14 genes: *Rgs4* — its protein interacts with the G-protein of metabotropic receptors, reducing calcium influx; Bestrophin-1 (*Best1*) is a calcium-activated anion channel that controls the transmission of calcium signals from glutamate [42, 43]); Glucose homeostasis (12 genes: *Serpinf1* — the gene for pigment epithelium-derived factor (PEDF), which maintains glucose homeostasis in neurons; *VGF* — a gene induced by neurotrophins that regulates energy balance and glucose metabolism [44, 45]); Cellular response to decreased oxygen levels (10 genes, including *Higd1a* (Hypoxia-inducible gene domain family-1a), a mitochondrial protein that plays a crucial role in ensuring neuronal survival under hypoxic stress [46]); Response to reactive oxygen species (10 genes, including the aforementioned peroxidase and thioredoxin genes); Negative regulation of apoptotic processes (9 genes: *Hspb1* (heat shock protein beta-1) — a critical chaperone gene that acts as a neuroprotectant by maintaining proper protein folding; *Ier3*, a gene encoding a protein activated by ER stress, which plays a role in protecting cells from apoptosis; *Uchl1* — a gene encoding a protein that contributes to the recovery process after neuronal injury [47, 48]); and ATP metabolic processes (8 genes). The increased expression of these gene groups ndicates that the presence of CM-GPC promotes the activation of protective mechanisms in response to oxidative stress and cell death against the background of glutamate toxicity. These mechanisms are associated with the stabilization of cellular respiration and ATP production, as well as the activation of antioxidant production, which facilitates the elimination of reactive oxygen species. Furthermore, genes were identified that reduce the influx of free calcium ions into the cytoplasm, thereby inhibiting the intracellular stress that leads to apoptosis.

Genes with increased expression related to the response to growth factors were also identified. Among the categories, the following can be highlighted: Regulation of cellular response to growth factor stimuli (10 genes: *Fstl3* (follistatin-like 3) — a secreted glycoprotein that functions as a regulator of TGF-β family signaling pathways; *Dok5* — a gene required for the NT-3 signaling pathway, which helps block TrkC-induced neuronal apoptosis; *Tgfb1i1* — the gene encoding the focal adhesion protein Hic-5, associated with the structural, neuroprotective, and regenerative role of the TGF-β1 signaling pathway; *Fgfbp3* (fibroblast growth factor-binding protein 3) — a secreted protein responsible for neurodevelopment, particularly for the formation and regeneration of motor neurons, through the modulation of FGF signals [49–52]). The activated genes also included 6 genes associated with the response to nerve growth factor, among them *Coro1A*, whose product is required for proper axon navigation and branching during development [53], and 6 genes involved in neuropeptide signaling. The increased expression of these gene groups demonstrates that the

addition of CM-GPC activated processes associated with the effects of various growth factors. This suggests that CM-GPC contains growth factors that activate intracellular signaling cascades within nerve cells, promoting survival and regeneration (Figure 2*a*).

For a more detailed investigation of the intracellular signaling pathways activated by the preparation, the upregulated genes were mapped onto canonical signaling cascades using the KEGG database. The analysis revealed significant enrichment in the categories "Phagosome" (10 genes), "Cell adhesion molecules (CAMs)" (7 genes), and "TGF-β signaling pathway" (6 genes). These results are consistent with the classification data based on biological processes, as the identified cascades are functionally associated with intercellular adhesion and growth factor signal transduction (Figure 2*b*).



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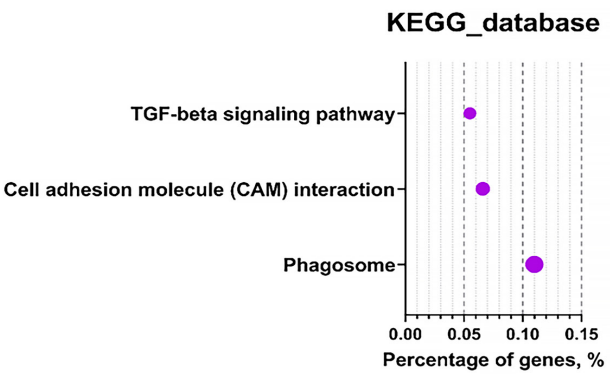


b

Figure 2. Results of classification of genes whose expression was increased in the group with the addition of CM-GPC

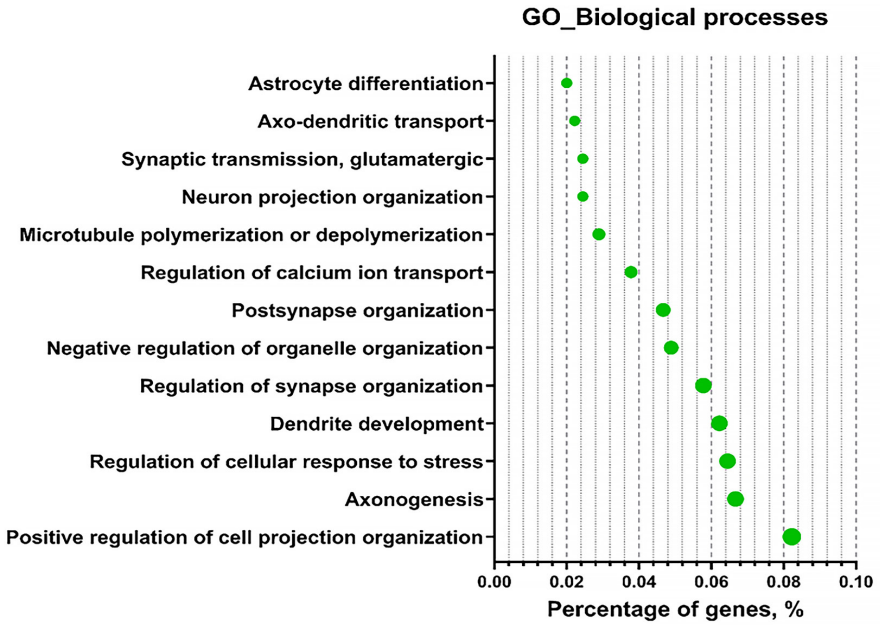
Note: ***a*** — classification with the Gene Onthology database; ***b*** — classification with the KEGG database. The number of genes is indicated on the X-axis, and the selected signaling pathways are shown on the Y-axis.

Conversely, the genes whose expression was found to be decreased in the group treated with CM-GPC were functionally grouped into categories associated with the processes of neuronal activation during synaptic transmission. Among these, the following categories stood out: positive regulation of cellular projection organization (37 genes, including *Clip1*, encoding the CLIP-170 protein, a multifunctional regulator of microtubule dynamics that tracks their plus ends; *Zeb2*, which is actively expressed in the peripheral nervous system and controls process branching; *Sirt1*, which stimulates neurite elongation and branching; *Enpp2*, also responsible for neurite branching; *Shtn1*, involved in the generation of asymmetric intracellular signals that determine neuronal polarization and neurite outgrowth [54–58]); axonogenesis (30 genes: *Tnr*, whose product modulates interactions with extracellular matrix components, which can lead either to stable adhesion and differentiation or to repulsion and inhibition of neurite growth; *Ephb1* — a regulator of directed axon migration; *Sema5a* — a bifunctional axon guidance signal whose activity depends on proteoglycan sulfation; *Plxnb3* (plexin-B3), a receptor for SEMA5A involved in axon growth direction and cell migration; *Nfasc* (neurofascin) — an ankyrin-binding cell adhesion protein implicated in neurite outgrowth, synaptogenesis, myelination, and neuro-glial interaction; *Robo1*, a receptor for SLIT1/SLIT2 that mediates responses to guidance cues during axonal navigation [59–64]); regulation of cellular response to stress (29 genes, including *Tmem33* — an ER stress-inducible molecule that modulates the unfolded protein response cascade and associated apoptosis; *Nfe2l1* — another gene activated under ER stress conditions; *Rock1* and *Rock2*, associated with the execution of the apoptotic program [65–67]); dendrite development (28 genes: *Mef2c* and *Mef2a*, which regulate the formation and elimination of dendritic spines, especially under conditions of excitotoxicity; *Ppp1r9a* (neurabin-1), which interacts with F-actin and is involved in neurite formation [68, 69]); regulation of synapse organization (26 genes: *Septin11*, which plays a role in neuronal cytoarchitecture, including dendritic branching and spine formation, as well as in GABAergic synaptic transmission; *Afdn* — a component of the adhesion system that presumably acts together with the cadherin-catenin complex in the formation of intercellular contacts [70, 71]); calcium ion transport (18 genes: *Cacna1c, Cacna1d, Cacna2d1, Ryr2*, encoding key ion channels and transporters that control Ca^{2+} entry into the neuronal cytoplasm [72]); postsynapse organization (21 genes: *Homer1*, a scaffolding protein of the postsynaptic density; *Tanc2*, a gene encoding a dendritic spine protein involved in the formation of stable postsynaptic contacts [73, 74]); negative regulation of organelle organization (22 genes); microtubule polymerization/depolymerization (17 genes: *Mapt, Map2, Map1a, Kif21a*, whose products regulate microtubule homeostasis and dynamic reorganization [75]); neuronal projection organization (13 genes); axo-dendritic transport (11 genes, predominantly encoding motor proteins of the kinesin family that mediate organelle transport along processes: *Kif5c, Kif3a, Kif21b, Kif2a, Kif21a, Kif5b*); as well as processes related to the glial component, in particular astrocyte differentiation (10 genes: *Plp1* — the major myelin proteolipid protein of the CNS; *Qki* — a regulator of myelination [76, 77]) (Figure 3*a*).

Analysis of signaling cascades using KEGG confirmed the suppression of a few key pathways: cAMP signaling pathway (15 genes), MAPK signaling pathway (15 genes), axon guidance (14 genes), regulation of actin cytoskeleton (14 genes), glutamatergic synapse (9 genes), dopaminergic synapse (9 genes), and long-term potentiation (6 genes) (Figure 3*b*).

The regenerative potential of the brain is extremely limited, as the complete restoration of lost nervous tissue remains unattainable to date. This circumstance significantly complicates the treatment of neurological diseases, including ischemic stroke, traumatic brain injury, traumatic spinal cord injuries, and progressive neurodegenerative pathologies [78, 79]. Existing therapeutic approaches for these conditions are primarily aimed at alleviating symptoms and improving the quality of life of patients but do not ensure the recovery of lost functions [80–82]. In this regard, there is an urgent need for the development of fundamentally new therapeutic strategies for

diseases of the central nervous system. Cell therapy is regarded as one of the most promising directions, largely driven by progress in the field of induced pluripotent stem cells (iPSCs) and the development of protocols for their directed differentiation into various cell types, including neuronal and glial lineages. Numerous studies have confirmed the efficacy of cell therapy with various routes of stem cell administration [83–85]. However, studies have shown that the therapeutic effect is largely attributable to the paracrine activity of the transplanted cells rather than their replacement of vacated cellular niches [86]. Thus, the use of conditioned medium may serve as a promising alternative to the direct implantation of stem cells. It contains growth factors, neurotrophins, and exosomes with therapeutic potential [87, 88].



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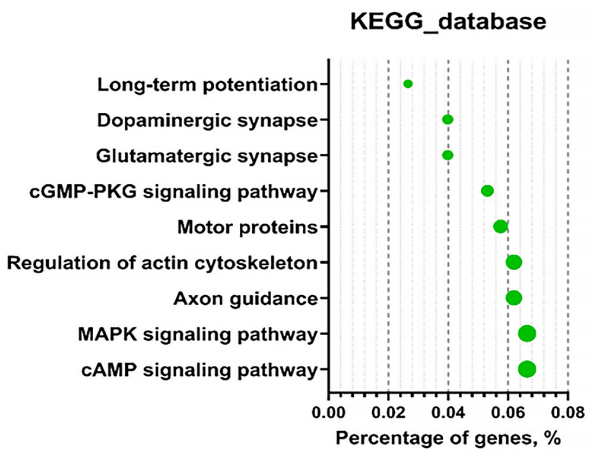

b

Figure 3. Results of classification of genes whose expression was decreased in the group with the addition of CM-GPC

Note: a — classification with the Gene Onthology database; *b* — classification with the KEGG database The number of genes is indicated on the X-axis, and the selected signaling pathways are shown on the Y-axis.

To date, the therapeutic effects of conditioned medium from many cell lines have been studied; however, the role of the glial cell conditioned medium in the restoration of brain function in neurological diseases remains insufficiently explored. One of the key characteristics of glia is their ability to exert neuroprotective effects through paracrine mechanisms mediated by the secretion of extracellular vesicles, cytokines, and other signaling molecules [89]. Through these factors, glial cells regulate CNS activity by performing metabolic, structural, and homeostatic functions, including the control of synaptic transmission, maintenance of neuronal homeostasis, and regulation of blood-brain barrier permeability [90]. The activation of these mechanisms contributes to the maintenance of normal neuronal activity and ensures the protection of nervous tissue from damaging influences. Consistent with previous studies on PC12 cells, neuroprotective properties of conditioned medium from glial progenitor cells against the toxic action of glutamate have also been demonstrated in rat cortical neurons. The decrease in the level of cell death observed in the presence of CM is consistent with the transcriptomic data, which revealed increased expression of genes associated with the negative regulation of apoptosis, including genes encoding chaperone proteins. Furthermore, it was previously shown that the presence of CM reduces oxidative stress in PC12 cells exposed to glutamate *in vitro* and promotes the increased expression of a number of genes responsible for the activation of the antioxidant defense system, among them *Hmox1* [91]. The results obtained in the present study are consistent with the transcriptomic data showing that the presence of CM under conditions of glutamate excitotoxicity is accompanied by increased expression of genes classified according to Gene Ontology into the categories “Response to oxidative stress” and “Response to reactive oxygen species”. Among the activated genes were those encoding glutathione peroxidases, thioredoxins, and a number of antioxidant proteins. Moreover, these results align with evidence on the protective functions of glial cells and their ability to stimulate the synthesis of antioxidants, leading to the protection of neurons from oxidative stress [92, 93]. Presumably, such a neuroprotective effect may be associated with the activation of TGF-beta growth factor signaling cascades in nerve cells, as the addition of CM increases the expression of the *Tgfb1* gene. According to studies, this growth factor is secreted by astrocytes and, by interacting with its receptors on the plasma membrane of neurons, activates the c-Jun/AP-1 signaling cascade, which leads to enhanced neuronal survival by activating the antioxidant defense system [94].

Furthermore, according to the transcriptomic data, the addition of CM reduces the expression of genes classified by the Gene Ontology database into the categories “Regulation of calcium ion transport into the cell”, “Regulation of synapse organization”, and “Postsynaptic organization”, which included genes encoding calcium channel proteins within the postsynaptic membrane. A number of genes with decreased expression upon CM addition were assigned by the KEGG database to the groups “Glutamatergic synapse” and “Long-term potentiation”, which included genes of glutamate receptors. These results are consistent with previously obtained data on the effect of CM proteins on calcium transport into neurons during glutamate exposure. Using real-time calcium imaging, it was demonstrated that proteins of the conditioned medium from glial progenitor cells reduce the efficiency of calcium ion entry into the neuronal cytoplasm upon glutamate exposure [95]. Such an effect may be associated with the decreased expression of genes encoding calcium transporters and glutamate receptors and a corresponding reduction in these channels on the postsynaptic membrane of cells, which also serves as a protective mechanism against glutamate hyperstimulation and the pathological accumulation of free calcium in the cell cytoplasm, consistent with the findings of other researchers. It has been shown that astrocytes protect neurons from excessive calcium influx into the cytosol and subsequent excitotoxicity by maintaining ionic homeostasis, especially after glutamate excitotoxicity, through the regulation of the ionic environment of neurons and the synthesis of neurotrophic factors that promote neuronal survival [94–97].

The investigation of the mechanisms underlying the neuroprotective action of conditioned media derived from various cellular sources remains one of the key challenges in modern cell biology and neurobiology. The data accumulated to date indicate that conditioned media possess a number of advantages over other therapeutic agents, which is attributable to their multi-target mode of action and their ability to modulate a broad spectrum of intracellular signaling cascades. A detailed elucidation of the mechanisms by which CM influences nerve cells provide a foundation for the development of innovative and effective therapeutic agents intended for the treatment of neurological diseases.

Conclusion

The obtained data indicate that human CM-GPC exerts a neuroprotective effect mediated through the activation of mechanisms associated with counteracting oxidative stress, the suppression of calcium ion entry into the cytosol, and the stimulation of growth factor signaling pathways, in particular transforming growth factor beta (TGF-β). The use of this therapeutic agent allows for the partial compensation of the consequences of pathological glutamate excitotoxicity, which opens up prospects for improving the efficacy of therapy for neurodegenerative diseases.

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Исследование нейропротективного действия кондиционированной среды от глиальных производных, полученных из индуцированных плюрипотентных стволовых клеток человека, на модели глутаматной эксайтотоксичности

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Аннотация. Актуальность. В современной биомедицине стратегии лечения неврологических расстройств все чаще фокусируются на разработке мультитаргетных терапевтических подходов. Среди перспективных направлений выделяются препараты на основе кондиционированных сред. В данной работе впервые осуществлен полногеномный транскриптомный анализ дифференциальной экспрессии генов клеток первичной культуры кортикальных нейронов, проинкубированных с кондиционированной средой от глиальных клеток-предшественников человека на фоне индукции глутаматной эксайтотоксичности. Данное исследование позволяет предположить потенциальные молекулярные механизмы наблюдаемого защитного действия. *Материалы и методы*. Кондиционированную среду собирали от культуры глиальных предшественников человека и подвергали очистке и концентрированию методом тангенциальной ультрафильтрации. Эксайтотоксическое воздействие глутамата моделировали на первичных кортикальных нейронах новорожденных (P0) крыс путем внесения натриевой соли глутамата в конечной концентрации 100 мкМ. Жизнеспособность клеток оценивали

с помощью МТТ-теста. Транскриптомные библиотеки секвенировали на платформе NextSeq 1000 (Illumina, США), контроль качества прочтений производили с помощью утилиты FastQC. Для оценки дифференциальной экспрессии генов был использован R-пакет edgeR (значение FDR менее 0,05). *Результаты и обсуждение*. Исследование показало, что добавление 45 мкг/мл кондиционированной среды восстанавливает уровень выживаемости клеток до контрольных значений, то есть препарат обладает дозозависимым нейропротекорным действием. По результатам тракскриптомного профилирования добавление среды на фоне глутаматного стресса приводило к повышению экспрессии 173 генов и подавлению 479 генов. Дифференциально экспрессированные гены статистически достоверно кластеризовались по функциональным группам. К числу наиболее представленных биологических процессов, связанных с активированными генами, относились регенерация, межклеточная адгезия, ответ на окислительный стресс, ингибирование апоптоза и активация TGF-β-сигнального каскада. Гены, экспрессия которых снижалась, преимущественно участвовали в транспорте ионов кальция, глитаматергической нейропередаче и регуляции роста и ветвления нейритов. Полученные данные позволяют предположить, что компоненты кондиционированной среды запускают в нейронах программы антиоксидантной защиты и восстановления, одновременно ограничивая чрезмерную активацию глутаматных рецепторов и приток ионов кальция. *Выводы.* Исследуемый препарат кондиционированной среды обладает нейропротекторными свойствами в условиях глутамат-индуцированной эксайтотоксичности. Предположительно, в основе данного эффекта лежит одновременная индукция экспрессии генов, способствующих выживанию, и подавление генов, обеспечивающих глутаматергическую нейротрансмиссию. Совокупность полученных данных подтверждает перспективность дальнейшей разработки лекарственных средств на базе кондиционированных сред глиальных клеток-предшественников человека для терапии неврологических расстройств.

Ключевые слова: кондиционированная среда, глутаматная эксайтотоксичность, транскриптомный анализ, глиальные клетки-предшественники, иПСК

Информация о финансировании. Работа выполнена при финансовой поддержке Министерства образования и науки Российской Федерации КБК: 075 0110 47 2 Ю8 70440 621. Код научной темы: FSSF-2025–0004.

Вклад авторов. Шеденкова М.О. — дизайн эксперимента, анализ данных и написание текста; Гурьянова А.А. — анализ данных; Судина А.К. — написание текста и перевод текста; Максимов Я.М. — перевод текста; Гугучин Е.П. — анализ данных; Карпулевич Е.А. — анализ данных; Фахудинов Т.Х. — идея исследования и дизайн; Гольдштейн Д.В. — идея исследования и дизайн; Салихова Д.И. — научное редактирование текста, идея исследования и дизайн. Все авторы внесли существенный вклад в проведение исследования и подготовку рукописи, прочитали и одобрили окончательную версию перед публикацией.

Информация о конфликте интересов: авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение. Эксперименты на животных проводились в соответствии с этическими принципами и нормативными документами, рекомендованными Европейским научным фондом (ESF) и Декларацией о гуманном обращении с животными, а также в соответствии с Приказом Минздравсоцразвития России от 23 августа 2010 г. № 708н «Об утверждении лабораторной практики». Уход за животными, их разведение и экспериментальные процедуры проводились в соответствии с требованиями Этического комитета Научно-исследовательского института морфологии человека имени академика А.П. Авцына ФГБНУ «Российский научный центр хирургии имени академика Б.В. Петровского». Протокол № 38(14) от 31.05.2022. На взятие биопсий кожи у доноров получено информированное согласие всех пациентов, участвовавших в исследовании. Процедура одобрена Институциональным этическим комитетом Медико-генетического научного центра (Протокол № 2019–2/3 от 13 октября 2020 г.).

Благодарности — неприменимо.

Информированное согласие на публикацию. У пациента было получено добровольное информированное согласие на участие в исследовании согласно Хельсинкской декларации Всемирной медицинской ассоциации (WMA Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects, 2013), обработку персональных данных и согласие на публикацию.

Поступила 13.04.2026. Принята 12.05.2026.

Для цитирования: *Shedenkova M., Gurianova A., Sudina A., Guguchin E., Karpulevich E., Fatkhudinov T., Goldshtein D., Salikhova D.* Study of the neuroprotective effect of conditioned medium from human iPSCs-derived glial progenitor cells in a model of glutamate excitotoxicity // Вестник Российского университета дружбы народов. Серия: Медицина. 2026;28(3):339–355. doi: 10.22363/2313-0245-2026-30-3-339-355 EDN: KOYSOO

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

МЕДИЦИНСКАЯ ГЕНЕТИКА
MEDICAL GENETICS

DOI 10.22363/2313-0245-2025-30-3-356-376
EDN KRHXOJ

ОБЗОР
REVIEW

Intronic enhancers: regulatory roles in tissue differentiation and human diseases

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Abstract. *Relevance*. Intronic enhancers are cis-regulatory DNA elements located within introns of protein-coding or non-coding genes. Although introns were historically regarded mainly as non-coding interruptions removed during pre-mRNA splicing, contemporary functional genomics has shown that they are repositories of regulatory information. Intronic enhancers can control the expression of their host genes, neighboring genes, or even distant genes. Their activity is usually cell-type-specific and is associated with open chromatin, transcription-factor binding, enhancer-associated histone modifications such as H3K4me1 and H3K27ac, recruitment of coactivators, enhancer-promoter looping, and in many cases transcription of enhancer RNAs. *Results and discussion*. This article reviews the biological significance of intronic enhancers with particular attention to two areas: tissue differentiation and disease. During differentiation, intronic enhancers integrate lineage-determining transcription factors, developmental signals and determine tissue specificity. They contribute to hematopoietic, endothelial, muscle, adipocyte, neuronal, and immune-cell identity by controlling the timing, intensity, and specificity of gene expression. In disease, intronic enhancers are vulnerable sites for pathogenic single-nucleotide variants, insertions, deletions, copy-number changes, epigenetic disruption, and aberrant enhancer hijacking. Examples include GATA2 enhancer mutations in immunodeficiency and myeloid malignancy predisposition, FTO intronic variants affecting IRX3/IRX5 regulation in obesity, and oncogenic enhancer activation near TAL1 in T-cell acute lymphoblastic leukemia. *Conclusion.* Thus intronic enhancers should not be treated as secondary regulatory elements merely because they lie inside genes. Instead, they represent a major layer of genome regulation, connecting non-coding variation to developmental biology, complex traits, and disease mechanisms. Their study is increasingly important

for precision medicine, functional interpretation of genome-wide association studies, and future enhancer-targeted therapeutic strategies.

Keywords: intronic enhancers; cis-regulatory elements; tissue differentiation; chromatin; enhancer RNA; GATA2; FTO; TAL1; non-coding variants; disease genetics

Funding. The authors received no financial support for the research, authorship, and publication of this article.

Author contributions. Radaev N. — idea of the research, text writing; Azova M.M. — idea of the research, text writing; Dzhunushalieva N.K. — text writing; Guryanova S.V. — design of the research, text writing and editing. All authors made significant contributions to the conception, conduct of the study and preparation of the article, read and approved the final version before publication.

Conflicts of interest statement. All the authors declare that they have no conflict of interest.

Ethics approval — not applicable.

Acknowledgements — not applicable.

Consent for publication — not applicable.

Received 03.12.2025 Accepted 29.12.2025.

For citation: Radaev NE, Azova MM, DzhunushalievaNK, Guryanova SV. intronic enhancers: regulatory roles in tissue differentiation and human diseases. *RUDN Journal of Medicine*. 2026;30(3):356–376. doi: 10.22363/2313-0245-2026-30-3-356-376 EDN: KRHXOJ

Introduction

The human genome contains approximately 20,000 protein-coding genes, yet protein-coding sequences occupy only 1,5% of total genomic DNA [1–3]. The larger biological question is not simply where genes are located, but how they are regulated in time and cellular microenvironment. A neuron, a hepatocyte, a myoblast, an endothelial cell, and a hematopoietic stem cell carry essentially the same genome, but they use different regulatory instructions. Enhancers are among the most important of these instructions. They are cis-regulatory elements that increase transcription of target genes independently of their orientation and often at considerable genomic distance from the promoters they regulate.

Importantly, the localization of enhancers within introns allows modulation of gene expression without altering the amino acid sequence of the genome. As a result, intronic enhancers are considered an important source of evolutionary variation in gene regulatory programs [4].

In addition to intronic enhancers, enhancers can be classified according to their genomic location into intergenic and promoter-associated enhancers. Unlike intronic enhancers, intergenic enhancers are located within non-coding regions between genes. Among intergenic enhancers, a subset known as distal enhancers can be distinguished. These regulatory elements are typically located at considerable distances from the promoters they regulate — up to 500–1000 kilobases in the human genome — whereas intronic enhancers are generally situated much closer to their target promoters, usually within 1–10 kilobases [5,6]. Promoter-associated enhancers are located in close proximity to the promoter of the gene whose transcription they enhance (Figure 1).

Intergenic distal enhancers are more frequently involved in the formation of complex regulatory networks, in which a single enhancer may simultaneously regulate the expression of multiple genes. In contrast, intronic enhancers generally exhibit a closer functional relationship with a specific gene or a group of closely related genes [6].

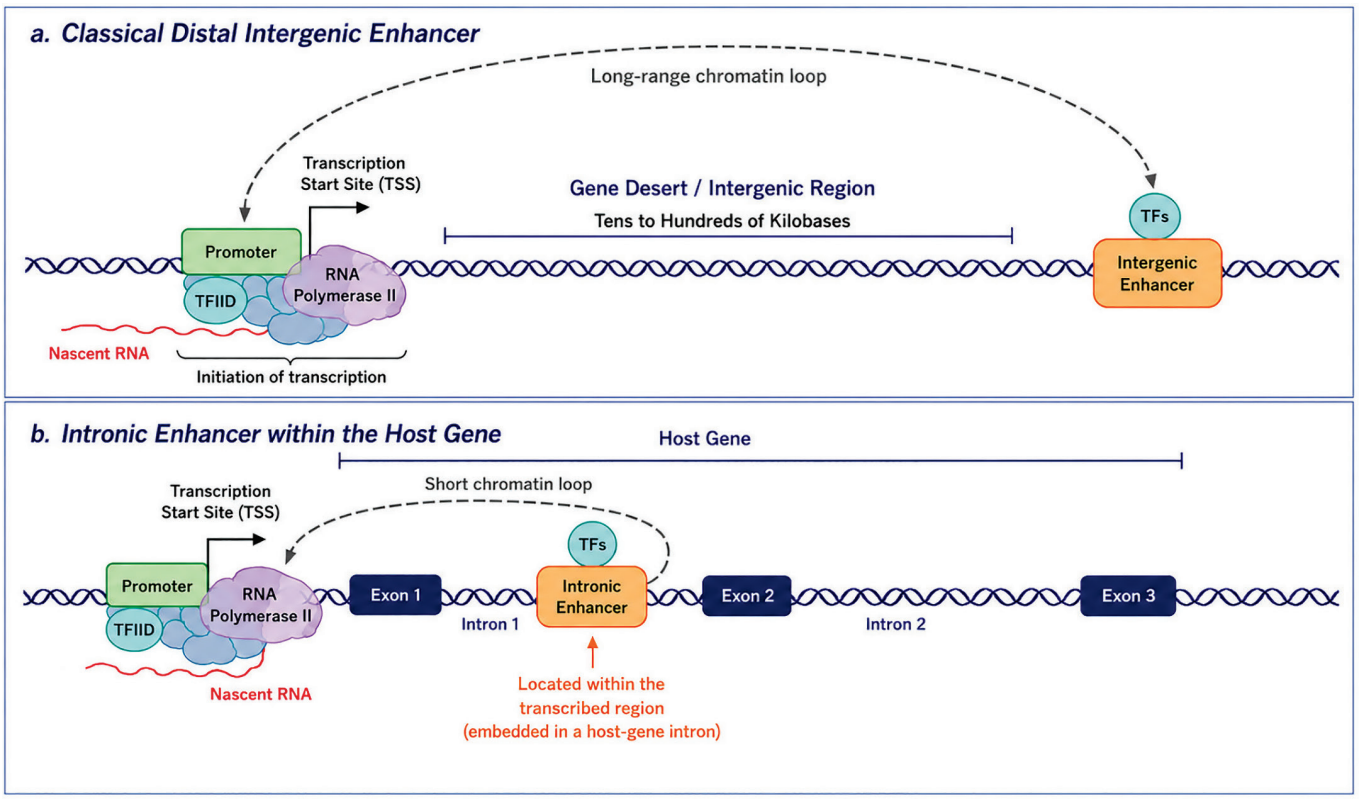

Figure 1. Distal intergenic and intronic enhancers. (*a*) Comperison of classical distal intergenic enchancer and entronic enhancer within the host gene. Classical intergenic enhancers are located in gene-poor genomic regions and regulate target promoters via long-range chromatin looping across large genomic distances. (*b*) Intronic enhancers are embedded within introns of protein-coding genes and operate within compact chromatin domains, forming short-range regulatory interactions with nearby promoters in the context of host gene transcription

Significantly, the functional differences associated with enhancer localization within the genome. Intronic enhancers have been shown to predominantly regulate the expression of tissue-specific genes involved in cellular differentiation and specialized biological functions, often referred to as luxury genes. In contrast, intergenic and promoter-associated enhancers are more commonly linked to genes responsible for cellular homeostasis and basic cellular functions, commonly known as housekeeping genes [6]. Notably, the proportion of intronic enhancers increases substantially from approximately 38–40% across the human genome as a whole to 63–74% in certain tissues characterized by complex cellular organization and a high degree of cellular differentiation, including blood, muscle, and nervous tissues. This observation was confirmed through bioinformatic analyses of publicly available genomic datasets from both humans (*Homo sapiens*) and rats (*Rattus norvegicus*) [5–9]. A more detailed comparison of intronic, intergenic and promoter-associated enhancers is presented in Table.

This comparison further demonstrates that enhancer function may depend on its genomic location and highlights the strong association between intronic enhancers and tissue-specific biological functions, particularly in tissues characterized by complex cellular differentiation.

Table

Comparison of intronic, intergenic, and distal enhancers

Enhancer category	Genomic location	Distance from promoter	Tissue specifi-city	Role in cell differen-tiation	Overall proportion in the human genome	Proportion in human neural tissue
Intronic	Within gene introns	From several hundred to tens of thousands of nucleotides	High	Often regulate tissue-specific genes	≈ 38–40%	≈ 63–74%
Intergenic	Between two genes in a non-coding region that is not intronic	From tens of kilobases to several megabases	Can be both high and low	More often involved in general processes (homeostasis maintenance)	≈ 50–58%	≈ 22–35%
Promoter-associated	Near promoters in exonic and intronic regions	Minimal — up to 1–2 kilobases	Usually low or moderate	Low	≈ 2–3%	-

More detailed analyses of embryonic and postnatal samples of blood, muscle, and nervous tissues revealed a shift in the proportion of predominant enhancer classes from intergenic enhancers during embryonic development to intronic enhancers in postnatal tissues. These findings suggest an important role for intronic enhancers in the establishment and maintenance of differentiated cellular identities [6].

Intronic enhancers should also be distinguished from intronic splicing enhancers. The latter are RNA sequence motifs that influence splice-site recognition after transcription. Intronic enhancers in the sense used in this article are DNA regulatory elements that influence transcription. The two categories can overlap because transcription, chromatin state, elongation, and splicing are coupled processes. Confusing those leads to a common but serious error: interpreting every intronic functional sequence as a splicing element when many intronic regions act at the chromatin and transcriptional level.

Intronic enhancers are biologically important for several reasons. First, their position within genes makes them efficient platforms for coordinating host-gene expression with broader regulatory networks. Second, because introns can be very large, they provide extensive sequence space in which lineage-specific transcription-factor binding sites can evolve. Third, intronic enhancers may be insulated within topologically associating domains, allowing them to contact specific promoters while avoiding inappropriate activation of unrelated genes. Fourth, they are frequent locations of disease-associated non-coding variants discovered by genome-wide association studies. A variant inside an intron may be mistakenly assumed to be harmless if analysis is restricted to protein sequence or canonical splice sites. In reality, such a variant can disrupt a transcription-factor motif, alter chromatin accessibility, change enhancer-promoter contact frequency, and modify disease risk.

Intronic enhancers are particularly important for the development and maintenance of the nervous system, especially the brain. A study published in 2014 demonstrated that disruption of enhancer activity in the genomes of embryonic mouse brain cells resulted in severe neurodevelopmental abnormalities and irreversible defects in brain formation that were incompatible with normal organismal development and survival [10]. Furthermore, accumulating evidence suggests that dysfunction of enhancers and other non-coding regulatory elements may contribute to the pathogenesis of various neuropsychiatric and neurodegenerative disorders [11–13].

Mechanisms of intronic enhancer function

It is known that introns occupy 24% of all DNA compared to 1.5% for exons [6]. Intronic enhancers are enhancers embedded within introns. The intron belongs structurally to a gene, but the enhancer within it may regulate the host gene, a neighboring gene, or a distant gene. A sequence may be "inside" one gene in genomic coordinates but functionally assigned to another gene through chromatin looping and three-dimensional genome organization.

This distinction has become central in modern genomics. Large-scale projects such as ENCODE, Roadmap Epigenomics, and FANTOM5 have demonstrated that active enhancers can be identified using combinations of chromatin accessibility, transcription-factor binding, histone marks, enhancer-associated transcription, and enhancer-promoter correlations. ENCODE classified candidate cis-regulatory elements using marks such as DNase hypersensitivity, H3K27ac, H3K4me3, and CTCF binding. FANTOM5 used cap analysis of gene expression to detect transcribed enhancers across a wide panel of human tissues and cell types. These resources have revealed that enhancers are not randomly distributed; many show strong tissue specificity, and substantial fractions occur within introns [14–16].

According to some researchers, enhancers represent the most abundant class of regulatory elements in the human genome, exceeding promoters, silencers, and insulators in number [17].

Importantly, intronic enhancers share many functional mechanisms with other classes of enhancers; however, their localization within introns confers several distinctive regulatory features.

Cells achieve differential gene expression through complex gene regulatory networks. In many cases, activation of a target gene requires the participation of specialized regulatory proteins known as transcription factors (TFs). These proteins recognize and bind specific DNA sequences within enhancer regions and recruit additional regulatory complexes involved in transcriptional activation [18].

The primary mechanism of intronic enhancer function is believed to be the enhancement of transcription through physical interactions with the promoter of a target gene. Experimental evidence indicates that enhancers can establish direct contacts with promoters even when located at considerable genomic distances from their target genes. These interactions are mediated by the formation of chromatin loops during transcriptional activation and were directly visualized using advanced microscopy techniques in 2022 [19].

The interaction between an enhancer and its target promoter is a highly complex process that remains incompletely understood despite extensive research conducted throughout the 2020s [20]. This process critically depends on the recruitment of transcription factors and numerous additional regulatory proteins. Many of these proteins are involved in altering chromatin conformation and include co-regulators, chromatin remodelers, and chromatin-modifying enzymes. In addition, RNA polymerase II (RNAPII) plays a central role in enhancer-mediated transcriptional activation. Conservative estimates suggest that enhancer function may involve interactions with up to 200 proteins [21]. Although not all of these proteins are likely to be indispensable, many of them cooperate to mediate a series of molecular interactions and biochemical processes that ultimately result in activation of the target promoter and increased transcriptional output.

The function of an enhancer is largely determined by its underlying nucleotide sequence, which typically contains dense clusters of transcription factor binding sites. The functional potential of an enhancer depends on several factors, including the types of transcription factors capable of binding to the enhancer sequence, their binding affinity, orientation, arrangement, number, and spacing of individual binding sites, as well as the surrounding DNA sequence context [5].

A single enhancer often contains multiple transcription factor binding sites. From an evolutionary perspective, the clustering of binding sites within the same regulatory element is advantageous because the cooperative binding of several transcription factors can substantially increase the transcriptional activity of a target gene. Furthermore, such an organization enables a single enhancer to function in different cell types by integrating distinct combinations of transcription factors. The availability of multiple transcription factor binding sites allows different cell types to utilize the same enhancer while regulating the timing, magnitude, and context of its activity according to their specific transcriptional programs. Importantly, a single enhancer may regulate multiple target promoters, while the promoter of a single gene can be controlled by several

enhancers acting simultaneously. This property of enhancers as cis-regulatory elements contributes to the precise and robust control of gene expression. In the case of intronic enhancers, such regulatory flexibility is thought to facilitate tissue-specific gene expression programs and cellular specialization. This phenomenon is particularly evident in blood, nervous, and muscle tissues, which are characterized by a high abundance of active intronic enhancers [6].

In addition to DNA and proteins, non-coding RNAs constitute another essential component of enhancer function. One example is enhancer RNA (eRNA), a class of non-coding transcripts produced from active enhancer regions. Researchers have reported that vertebrate cells contain tens of thousands of distinct eRNAs, with their number potentially exceeding that of matrix RNAs (mRNAs) [5]. The transcription of eRNAs often precedes promoter activation or occurs simultaneously with transcription of the target gene. eRNAs are generally short-lived molecules, and both their biogenesis and abundance are regulated by other classes of RNA [22]. Notably, a subset of polyadenylated eRNAs has been reported to interact with promoters located on different chromosomes and contribute to the regulation of target gene transcription [21]. Some researchers have proposed that eRNAs may serve as markers of enhancer activity. The prevailing view is that eRNAs contribute to maintaining an open chromatin conformation at active enhancers, thereby facilitating transcription. However, alternative hypotheses suggest that eRNAs may represent a by-product of enhancer activation rather than functional regulatory molecules [21]. Several studies have demonstrated that enhancers producing eRNAs tend to exhibit stronger regulatory activity than those that do not [23]. Furthermore, eRNAs have been shown to interact with key components of enhancer-mediated transcriptional regulation, including the mediator complex, transcriptional activators and co-activators, as well as proteins involved in epigenetic chromatin remodeling [21].

It is important to note that transcriptional activity is also influenced by the efficiency of pre-mRNA splicing. Splicing and transcription are functionally interconnected processes, and accumulating evidence suggests that splicing can enhance transcription through a positive feedback mechanism. In general, genes undergoing more efficient splicing tend to exhibit higher transcriptional activity. One proposed mechanism is that splicing factors associated with nascent RNA transcripts interact with RNA polymerase II (RNAPII), thereby promoting the formation and stabilization of the transcription initiation complex and enhancing transcriptional output.

Enhancers can be either inducible (active only in response to specific stimuli) or constitutive (active continuously). Constitutive enhancers most commonly promote the expression of so-called housekeeping genes, which are expressed in virtually all cell types regardless of their differentiation status [24]. Inducible enhancers are considerably more abundant in the human genome than constitutive ones [24]. The interaction of extracellular signaling molecules with specific cell-surface receptors initiates a variety of intracellular signaling cascades. The ultimate outcome of these pathways is the activation of transcription factors that bind to enhancer elements. In some cases, these signaling molecules are hormones, including steroid hormones. It is well established that nuclear hormone receptors, such as the progesterone receptor, androgen receptor, glucocorticoid receptor, and estrogen receptor, undergo conformational changes upon ligand binding and subsequently interact with specific hormone response elements located within enhancers [25]. However, their strong association with tissue-specific gene expression suggests that a substantial proportion of intronic enhancers are activated only in particular cellular contexts and at specific stages of organismal development. Such activation promotes the expression of luxury genes, thereby contributing significantly to cell differentiation and tissue-specific gene regulation.

Therefore, intronic enhancers regulate gene expression through interactions with transcription factors, the formation of physical contacts with promoters, and participation in maintaining an open chromatin state. Their activity may be associated with co-transcriptional splicing processes. Enhancer RNAs (eRNAs) also play an important role in transcriptional regulation. A substantial proportion of

intronic enhancers are activated only under specific conditions, such as hormonal stimulation. Collectively, these mechanisms ensure precise spatiotemporal regulation of gene expression, which is essential for cell differentiation and the establishment of tissue-specific functions.

The logic of intronic enhancer function is particularly visible during tissue differentiation. Differentiation requires a stable but flexible transition from one transcriptional state to another. A progenitor cell must silence some genes, activate others, and maintain a subset in a poised state. Enhancers mediate this by responding to lineage-determining transcription factors and extracellular signals. Intronic enhancers are prominent in this process because they often lie within genes that are themselves involved in development, signaling, chromatin control, or transcriptional regulation. For example, the GATA2 locus contains intronic enhancer elements required for hematopoietic regulation; the FTO locus contains intronic regulatory sequences that influence IRX3 and IRX5 during adipocyte biology; and muscle differentiation involves extensive enhancer activation by MyoD and related transcription factors [26].

The role of intronic enhancers in disease follows naturally from their developmental role. If an enhancer determines when and where a gene is expressed, then mutations in that enhancer can cause disease without altering protein-coding sequence. Disease mechanisms include loss of enhancer activity, gain of enhancer activity, creation of novel transcription-factor binding sites, disruption of chromatin loops, enhancer adoption by oncogenes, and epigenetic reprogramming. These mechanisms are particularly important in cancer, immunological disorders, developmental syndromes, metabolic and neurodegenerative diseases.

Intronic enhancers in tissue differentiation

General principles of enhancer-mediated differentiation

The proper functional activity of tissues is ensured by the stepwise differentiation of cells from less differentiated progenitor cells. It is evident that all cells contain an identical set of genetic material, whereas their specialization is achieved through epigenetic regulation of the genome. Intronic enhancers play a key role in the epigenetic regulation of cellular differentiation.

Enhancers exhibiting tissue-specific activity are highly enriched within non-coding intronic regions and regulate the expression of genes involved in tissue-specific functions, whereas housekeeping genes are more frequently controlled by intergenic enhancers shared across multiple tissues. Notably, during the transition from developmental to adult stages, the predominant class of active enhancers shifts from intergenic to intronic. The most highly differentiated tissues contain a greater proportion of intronic enhancers responsible for the establishment of tissue-specific characteristics and the activation of the corresponding luxury genes, whereas the lowest proportion of intronic enhancers is observed in embryonic stem cells. Researchers have concluded that the genomic location of active enhancers is a key determinant of tissue-specific gene expression control. This concept was demonstrated by the research group led by Beatrice Borsari through the analysis of genomic data from 70 human cell types generated within the Encyclopedia of DNA Elements (ENCODE) project using computational and bioinformatic approaches [6, 27]. It is also well established that epigenetic features, including enhancer activity, can change throughout an individual's lifetime. During development, embryos undergo extensive morphological and functional transformations. These changes determine cell fate and cellular identity through tightly regulated transcriptional programs, thereby generating the remarkable diversity of cell types characteristic of multicellular organisms [28]. Most genes associated with tissue-specific functions are actively transcribed in more than one tissue; however, they differ in their expression levels as well as their temporal and spatial expression patterns, suggesting that their regulatory mechanisms vary among tissues. Nevertheless, approximately 10–20% of all genes are ubiquitously expressed housekeeping genes that participate in the fundamental processes required for cellular maintenance and survival [29].

During differentiation, enhancers often are modified in different ways. A future enhancer may be in a closed or inactive state. It may then become primed, marked by factors such as H3K4me1 but

lacking strong H3K27ac. When differentiation cues activate lineage-determining transcription factors, the enhancer may gain chromatin accessibility, recruit p300/CBP coactivators, acquire H3K27ac, and contact a promoter through chromatin looping. This transition from inactive or primed enhancer to active enhancer is one of the molecular foundations of cell-fate commitment [30].

Intronic enhancers participate in this process in several ways. Some regulate the same gene in whose intron they reside. This arrangement may allow feedback or feed-forward regulation, especially when the host gene encodes a transcription factor or signaling protein. Other intronic enhancers bypass the host gene and regulate a different gene. This is common in loci where three-dimensional chromatin architecture brings an intronic element into contact with a distal promoter. Therefore, the physical position of an intronic enhancer does not automatically determine its target.

Tissue-specific intronic enhancers are often enriched for motifs recognized by lineage-determining transcription factors. In myogenesis, these include MyoD, MYF5, myogenin, MEF2, and related regulators. In hematopoiesis, GATA, RUNX, TAL1, LMO2, and ETS-family factors are important. In adipogenesis, C/EBP and PPAR-family factors play major roles [31]. In neuronal differentiation, proneural factors, bHLH proteins, and activity-dependent transcription factors contribute to enhancer selection. Intronic enhancers act as genomic landing platforms for these factors.

A useful way to understand intronic enhancers is to view them as regulatory "switchboards". A promoter may define where transcription begins, but enhancers determine whether transcription occurs strongly, weakly, transiently, or in a specific lineage. Intronic enhancers can also help fine-tune expression rather than simply turning genes on or off. This is critical in differentiation, where dosage matters. Too little expression of a developmental transcription factor may prevent lineage commitment; too much may produce aberrant proliferation or inappropriate differentiation.

Intronic enhancers and three-dimensional genome organization

Enhancer function cannot be fully understood from linear genome coordinates alone. Enhancers usually regulate genes through physical proximity created by chromatin folding [32]. The genome is organized into compartments, topologically associating domains, and smaller enhancer-promoter loops [33]. Intronic enhancers operate inside this architecture (Figure 2).

A major implication is that an intronic enhancer may regulate a gene located tens or hundreds of kilobases away. It may even skip the promoter of its host gene. This is not an exception but a basic property of enhancer biology. The target gene is determined by chromatin contacts, boundary elements, promoter compatibility, transcription-factor context, and cell type. Thus, assigning intronic enhancers to genes solely by nearest-gene annotation is often misleading.

This matters during differentiation because chromatin architecture itself changes. As cells commit to a lineage, some enhancer-promoter contacts are strengthened while others weaken. A progenitor cell may contain poised enhancer-promoter configurations that become active only after differentiation signals. In other cases, differentiation reorganizes the local chromatin environment, allowing intronic enhancers to contact promoters that were previously inaccessible.

Intronic enhancers may also contribute to regulatory insulation. If an enhancer lies inside a gene within a particular chromatin domain, it may act only on promoters inside that domain. Boundary proteins such as CTCF and cohesin help shape these domains. Disruption of boundaries can allow intronic enhancers to activate inappropriate genes, a mechanism that becomes especially important in disease [34].

Enhancer RNAs and intronic enhancer activity

Many active enhancers are transcribed into short non-coding RNAs called enhancer RNAs, or eRNAs [23]. These RNAs are often unstable, bidirectional, and non-polyadenylated, although exceptions exist. The presence of eRNA transcription

is frequently correlated with enhancer activity. In the context of intronic enhancers, eRNA biology is especially complex because enhancer transcription occurs within or near a host gene transcription unit.

There are at least three possible relationships between intronic enhancer transcription and host-gene transcription. First, the enhancer may produce its own eRNA independently of host-gene transcription. Second, the enhancer may influence transcription elongation through the host gene. Third, enhancer-associated transcription may interact with splicing, RNA polymerase II pausing, or local chromatin modifications.

The functional role of eRNAs remains debated, but several mechanisms have been proposed. eRNAs may stabilize enhancer-promoter loops, recruit coactivators, facilitate transcription-factor residence, modulate chromatin accessibility, or help release paused RNA polymerase II at target promoters [35, 36]. In differentiation, eRNA production can serve as a sensitive marker of enhancer activation, sometimes preceding measurable changes in mRNA abundance. This makes eRNA profiling useful for identifying lineage-specific enhancers.

Intronic eRNAs also create interpretive challenges. RNA-seq signals inside introns may be dismissed as unspliced pre-mRNA or transcriptional noise. However, some intronic transcription signals represent active enhancers. Distinguishing these requires integration of chromatin marks, strand specificity, and enhancer-associated histone modifications.

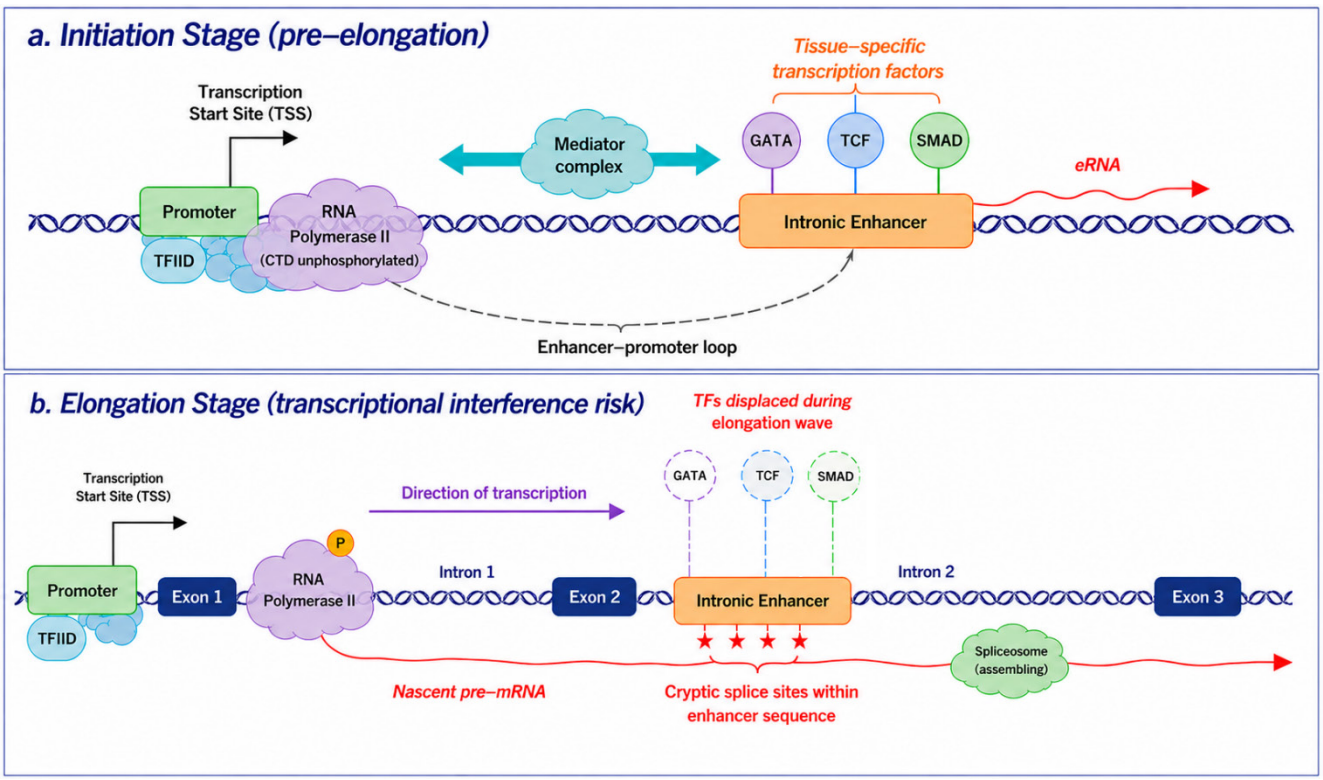

Figure 2. Transcription–splicing conflict at an intronic enhancer. (*a*) During the initiation stage, tissue-specific transcription factors bind the intronic enhancer, which communicates with the pre-initiation complex at the promoter via the Mediator complex. The enhancer transcribes a single eRNA, while splicing is inactive. (*b*) During elongation, phosphorylated RNA Polymerase II transcribes the gene, producing nascent pre-mRNA. The elongation wave displaces transcription factors from the enhancer. The spliceosome assembles on nascent RNA but must ignore cryptic splice sites within the enhancer sequence; SR proteins and hnRNPs mask these sites to prevent aberrant splicing

Hematopoietic differentiation and the GATA2 locus

One of the best-studied examples of intronic enhancer function in differentiation is the GATA2 locus. GATA2 encodes a transcription factor essential for hematopoietic stem and progenitor cells, vascular biology, and immune-cell development. Its expression must be precisely regulated: insufficient GATA2 impairs hematopoietic stem-cell function, while dysregulated expression can contribute to malignancy [37].

The GATA2 locus contains conserved intronic enhancer elements, including the well-known +9.5 or +9.8 kb enhancer region, depending on species and coordinate convention. This enhancer binds hematopoietic transcription factors and is necessary for proper GATA2 expression in specific developmental contexts. Experimental and clinical evidence shows that disruption of this enhancer can cause GATA2 haploinsufficiency [38]. Patients with GATA2 deficiency may develop immunodeficiency, monocytopenia, susceptibility to infections, lymphedema, myelodysplastic syndrome, or acute myeloid leukemia.

The importance of this example is broader than one gene. It demonstrates that an intronic enhancer can be clinically equivalent to a coding mutation. If an enhancer is required for expression of a dosage-sensitive transcription factor, then enhancer disruption can produce disease even when the coding sequence is intact. It also shows why non-coding regions must be included in genetic diagnosis when phenotype strongly suggests a regulatory defect.

During hematopoietic differentiation, GATA2 regulation is stage-specific. Hematopoietic stem cells and progenitors require GATA2, but differentiation toward mature lineages involves coordinated changes in GATA-factor expression. Intronic enhancers help implement this timing. They allow transcription-factor networks to maintain progenitor identity while preparing for lineage-specific transitions. In this sense, intronic enhancers do not merely activate genes; they help define developmental competence.

Muscle differentiation and myogenic enhancer networks

Skeletal muscle differentiation provides another clear model of enhancer-driven lineage commitment. Myogenesis is controlled by a hierarchy of transcription factors, including MyoD, MYF5, myogenin, and MEF2 [39]. These factors bind to enhancers and promoters to activate muscle-specific genes while repressing alternative lineage programs. Genome-wide studies have shown that myogenic differentiation involves widespread enhancer activation, including enhancers located in intronic regions.

MyoD is especially important because it can act as a pioneer-like factor in muscle gene regulation. It binds E-box motifs and recruits chromatin modifiers to establish active enhancer states. During the transition from myoblasts to myotubes, many enhancers gain H3K27ac and show increased accessibility. Some of these enhancers are located within introns of genes involved in muscle structure, metabolism, and differentiation.

Intronic muscle enhancers often contribute to fiber-type specificity and developmental timing. For example, enhancers within or near muscle genes can distinguish expression in slow, fast, or intermediate fibers. This is biologically important because skeletal muscle is not one uniform tissue. It contains fibers with different contractile properties, metabolic profiles, and responses to exercise or injury. Intronic enhancers help encode this diversity by responding to transcription-factor combinations and physiological cues.

Another important feature of muscle enhancer biology is regeneration. Adult skeletal muscle contains satellite cells that can activate myogenic programs after injury. Enhancers involved in embryonic or developmental myogenesis may be reused, modified, or supplemented by regeneration-specific enhancers [40]. Intronic enhancers therefore contribute not only to initial differentiation but also to tissue repair.

Adipocyte differentiation and the FTO/IRX regulatory landscape

The FTO locus is one of the most famous examples of non-coding variation associated with a complex trait. Variants within introns of FTO are strongly associated with body mass index and obesity risk. For years, the intuitive assumption was that these variants affected FTO itself. Functional studies later showed that the regulatory story is more complex: intronic sequences within FTO can form long-range contacts with IRX3 and IRX5, genes involved in adipocyte biology and energy balance [26, 41].

This example is important for differentiation because the disease-associated regulatory effects are linked to adipocyte precursor cells. The risk-associated enhancer state can influence the balance between energy-dissipating and energy-storing adipocyte programs. In simplified terms, non-coding variants in the FTO intronic region can alter enhancer activity and change expression of IRX3 and IRX5, thereby affecting adipocyte function [41].

The FTO case illustrates three general principles. First, an intronic enhancer does not necessarily regulate its host gene. Second, disease-associated variants can act during a specific developmental window, such as precursor-cell differentiation, rather than in mature tissue alone. Third, enhancer-mediated disease risk may involve quantitative shifts in cell-state programming rather than complete loss of gene function.

Adipogenesis depends on transcription factors such as PPARγ and C/EBPα, which establish and maintain adipocyte identity. Intronic enhancers in adipogenic genes contribute to this regulatory program by integrating hormonal, metabolic, and developmental signals. The result is a transcriptional network that determines whether precursor cells become mature adipocytes and what functional subtype they adopt.

Endothelial, neural and immune differentiation

Intronic enhancers also play important roles in endothelial, neural, and immune-cell differentiation. Endothelial cells require precise regulation of genes controlling vascular identity, angiogenesis, barrier function, and response to shear stress. Intronic enhancers within vascular regulatory genes can confer endothelial-specific expression by binding ETS-family transcription factors and other endothelial regulators [42].

In neural development, intronic enhancers contribute to the differentiation of neurons and glia. Neural genes are often large, with long introns that contain regulatory elements. These intronic enhancers may regulate temporal waves of neurogenesis, regional identity, synapse formation, and activity-dependent transcription. Because the nervous system requires exceptional cell-type diversity, it relies heavily on combinatorial enhancer logic. Intronic enhancers provide sequence space for this complexity.

In immune-cell differentiation, intronic enhancers are involved in lineage specification, activation, and memory formation. B cells, T cells, macrophages, dendritic cells, and innate lymphoid cells each use distinct enhancer landscapes. Activation of immune cells by fragments of bacterial cell wall or viruses can rapidly remodel enhancer states, creating *de novo* enhancers or activating latent enhancers [43–50]. Some of these elements reside in introns of cytokine genes, transcription factors, or signaling molecules. This allows immune cells to respond quickly to environmental stimuli while maintaining lineage identity.

Intronic enhancers as fine-tuners rather than simple switches

A simplistic model would describe enhancers as switches that turn genes on or off. This is sometimes useful, but it is incomplete. Many intronic enhancers act as fine-tuners. They regulate expression level, timing, cell-type specificity, responsiveness to signals, and robustness against transcriptional noise.

Fine-tuning is especially important in differentiation. A developmental gene may need to be expressed at low levels in progenitors, high levels during commitment, and reduced levels after maturation. A single promoter cannot easily encode all these states. Multiple enhancers, including intronic enhancers, can provide modular control. Some enhancers respond to

early signals, others to late differentiation factors, and others to tissue-specific physiological inputs [51].

This modularity also creates redundancy. Several enhancers may regulate the same gene, forming shadow enhancers or enhancer clusters. Redundancy can buffer development against mutations or environmental variation. However, it can also complicate functional interpretation: deleting one enhancer may have modest effects under laboratory conditions but strong effects under stress, during development, or in a specific tissue [45].

Intronic enhancers are therefore part of the regulatory grammar that allows multicellular organisms to generate stable but adaptable cell identities. Their location inside introns is not incidental; it reflects the dense regulatory use of gene as platforms for chromatin-based control.

The influence of intronic enhancers in disease

Non-coding variation and the disease problem

Most disease genetics initially focused on coding mutations because they are easier to interpret. A missense, nonsense, or frameshift mutation can be linked directly to protein structure. Intronic variants, by contrast, were often treated as variants of uncertain significance unless they affected canonical splice sites. This approach is no longer adequate. Many disease-associated variants identified by genome-wide association studies lie in non-coding regions, including introns. A substantial fraction of these variants likely influence gene regulation rather than protein sequence [52].

Intronic enhancer variants can cause disease through several mechanisms. A variant may disrupt a transcription-factor binding motif, reducing enhancer activity. It may create a new motif, increasing enhancer activity or changing the cell type in which the enhancer functions. It may alter chromatin accessibility, DNA methylation, nucleosome positioning, or histone modification. It may affect enhancer RNA transcription or enhancer-promoter looping. Structural variants may delete an enhancer, duplicate it, reposition it, or place it near an oncogene.

These mechanisms are difficult to interpret computationally because enhancer function is context-dependent. A variant may have no effect in one cell type but strong effects in another. A variant may affect a gene that is not the nearest gene [53]. A variant may act only during differentiation, inflammation, hypoxia, hormonal stimulation, or other conditions. Therefore, functional validation often requires disease-relevant cell types, developmental stages, and perturbation methods [54].

GATA2 intronic enhancer mutations and hematological disease

GATA2 enhancer disruption is one of the clearest examples of intronic enhancer disease. Germline mutations in the conserved intronic enhancer of GATA2 can lead to GATA2 haploinsufficiency [38, 55]. Clinically, this is associated with immunodeficiency, monocytopenia, dendritic-cell and natural-killer-cell defects, pulmonary alveolar proteinosis, lymphedema, myelodysplastic syndrome, and acute myeloid leukemia predisposition.

The mechanistic logic is direct. GATA2 is dosage-sensitive. If an intronic enhancer required for GATA2 expression in hematopoietic cells is disrupted, the coding sequence may remain normal but total functional expression is insufficient. This produces a phenotype similar to loss-of-function coding mutations. In diagnostic practice, this means that sequencing only exons may miss pathogenic regulatory variants [56].

The GATA2 example also reveals why enhancer disease can be pleiotropic. Because GATA2 acts in multiple developmental and immune contexts, enhancer disruption can affect several tissues and cell lineages. The phenotype may vary depending on the exact mutation, genetic background, environmental exposures, and stochastic effects in hematopoietic stem-cell populations.

From a broader perspective, GATA2 disease demonstrates that intronic enhancers can be essential developmental elements. They are not optional decorations of gene architecture. In some loci, they are required for life-long maintenance of stem-cell systems.

Intronic enhancers in cancer

Cancer is fundamentally a disease of dysregulated growth, survival, and cell identity. Enhancers are deeply involved in all three. Cancer cells can acquire new enhancer activity, amplify enhancer regions, hijack existing enhancers, or become dependent on super-enhancers that drive oncogene expression. Intronic enhancers contribute to this process in multiple cancers [57].

One prominent example is TAL1 activation in T-cell acute lymphoblastic leukemia. TAL1 is normally involved in hematopoiesis, but aberrant TAL1 expression in T cells contributes to leukemogenesis. Studies have shown that non-coding mutations can create or strengthen enhancer activity near TAL1, including enhancer mechanisms involving transcription-factor recruitment and monoallelic expression. Such mutations can generate oncogenic regulatory elements without changing the TAL1 protein sequence [58].

Cancer-associated enhancer activation may involve several molecular events. A small insertion or point mutation may create a binding motif for a transcription factor such as MYB or ETS-family proteins. A structural variant may bring an enhancer into contact with an oncogene. Epigenetic reprogramming may activate enhancers that are normally silent in the relevant tissue. Enhancer clusters or super-enhancers may drive very high expression of oncogenic transcription factors [59].

Intronic enhancers are also relevant to tumor suppressor genes. Loss or methylation of an intronic enhancer can reduce expression of a gene needed for differentiation, DNA repair, apoptosis, or immune recognition. In this case, the disease mechanism is not enhancer gain but enhancer loss. Because many tumor suppressors are dosage-sensitive, partial regulatory reduction may contribute to cancer risk or progression.

Another major issue is enhancer dependency. Cancer cells may depend on specific enhancer-driven transcriptional circuits. This creates therapeutic possibilities. Drugs targeting chromatin regulators such as BET proteins, CDK7, CDK9, or p300/CBP may preferentially affect enhancer-dependent oncogenic programs [60]. However, enhancer-targeted therapy remains difficult because enhancers are not proteins with simple active sites. The more realistic near-term approach is to target coactivators or transcriptional dependencies rather than individual DNA elements.

FTO intronic enhancer variants and metabolic disease

The FTO obesity locus is a classic case of how intronic regulatory variants can influence complex disease. Obesity-associated variants within FTO introns were initially interpreted as implicating FTO. Later functional work showed that these variants are part of a regulatory landscape affecting IRX3 and IRX5. These genes influence adipocyte differentiation and energy metabolism [26].

The disease mechanism is not a simple Mendelian loss of function. Instead, risk alleles modify enhancer activity in adipocyte precursor cells, shifting transcriptional programs related to thermogenesis, lipid storage, and adipocyte identity [61]. This is characteristic of many common disease variants: they produce modest but biologically meaningful changes in gene regulation, often in a specific cell type or developmental state.

The FTO example has several implications. First, intronic location does not identify the disease gene. The disease-relevant target may be distant. Second, enhancer variants can affect disease through developmental programming, not merely adult tissue function. Third, functional genomics can revise gene-trait assumptions produced by association studies. Without chromatin interaction data and enhancer assays, the regulatory connection to IRX3 and IRX5 would be difficult to infer.

This case also illustrates why intronic enhancers are important for metabolic medicine. Metabolic traits are often polygenic and regulated through tissue-specific networks in adipose tissue, liver, pancreas, muscle, and brain. Intronic enhancers are likely to explain many risk loci whose coding consequences are absent or weak [62].

Intronic enhancers in autoimmune and inflammatory disorders

Autoimmune and inflammatory diseases are strongly enriched for non-coding risk variants [63]. Many of these variants map to enhancers active in

immune cells. Because immune-cell activation involves rapid enhancer remodeling, intronic enhancer variants can alter immune responsiveness without causing obvious developmental defects [64].

For example, a variant in an intronic enhancer of an immune regulatory gene may increase transcription-factor binding after cytokine stimulation [65–69]. The result may be excessive inflammatory gene expression. Another variant may weaken enhancer activity and impair tolerance, pathogen response, or immune-cell differentiation. Because immune enhancers are highly cell-state-specific, a variant may act only in activated T cells, macrophages exposed to microbial products, or B cells undergoing differentiation.

Intronic enhancers also participate in the formation of immunological memory. After stimulation, some enhancers remain epigenetically primed, allowing faster response to future stimuli [70]. Variants that alter such enhancers may influence chronic inflammation, autoimmunity, or vaccine response. This area remains challenging because disease-relevant cell states are often transient and difficult to capture in patient samples.

Neurological and developmental disorders

The nervous system is particularly sensitive to regulatory variation. Neural genes are often large and contain long introns enriched for regulatory elements. Brain development requires precise temporal and spatial gene expression across many cell types. Consequently, intronic enhancers are plausible contributors to neurodevelopmental disorders, psychiatric disease, epilepsy, and neurodegeneration.

Enhancer disruption can affect neuronal proliferation, migration, axon guidance, synapse formation, neurotransmitter identity, and activity-dependent plasticity [71]. In developmental disorders, enhancer mutations may alter gene expression during a narrow embryonic window, leaving only indirect traces in adult tissue. This creates a major diagnostic challenge: the pathogenic effect may occur in a cell type that is unavailable for testing.

Structural variants are especially important in neurodevelopmental disease [72]. A deletion may remove an intronic enhancer; a duplication may increase enhancer dosage; an inversion may separate enhancer and promoter; a translocation may expose a gene to an inappropriate enhancer. These mechanisms can produce disease even when all coding exons are intact.

Because brain tissue is difficult to sample, induced pluripotent stem cells, organoids, and single-cell epigenomic methods are increasingly used to model regulatory disease. These approaches are imperfect, but they allow functional testing of intronic enhancer variants in more relevant developmental contexts than blood-derived DNA alone.

Copy-number variants, enhancer dosage, and structural rearrangements

Intronic enhancers are not affected only by single-nucleotide variants. Copy-number variants and structural rearrangements can have strong effects [73]. A deletion may remove an enhancer, reducing target-gene expression. A duplication may increase enhancer dosage or create ectopic contacts. An inversion may change regulatory topology. A translocation may place an enhancer near a new promoter.

Enhancer dosage is particularly important for genes that require precise expression levels [74]. Developmental transcription factors, signaling molecules, and chromatin regulators often fall into this category. Too little expression may cause haploinsufficiency; too much may cause overgrowth, cancer, or inappropriate lineage specification. Intronic enhancer CNVs can therefore produce both loss-of-function and gain-of-function regulatory phenotypes.

Structural variants can also disrupt topological domains. If a boundary is deleted, an intronic enhancer may escape its normal regulatory neighborhood and activate a gene in an adjacent domain. This mechanism is sometimes called enhancer adoption or enhancer hijacking. It is well established in developmental disorders and cancer [75].

Epigenetic dysregulation of intronic enhancers

Not all enhancer disease is caused by DNA sequence mutation. Enhancers can also be dysregulated epigenetically. DNA methylation,

histone modifications, chromatin accessibility, and nucleosome positioning can alter intronic enhancer activity. In cancer, enhancer landscapes are often globally reprogrammed [76]. In inflammatory disease, chronic stimulation can reshape enhancer states. In aging, enhancer activity may drift, contributing to altered tissue function.

Illustration of results of epigenetic changes and mutations in intron enhancers shown in figure 3.

Epigenetic enhancer dysregulation is difficult to classify because cause and consequence often overlap. A cancer cell may activate an intronic enhancer because of oncogenic transcription factors, but the enhancer may then help maintain the malignant state. Similarly, inflammation may activate enhancers that further promote inflammatory gene expression, creating a self-reinforcing loop.

Epigenetic reversibility makes these mechanisms therapeutically attractive [77]. In theory, enhancer activity can be modified without changing DNA sequence. In practice, current epigenetic drugs are broad and can affect many genes. More precise strategies, such as CRISPR-based epigenome editing, may eventually allow targeted repression or activation of disease-relevant intronic enhancers [78]. However, delivery, specificity, durability, and safety remain major barriers.

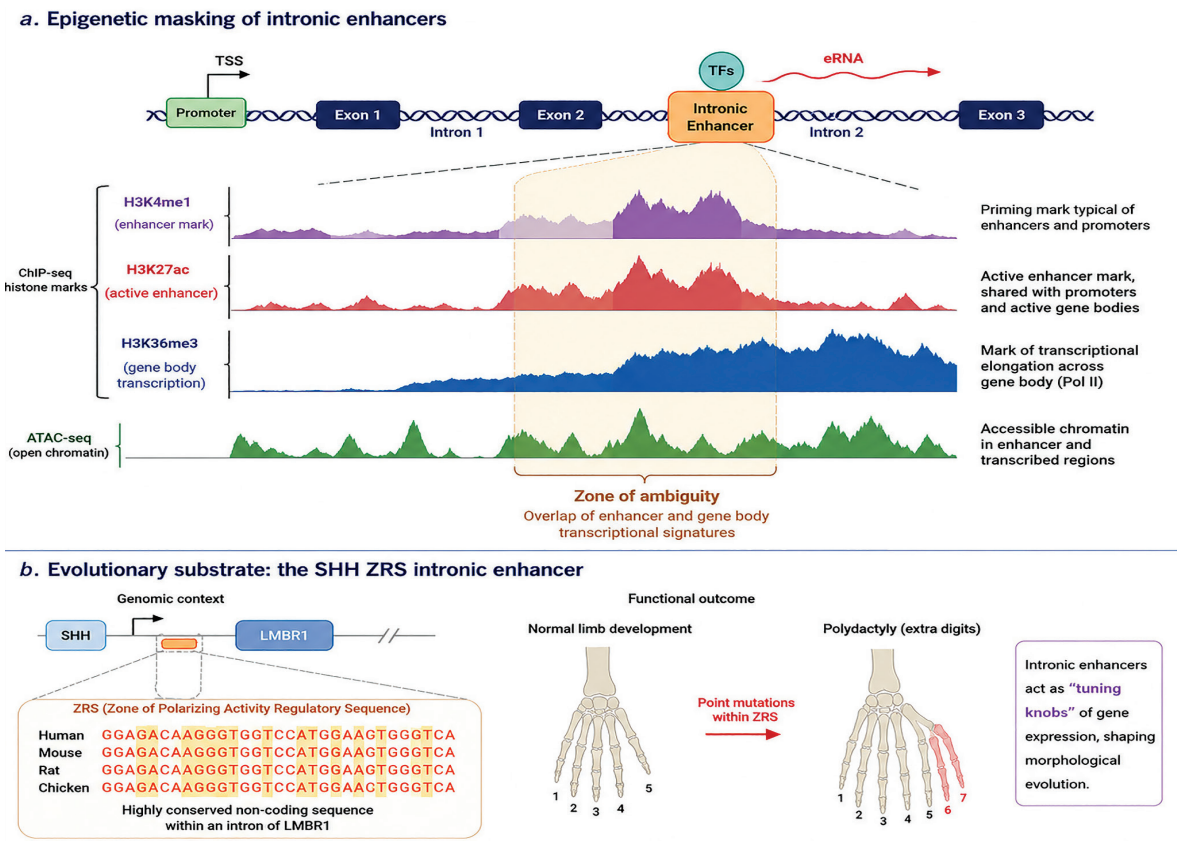

Figure 3. Epigenetic and evolutionary landscape of intronic enhancers. (*a*) Intronic enhancers show overlapping enhancer-associated and transcription-associated chromatin signatures, creating a zone of epigenetic ambiguity that complicates functional annotation. (*b*) The evolutionarily conserved SHH ZRS intronic enhancer illustrates how mutations in non-coding intronic regulatory elements can drive major morphological changes such as polydactyly, highlighting their role as key modulators of vertebrate evolution

Methods for studying intronic enhancers in disease

Interpreting intronic enhancer function requires multiple methods. No single assay is sufficient. Chromatin accessibility assays such as ATAC-seq and DNase-seq identify open regulatory regions [79]. ChIP-seq or CUT&Tag for H3K27ac, H3K4me1, p300, and transcription factors helps distinguish active or poised enhancers [80]. CAGE, GRO-seq, PRO-seq, and related methods detect enhancer-associated transcription. Hi-C, Capture-C, promoter capture Hi-C, and related methods identify physical enhancer-promoter contacts [81].

Functional validation is essential. Reporter assays can test whether a sequence has enhancer activity, but they remove the element from its native chromatin context. Massively parallel reporter assays allow high-throughput testing of many variants, but they also have context limitations. CRISPR deletion, CRISPR interference, CRISPR activation, base editing, and prime editing can test enhancer function at the endogenous locus [82]. These approaches are especially valuable for intronic enhancers because the native genomic position strongly influences target-gene selection.

Expression quantitative trait locus analysis can link genetic variants to gene expression, but cell-type specificity is a limitation. A variant may show no eQTL effect in bulk blood but strong effects in a rare immune subset or during differentiation. Single-cell multi-omics is beginning to address this by connecting genotype, chromatin accessibility, and gene expression in specific cell populations.

Disease interpretation increasingly requires an integrated framework: variant location, chromatin state, transcription-factor motifs, evolutionary conservation, enhancer-promoter contacts, gene expression, phenotype relevance, and experimental perturbation. Intronic enhancer variants should not be dismissed because they are non-coding. They should be prioritized when they overlap active enhancers in disease-relevant cells and connect to plausible target genes.

Databases are being created using systems biology approaches to systematize molecular genetic features in various diseases for accurate diagnostics and precision medicine [83–85].

Therapeutic implications

Intronic enhancers create both diagnostic and therapeutic opportunities. Diagnostically, they expand the search space for pathogenic variants. Whole-genome sequencing is superior to exome sequencing for detecting enhancer mutations, structural variants, and deep intronic regulatory changes [86]. However, interpretation remains the bottleneck. Many intronic variants are found; few can be confidently classified.

Therapeutically, several strategies are possible. One is indirect targeting of enhancer-dependent transcription through chromatin regulators. This is already being explored in cancer. Another is correction of causal variants through genome editing, although this remains technically and ethically complex. A third is epigenome editing, in which catalytically inactive Cas proteins are fused to repressors or activators to modify enhancer states without cutting DNA [87]. A fourth is oligonucleotide-based intervention when enhancer-associated transcription or splicing interactions are involved.

The most realistic near-term impact is improved diagnosis and patient stratification. For example, identifying a pathogenic GATA2 intronic enhancer mutation can change clinical surveillance, family testing, and transplant decisions [88]. In complex disease, enhancer variants may help define molecular subtypes or predict response to therapy. Over time, enhancer maps may become as clinically important as coding-gene panels are today.

Conclusion

Intronic enhancers are central components of the regulatory genome. Their location within introns once made them easy to overlook, but functional genomics has shown that introns contain dense regulatory information. Intronic enhancers can regulate host genes, neighboring genes, or distant genes through chromatin looping and three-dimensional genome organization. Their activity is highly dependent on cell type, developmental stage, chromatin state, and transcription-factor context.

During tissue differentiation, intronic enhancers help establish and stabilize cell identity. They integrate lineage-determining transcription factors, extracellular signals, enhancer-associated transcription, and chromatin remodeling. Examples from hematopoiesis, myogenesis, adipogenesis, endothelial biology, neural differentiation, and immune-cell activation show that intronic enhancers are not rare exceptions but a recurring regulatory strategy.

In disease, intronic enhancers provide a mechanistic explanation for many non-coding variants. They can be disrupted by point mutations, indels, copy-number changes, structural rearrangements, enhancer hijacking, and epigenetic reprogramming. GATA2 enhancer mutations demonstrate that intronic enhancer disruption can cause severe Mendelian disease. FTO intronic variants illustrate how enhancer variation can influence complex metabolic traits through distal gene regulation. TAL1 enhancer activation in leukemia shows how non-coding enhancer changes can drive oncogene expression.

The main conclusion is that intronic enhancers should be treated as functional genomic elements with direct biological and clinical relevance. Exome-centered thinking is insufficient for many diseases. Future research should combine whole-genome sequencing, single-cell epigenomics, chromatin interaction mapping, and endogenous enhancer perturbation. The long-term goal is not merely to annotate intronic enhancers but to understand their causal roles in development and disease. Once this is achieved, intronic enhancers may become important targets for precision diagnostics, risk prediction, and regulatory therapeutics.

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Интронные энхансеры: регуляторная роль в дифференцировке тканей и влияние на заболевания человека

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Аннотация. *Актуальность*. Интронные энхансеры — это цис-регуляторные элементы ДНК, локализованные внутри интронов генов, кодирующих белки, либо внутри интронов некодирующих генов. Хотя интроны исторически рассматривались в основном как не кодирующие последовательности, удаляемые во время сплайсинга пре-мРНК, современные данные функциональной геномики показали, что они являются регуляторами транскрипции. Интронные энхансеры могут контролировать экспрессию своих генов, соседних или даже дальних генов. Их активность обычно специфична для каждого типа клеток и ассоциирована с открытым хроматином, связыванием транскрипционных факторов, энхансер-ассоциированными метками гистона, такими как H3K4me1 и H3K27ac, рекрутированием коактиваторов, формированием петлей между энхансером и промотором, и во многих случаях транскрипцией энхансерной РНК (эРНК). *Результаты и обсуждение*. Эта статья рассматривает биологическую значимость интронных энхансеров, уделяя особое внимание двум областям: дифференциации тканей и заболеваниям. Во время дифференциации интронные энхансеры интегрируют факторы транскрипции, определяющие направление развития, регуляторные сигналы и формируют тканеспецифичность. Они способствуют формированию идентичности клеток гемопоэза, эндотелия, мышц, адипоцитов, нейронов и иммунной системы, контролируя время, интенсивность и специфику экспрессии генов. При заболеваниях в интронных энхансерах обнаруживаются одиночные нуклеотидные замены, вставки, делеции, изменения числа копий, эпигенетические нарушения и аномальный перехват энхансера. Примеры включают мутации энхансера GATA2 при иммунодефицитах и предрасположенности к миелоидным злокачественным опухолям, интронные варианты гена FTO, влияющие на регуляцию IRX3/IRX5 при ожирении, и онкогенный активатор энхансера возле TAL1 при остро-клиническом лимфобластном лейкозе Т-клеток. Выводы. Интронные энхансеры не следует рассматривать как вторичные регуляторные элементы лишь потому, что они располагаются внутри генов. Они скорее представляют собой важные элементы регуляции

генома, связывающие не кодирующую вариацию с развитием, сложными признаками и механизмами заболеваний. Их изучение становится все более важным для прецизионной медицины, функциональной интерпретации исследований ассоциаций генома на уровне всего генома (GWAS) и будущих стратегий терапии, нацеленных на энхансеры.

Ключевые слова: интронные энхансеры, цис-регуляторные элементы, дифференцировка тканей, хроматин, энхансерная РНК, GATA2, FTO, TAL1, некодирующие варианты, генетика заболеваний

Информация о финансировании. Авторы заявляют об отсутствии финансирования.

Вклад авторов. Радаев Н.Е. — идея исследования, написание текста; Азова М.М. — идея исследования, написание текста; Джунушалиева Н.К. — написание текста; Гурьянова С.В. — дизайн исследования, написание и редактирование текста. Все авторы внесли значительный вклад в разработку концепции, проведение исследования и подготовку статьи, ознакомились и одобрили окончательный вариант перед публикацией.

Информация о конфликте интересов: авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение — неприменимо.

Благодарности — неприменимо.

Информированное согласие на публикацию — неприменимо.

Поступила 03.12.2025 Принята 29.12.2025.

Для цитирования: *Radaev N.E., Azova M.M., Dzhunushalieva N.K., Guryanova S.V.* Intronic enhancers: regulatory roles in tissue differentiation and human disease // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 356–376. doi: 10.22363/2313-0245-2026-30-3-356-376 EDN: KRHXOJ

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

ФИЗИОЛОГИЯ
PHYSIOLOGY

DOI 10.22363/2313-0245-2025-30-3-377-385
EDN KSOEWZ

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
ORIGINAL RESEARCH

Obstructive sleep apnea among commercial drivers in North India

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Abstract. *Relevance.* Obstructive sleep apnea (OSA) is the most common medical cause of excessive daytime sleepiness and is recognised worldwide as a significant risk factor for road traffic accidents when undiagnosed or untreated. OSA presents a critical occupational health concern in the transportation industry, affecting a substantial proportion of today's transport operators, especially in the trucking sector. *Aim of the* study was to estimate the risk of OSA among commercial drivers in North India and investigate its association with vehicle accidents. *Materials and methods*. A cross-sectional study was conducted involving 153 commercial drivers aged between 24 and 65 years, each with at least 5 years of driving experience. Participants were selected through simple random sampling after giving their consent. The collected data included demographic information, driving history (total years of driving, vehicular collisions due to sleepiness, and vehicular incidents), anthropometric measurements (height, weight, and neck circumference), and health history. Sleep quality, particularly difficulty in falling asleep or maintaining restful sleep, was assessed using the Pittsburgh Sleep Quality Index (PSQI). The risk of OSA and excessive daytime sleepiness was evaluated using the STOP-BANG questionnaire and the Epworth Sleepiness Scale, respectively. *Results and discussion.* Among participants, 21.56% were at risk of OSA, while 28.10% exhibited poor sleep quality as per the PSQI, and 15.16% of high-risk OSA drivers experienced excessive daytime sleepiness. A significant association was observed between the high-risk OSA group and vehicle collisions ($p < 0.05$). *Conclusion*. The prevalence of OSA risk is notably high among commercial drivers, who also display poor sleep quality. In a country with a population of 1.4 billion, studies on OSA among commercial drivers are limited. Currently, there are no guidelines for OSA detection in Indian commercial drivers, despite its recognised prevalence in studies from other countries and related hazards such as an increased risk of motor vehicle collisions**.**

Keywords: obstructive sleep apnea, commercial drivers, STOP-BANG, Epworth sleepiness scale, PSQI, road traffic accidents, neck circumference

Funding. This work was financed.

Author contributions. Bhardwaj M.B. — concept and writing of the final manuscript text; Mallick H. — intellectual content of the manuscript; approved the final version of the manuscript; Kaur N. — intellectual content of the manuscript; approved the final version of the manuscript. All authors made significant contributions to the conception, conduct of the study and preparation of the article, read and approved the final version before publication.

Conflict of interest statement. The authors declare no conflict of interest.

Ethics approval. Approval No. IEC/FMHS/MD/MS/2023.

Acknowledgements. We wish to thank our study participants.

Consent for publication. All participants of the investigation provided informed voluntary consent to participate in the study according to the Helsinki Declaration of the World Medical Association (WMA Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects, 2013) and personal data processing.

Received 29.06.2025. Accepted 21.07.2025.

For citation: Bhardwaj MB, Mallick H, Kaur N. Obstructive sleep apnea among commercial drivers in North India. *RUDN Journal of Medicine.* 2026;30(3):377–385. doi: 10.22363/2313-0245-2026-30-3-377-385 EDN: KSOEWZ

Introduction

Obstructive sleep apnea (OSA) is a common sleep disorder characterized by excessive daytime sleepiness, loud snoring, witnessed breathing pauses, or frequent arousals in the presence of at least five obstructive respiratory events per hour of sleep [1]. There is a partial or complete cessation of breathing during sleep. OSA is a critical occupational health concern in the transportation industry that is underdiagnosed, which affects a substantial percentage of today's transportation operators, mainly in the trucking sector. Excessive daytime sleepiness (EDS) is the most common symptom and is considered the most critical medical cause of OSA. Lack of sleep usually leads to impairment in a person's judgment and hampers their abilities, which is of utmost importance for driving and hence can cause motor vehicle crashes [2]. According to Parks et al, there are 2–7-fold increased chances of vehicular crashes in drivers with OSA, with an estimated prevalence of as high as 17% to 28% [3]. According to a systematic review, subjects having OSA on average have an increased risk of motor vehicle accidents (MVA) by 1.2 to 4.9-fold [4]. The International Road Federation World Road Statistics 2022 suggested that India has one of the highest numbers of total deaths due to road traffic accidents [5]. In one of the studies, the prevalence of OSA in the Indian male population is estimated to range between 13% and 19% [6].Although the prevalence in Indian drivers is mainly unknown. This study aimed to assess the risk of OSA in commercial drivers in North India and to find its correlation with vehicle accidents or near-miss incidents.

Materials and methods

One hundred fifty-three commercial drivers were enrolled after their consent by a simple random sampling technique. The study design was cross-sectional. The study was conducted after institutional approval from the ethical committee. Inclusion criteria consist of male, commercial drivers (truck, bus, interstate taxi service) with a minimum 5 years of driving experience, age group (25–65 years) who gave informed consent to participate in the study whereas participants already being diagnosed as OSA or any known sleep disorders, psychiatric disorders, drivers who didn't give consent for the study were being excluded. All the Demographic, anthropometric, and historical data were obtained. Sleep

quality, i.e., whether the subject is having difficulty in falling asleep or maintaining a sound sleep, was assessed using PSQI. The risk of OSA and excessive daytime sleepiness (EDS) was assessed using the STOP-BANG questionnaire and Epworth Sleepiness Scale (ESS), respectively.

Anthropometric measurements, such as height, weight, and neck circumference, were measured. Study questionnaires consisting of demographic characteristics, clinical history (if any), sleep habits and quality, driving history including total years of driving and average hours of driving per day, vehicular collisions occurred due to sleepiness, near-miss accidents due to sleep, etc., were obtained. The STOP BANG questionnaire was used to assess the risk of OSA, and a score >3 was taken as the cut-off for high risk.

Excessive Daytime Sleepiness was assessed using the ESS, a self-administered questionnaire with eight questions about the likelihood of dozing or falling asleep during eight different activities. Commercial drivers were asked to rate, on a 4-point scale (0–3), their usual chances of dozing while engaged in these activities. An ESS score above 10, i.e., 11–24, indicates a high risk of EDS. Sleep quality among commercial drivers was evaluated using the PSQI questionnaire, which measures aspects such as sleep latency, sleep duration, and habitual sleep efficiency. It also considers other factors related to sleep disturbances, including the use of sleeping medication and daytime dysfunction. The scores from seven components in PSQI are combined to produce a single global score that reflects both sleep quality and quantity. A global score exceeding 5 suggests poor sleep quality. The association of high-risk OSA with accidents were evaluated. All participants of the investigation provided informed voluntary consent to participate in the study according to the Helsinki Declaration of the World Medical Association (WMA Declaration of Helsinki — Ethical Principles for Medical Research Involving Human Subjects, 2013) and personal data processing.

The data obtained were analysed using SPSS statistical software (version 24). Quantitative data are presented as mean ± SD or median and compared with a Fisher t-test, while qualitative data are expressed as frequencies and percentages and compared using a chi-square test. A p-value of less than 0.05 was considered statistically significant.

Results and discussion

Among 153 commercial drivers, the average age of the participants was 39.9±9.10 years, ranging from 25 to 65 years. Hypertension was observed in 19 (12.2%), Diabetes mellitus in 7 (4.6%), and hypothyroidism in 3 (2%) participants. Normal Body mass index (BMI) was identified in 93 (60.78%) participants;12 (7.8%) were underweight, 42 (27.4%) were overweight, and 6 (3.9%) were obese as per World Health Organisation(WHO) classification. Among drivers, 11 reported accidents, whereas 15 reported near-missed accidents (Table 1).

Table 1

Descriptive characteristics of participants enrolled in the study

Variables	Frequency (n)	Percentage, %	Median	IR
Age			38 years	15 years
≤50 years	128	83.7	–	–
≥51 years	25	16.3	–	–
Observed apnea				
No	149	97.4	–	–
Yes	4	2.6	–	–

Ending tabl. 1

Variables	Frequency (n)	Percentage, %	Median	IR
Age			38 years	15 years
Snoring				
No	113	72.4	—	—
Yes	40	25.6	—	—
Daytime tiredness				
No	40	25.6	—	—
Yes	113	72.4	—	—
Accident / NMA				
No	131	85.6	—	—
Yes	11/15	7.1/9.8	—	—
Alcohol consumption				
No	58	37.9	—	—
Yes	95	62.1	—	—
OSA risk				
Low risk	120	78.5	—	—
High risk	33	21.5	—	—
Smoking				
No	61	39.9	—	—
Yes	92	60.1	—	—
Comorbidities				
HTN	19	12.2	—	—
DM	7	4.6	—	—
Hypothyroidism	3	2	—	—
BMI				
< 18.5	12	7.8	—	—
18.5–24.9	93	60.7	—	—
25–29.99	42	27.4	—	—
>30	6	3.9	—	—
Neck circumference				
31–40 cm	89	58.2	—	—
>41 cm	64	41.8	—	—
STOP BANG				
≤3	120	78.4	—	—
≥4	33	21.6	—	—
ESS				
0–7	108	70.6	—	—
8–9	30	19.6	—	—
10–15	15	9.8	—	—
16–24	0	0	—	—
PSQI				
0–5	110	71.9	—	—
6–21	43	28.1	—	—

33 among 153 commercial drivers were at high-risk of OSA whereas 120 drivers were at low-risk based on STOP BANG score >3. The bivariate analysis indicates that larger neck circumference, BMI, blood pressure, alcohol consumption, incidents or missed incidents, tiredness, hypertension (HTN), and snoring were significantly more common in the high-risk OSA group compared to the low-risk group. No significant difference was observed among subjects with diabetes mellitus and hypothyroidism (Table 2).

Association of OSA risk with accidents

Among participants with a high risk of OSA, 30.30% were involved in motor vehicle crashes compared to 13.13% in the low-risk OSA group ($p<0.05$). An ESS score (>10) was seen in 15.15% of the high-risk group ($p<0.05$) (Table 2).

Table 2

Bivariate analysis between OSA risk (dependent factor) and independent factors

Variables	OSA Risk		Total	*p* value
	Low riskN=120 (%)	High riskN=33 (%)		
Age median (Range)	35(24–65)	49 (32–62)	—	—
≤50 years	112 (93.33)	16 (48.48)	128	—
≥ 51 years	8 (6.66)	17 (51.51)	25	—
Observed apnea	1 (0.83)	3 (9.09)	4	0.05
Snoring	13 (10.83)	27 (81.81)	40	<0.001*
Tiredness	81 (67.5)	32 (96.96)	113	<0.001*
Accident or near miss accidents	16 (13.33)	10 (30.30)	26	<0.001*
Alcohol consumption	72 (60)	23 (69.69)	95	<0.001*
Hypertension	6 (5)	13 (39.39)	19	<0.001*
Diabetes Mellitus	2 (1.66)	5 (15.15)	7	0.02
Hypothyroidism	2 (1.66)	1 (3.03)	3	0.001
Epworth Sleepiness Scale				
≤ 10	118 (98.33)	28 (84.84)	146	—
> 10	2 (1.66)	5 (15.15)	7	<0.001*
Neck circumferenceMean (SD)	38.53 (1.9)	41.12 (1.34)	—	—
≤ 40 cm	110 (91.66)	10 (30.30)	120	—
≥ 41 cm	10 (8.33)	23 (69.69)	33	<0.001*
Body mass indexMean (SD)	22.7 (3.3)	25.3 (2.5)	—	—
< 18.5	12 (10)	-	12	—
18.5–24.9	78 (65)	15 (45.45)	93	—
25–29.9	26 (21.66)	16 (48.48)	42	—
> 30	4 (3.33)	2 (6.06)	6	<0.001*

This study revealed that 33 (21.5%) of commercial drivers were in a high-risk group for OSA based on the STOP-BANG questionnaire, which is an internationally validated tool having high sensitivity and specificity for OSA screening [7]. Obstructive sleep apnea is a significant health concern in the transportation sector, which affects a considerable number of commercial drivers, causing significant morbidity. According to a previous study in North America, 17–28% of commercial drivers were expected to have OSA [8]. In a recent survey done in Lusaka, Zambia, 22.8% of commercial drivers were found to have a high risk for OSA [9]. The estimated prevalence of OSA in the Indian population, based on a meta-analysis by Suri TM et al, was 11% [10]. In another study, commercial drivers were found to have 2–7 times higher prevalence of OSA compared to the general population [11]. In our study, a significant number of commercial drivers were overweight and obese and had a neck circumference of more than 40 cm. This can be due to the sedentary lifestyle, long working hours, and eating habits of commercial drivers, exposing them to many non-communicable diseases [12]. The average working hours in our study were 10.30 hours per day. These long working hours make drivers vulnerable to prolonged immobility and morbidity. A study among Australian drivers discovered that 44% of drivers in the study were obese and 24% were diagnosed with OSA [13]. In our study, 45% of the participants from the high-risk group for OSA were under the normal BMI range; the same findings were also seen in a Japanese study, where most drivers with OSA were not obese per WHO international criteria (BMI ≥30 kg/m^2) [14]. Villaneuva et al reviewed the existing literature, which shows that the Asian population is at a higher risk of having OSA at the lower level of obesity as compared to the Caucasian population, showing a high propensity of OSA in the Asian population [15]. In another study, weight gain as an anatomical factor plays a vital role in OSA pathogenesis, along with the contribution of non-anatomical traits such as low arousal threshold or poor sleep-related upper airway muscle tone/low pharyngeal muscle responsiveness [16]. Among the studied commercial drivers**,** snoring, BMI and neck circumference were significantly higher among high-risk groups of OSA. These findings are comparable to the study done by Wahida et al [17].

It has been reported that ~50% of OSA patients have hypertensionand that ~30% of hypertensive patients suffer from OSA [18]. In our study, a significant number of respondents in the high-risk OSA (39.39%) category were hypertensive. A cohort study from Brazil included 10,101 truck drivers and determined that factors such as obesity and hypertension that were associated with OSA were common among drivers [19].

Excessive daytime sleepiness (EDS) is the most crucial consequence of OSA in the context of occupational health. It is also a significant cause of motor vehicle collisions (MVCs) among commercial drivers. In this study, the Epworth sleepiness scale (ESS) was used to determine EDS among commercial drivers, where an ESS score >10 was significantly associated with high-risk OSA respondents as compared to the low-risk group. Long-haul truck drivers are a high-risk population due to their high driving distance/year, i.e., increased exposure, frequent sleep debt, and a high prevalence of OSA[20]. The study by Burks and co-workers in truck drivers in the United States showed that drivers with OSA who were non-adherent to CPAP treatment had a fivefold risk of serious preventable crashes. In contrast, those adherent to CPAP treatment showed a crash rate similar to the controls [21]. Between 10–30% of all vehicle crashes are estimated to have sleepy drivers as a root cause [22]. However, in this study, almost 85% of high-risk OSA drivers had an ESS score of less than 10.

In trying to associate OSA with RTAs, we discovered that out of the 33 drivers that were classified with a high risk for OSA, 10 had experienced at least one MVC. These findings show us that the possibility of accidents happening because of the dozing of commercial drivers at the wheel and not being able to concentrate fully on the road could very likely be because of OSA and EDS. Consistent with the reported literature, we found a statistically significant association (p<0.05) between the STOP-Bang score and the number of accidents and near accidents. However, several studies depict that the vast majority of OSA patients

without complaints of EDS were also found to exhibit attention defects that may potentially expose them to specific risks of accidents while driving [22, 23].

Our study found that 28.1% of commercial drivers experienced poor sleep quality, with a PSQI score above 5, including 12 (36.36%) in the high-risk group and 31 (25.83%) in the low-risk group. This aligns with the study by Kalcina et al., where Obstructive sleep apnoea patients had higher overall PSQI scores, indicating poorer sleep compared to a healthy control group [24]. Commercial drivers often work long hours, perform shift work, lack proper resting facilities during work, and do not adhere to their permitted daily working hours.

Road traffic accidents impose a significant burden on the nation's economy and healthcare systems. Obstructive sleep apnea and EDS are major risk factors for RTA. Therefore, there should be appropriate screening and awareness programs, and strict rules should be followed while issuing or renewing driving licenses by local transport organizations. Polysomnography is a gold standard test for diagnosing OSA, and may be included in the case of individuals with high-risk factors for OSA before issuing a vehicular licence.

Limitations

The study is entirely based on self-reporting and not on any documentary evidence or driving simulation-based assessments. In addition, the entire data has been collected at a single time point and not a longitudinal analysis. The data regarding accidents and near accidents were collected retrospectively from participants, which may have led to recall bias.

Conclusion

The prevalence of OSA among commercial drivers is high in India. Also, the sleep quality is poor among them. Therefore, there are high chances of road traffic accidents. There should be guidelines for OSA screening among commercial drivers for early detection and treatment of OSA, thereby decreasing fatal events.

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Обструктивное апноэ сна среди водителей коммерческого транспорта в Северной Индии

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Аннотация. *Актуальность*. Обструктивное апноэ сна (ОАС) является наиболее распространенной медицинской причиной чрезмерной дневной сонливости и признано во всем мире как значительный фактор риска дорожно-транспортных происшествий, если оно не диагностировано или не лечится. ОАС представляет собой серьезную проблему для здоровья работников транспортной отрасли, затрагивая значительную часть современных транспортных операторов, особенно в секторе грузоперевозок. Цель исследования состояла в том, чтобы оценить риск ОАС среди водителей коммерческого транспорта в Северной Индии и изучить его связь с дорожно-транспортными происшествиями. *Материалы и методы.* Было проведено поперечное исследование с участием 153 водителей коммерческого транспорта в возрасте от 24 до 65 лет, каждый со стажем вождения не менее 5 лет. Участники были отобраны методом простой случайной выборки после получения их согласия. Собранные данные включали демографическую информацию, историю вождения (общее количество лет вождения, дорожно-транспортные происшествия из-за сонливости и другие ДТП), антропометрические измерения (рост, вес и окружность шеи) и историю болезни. Качество сна, в частности трудности с засыпанием или поддержанием спокойного сна, оценивалось с помощью Питтсбургского индекса качества сна (PSQI). Риск синдрома обструктивного апноэ сна (СОАС) и чрезмерной дневной сонливости оценивался с помощью опросника STOP-BANG и шкалы сонливости Эпворта соответственно. *Результаты и обсуждение*. Среди участников 21,56 % находились в группе риска СОАС, в то время как 28,10 % демонстрировали плохое качество сна по данным PSQI, а 15,16 % водителей с высоким риском СОАС испытывали чрезмерную дневную сонливость. Была выявлена значительная связь между группой высокого риска обструктивного апноэ сна (ОАС) и дорожно-транспортными происшествиями ($p < 0,05$). *Выводы*. Распространенность риска ОАС особенно высока среди водителей коммерческого транспорта, которые также демонстрируют плохое качество сна. В стране с населением 1,4 миллиарда человек исследования ОАС среди водителей коммерческого транспорта ограничены. В настоящее время отсутствуют рекомендации по выявлению ОАС у индийских водителей коммерческого транспорта, несмотря на признанную распространенность этого заболевания в исследованиях из других стран и связанные с ним риски, такие как повышенный риск дорожно-транспортных происшествий.

Ключевые слова: обструктивное апноэ сна, водители коммерческого транспорта, STOP-BANG, шкала сонливости Эпворта, PSQI, дорожно-транспортные происшествия, окружность шеи

Информация о финансировании. Данная работа была профинансирована.

Вклад авторов. Бхардвадж М.Б. — концепция и написание окончательного текста рукописи; Маллик Х. — интеллектуальное содержание рукописи; утвердил окончательную версию рукописи; Каур Н. — интеллектуальное содержание рукописи;

утверждение окончательной версии рукописи. Все авторы внесли значительный вклад в концепцию, проведение исследования и подготовку статьи, прочитали и одобрили окончательную версию перед публикацией.

Заявление о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

Информация о конфликте интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией данной статьи.

Этическое утверждение. Номер одобрения: IEC/FMHS/MD/MS/2023.

Благодарности. Мы выражаем благодарность участникам нашего исследования.

Информированное согласие на публикацию. Все участники исследования предоставили информированное добровольное согласие на участие в исследовании в соответствии с Хельсинкской декларацией Всемирной медицинской ассоциации (Хельсинкская декларация ВМА — Этические принципы медицинских исследований с участием человека, 2013 г.) и обработкой персональных данных.

Поступила 29.06.2025. Принята 21.07.2025.

Для цитирования: *Bhardwaj M.B., Mallick H., Kaur N.* Obstructive sleep apnea among commercial drivers in North India // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 377–385. doi: 10.22363/2313-0245-2026-30-3-377-385 EDN: KSOEWZ

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

DOI 10.22363/2313-0245-2025-30-3-386–395
EDN KXZLKL

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
ORIGINAL RESEARCH

Различный эффект влияния изомеров метионина на биохимические и цитологические показатели крови экспериментальных животных

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Аннотация. *Актуальность*. Избыток метионина, поступающего в организм (с пищей, лекарствами, биодобавками), сопровождается токсическим накоплением гомоцистеина и неблагоприятными эффектами, приводящими к формированию патологии ряда систем. Сравнительному влиянию изомеров метионина посвящено незначительное количество работ. *Цель исследования*: установить особенности биологического действия изомеров метионина, используемого в качестве пищевой биодобавки, на биохимические и цитологические показатели крови экспериментальных животных. *Материалы и методы*. Экспериментальные крысы получали добавку к корму с повышенным содержанием метионина в виде DL-формы (рацемат DL-метионина, Бельгия), либо L-формы (100 % L-метионин, Fingres Biotech Inc, Китай) в количестве 0,15г/100г массы животного. У контрольных животных прикорм не содержал добавок. Через 25 дней у животных под эфирным наркозом транскардиальной пункцией забирали кровь. Определяли: концентрацию гомоцистеина (Axis-Shield PLC (Великобритания)); проводили цитологический анализ белой крови и красной крови (гематологический анализатор System XS-500i (Япония)); для качественной оценки мазки крови окрашивали с помощью гематологического красителя Лейкодиф-200 (Lachema, Чехия) и просматривали в микроскопе Carl Zeiss (Германия) при увеличении × 1000. Рассчитывали индекс аллергизации организма и нагрузочный эритроцитарный коэффициент. Статистический анализ проводили с использованием методов анализа на нормальность распределения данных по Шапиро-Уилка, однофакторный дисперсионный анализ ANOVA, множественные сравнения проводились с помощью теста Тьюки. *Результаты и обсуждение*. Введение рацемической смеси DL-метионина привело к повышению гомоцистеина в крови до 28,9 ± 2,65 (p = 0,05), L-формы до 79,38 ± 3,91 (p = 0,05) мкмоль/л (в контроле 8,5 ± 0,6 мкмоль/л). В крови животных 1-й группы было выявлено повышение абсолютного числа гранулоцитарных форм лейкоцитов (в 1,4 раза, p = 0,049), отмечался нейтрофилез. При введении L-формы метионина у животных было резко снижено как абсолютное (в 3,7 раза в сравнении с контрольной группой, p = 0,01), так и относительное количество нейтрофилов, отмечено появление гиперсегментированных форм нейтрофилов и незрелых форм эозинофилов. В обеих группах проявилась аллергическая настроенность организма,

более выраженная у животных с введением L-формы метионина, индекса аллергизации был повышен на 49,2 ± 3,61 % (p = 0,01). При введении L-формы снижалось количество тромбоцитов; увеличивалась СОЭ; на 15,7 % (p = 0,043) повышался нагрузочный эритроцитарный коэффициент. *Выводы*. Таким образом, выраженность гипергомоцистеинемии, направленность ответных реакций белой и красной крови, коэффициентов, отражающих аллергическую настроенность организма и функциональную эритроцитарную нагрузку при избыточном введении метионина в качестве биодобавки, определяются формой изомера, входящего в ее состав.

Ключевые слова: DL-метионин, L-метионин, гипергомоцистеинемия, клинический анализ крови

Информация о финансировании. Авторы заявляют об отсутствии внешнего финансирования.

Вклад авторов. Чучкова Н.Н. — концепция и дизайн исследования, написание и оформление текста; Сметанина М.В. — концепция и дизайн исследования, написание и оформление текста; Кормилина Н.В. — эксперимент; Сметанина М.В. — эксперимент; Пазиненко К.А. — эксперимент, статистический анализ. Все авторы внесли существенный вклад в проведение исследования и подготовку рукописи, прочитали и одобрили окончательную версию перед публикацией.

Информация о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение. Исследование одобрено комитетом по биомедицинской этике ФГБОУ ВО «Ижевский государственный медицинский университет» Минздрава РФ (аппликационный № 610), г. Ижевск.

Благодарности — неприменимо.

Информированное согласие на публикацию — неприменимо.

Поступила 01.10.2025. Принята 30.10.2025.

Для цитирования: *Чучкова Н.Н., Пазиненко К.А., Сметанина М.В., Кормилина Н.В., Пазиненко О.А.* Различный эффект влияния изомеров метионина на биохимические и цитологические показатели крови экспериментальных животных // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 386–395. doi: 10.22363/2313-0245-2026-30-3-386-395 EDN: KXZLKL

Different effects of methionine isomers on biochemical and cytological indices of blood in experimental animals

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Abstract. *Relevance.* Excess methionine entering the body (with food, drugs, dietary supplements) is accompanied by toxic accumulation of homocysteine and adverse effects leading to the formation of pathology of some systems. Small amount of studies are devoted to the comparative effect of methionine isomers. *Objective*: to establish the features of the biological effect of methionine isomers used as a dietary supplement on biochemical and cytological parameters of the blood of experimental

animals. *Materials and methods.* Experimental rats received a feed additive with an increased content of methionine in the form of DL-form (racemate DL-methionine, Belgium) or L-form (100% L-methionine, Fingres Biotech Inc, China) for 0.15 g / 100 g of animal weight. In control animals, the feed did not contain additives. After 25 days, blood was taken from the animals under ether anesthesia by transcardial puncture. The following was determined: homocysteine concentration (Axis-Shield PLC (UK)); cytological analysis of white and red blood was performed (System XS-500i hematological analyzer (Japan)); for qualitative assessment, blood smears were stained with the hematological dye Leukodif-200 (Lachema, Czech Republic) and viewed under a Carl Zeiss microscope (Germany) at a magnification of × 1000. The body's allergization index and the erythrocyte loading coefficient were calculated. Statistical analysis was performed using the Shapiro-Wilk normality analysis of data distribution, one-way ANOVA analysis of variance, multiple comparisons were performed using the Tukey test. *Results and discussion*. Introduction of racemic mixture of DL-methionine resulted in increase of homocysteine in blood to 28.9 ± 2.65 ($p = 0.05$), L-form to 79.38 ± 3.91 ($p = 0.05$) μmol/l (in control 8.5 ± 0.6 μmol/l). In blood of animals of group 1 increase of absolute number of granulocytic forms of leukocytes (by 1.4 times, $p = 0.049$) was revealed, neutrophilia was noted. When introducing L-form of methionine in animals there was sharp decrease of both absolute (by 3.7 times in comparison with control group, $p = 0.01$) and relative number of neutrophils, appearance of hypersegmented forms of neutrophils and immature forms of eosinophils was noted. In both groups, allergic predisposition of the organism was manifested, more pronounced in animals with the introduction of the L-form of methionine, the allergization index was increased by 49.2 ± 3.61% ($p = 0.01$). With the introduction of the L-form, the number of platelets decreased; ESR increased; the erythrocyte loading coefficient increased by 15.7% ($p = 0.043$). *Conclusion.* Thus, the severity of hyperhomocysteinemia, the direction of the responses of white and red blood, the coefficients reflecting the allergic disposition of the body and the functional erythrocyte load with excessive administration of methionine as a dietary supplement are determined by the form of the isomer included in its composition.

Keywords: DL-methionine, L-methionine, hyperhomocysteinemia, clinical blood test

Funding. The authors received no financial support for the research, authorship, and publication of this article.

Author contributions. Chuchkova N.N. — study concept and design, writing and formatting; Smetanina M.V. — study concept and design, writing and formatting; Kormilina N.V. — experiment; Smetanina M.V. — experiment; Pazinenko K.A. — experiment, statistical analysis. All authors made a significant contribution to the study and preparation of the manuscript, read and approved the final version before publication.

Conflicts of interest statement. The authors declare that there is no conflict of interest.

Ethics approval. The study was approved by the Biomedical Ethics Committee of the Izhevsk State Medical Academy of the Ministry of Health of the Russian Federation (application No. 610), Izhevsk.

Acknowledgements — not applicable.

Consent for publication — not applicable.

Received 01.10.2025. Accepted 30.10.2025.

For citation. Chuchkova NN, Smetanina MV, Pazinenko KA, Kormilina NV, Pazinenko OA. Different effects of methionine isomers on biochemical and cytological indices of blood in experimental animals. *RUDN Journal of Medicine.* 2026;30(3):386–395. (In Russ.). doi: 10.22363/2313-0245-2026-30-3-386-395 EDN: KXZLKL

Введение

Метионин — незаменимая аминокислота, избыток которой приводит к повышению гомоцистеина (ГЦ) в плазме крови. Токсическое накопление ГЦ играет ключевую роль в возникновении и прогрессировании различных патологических состояний: атеросклеротических повреждений, сердечно-сосудистой и нейродегенеративной патологии, тромботических изменений [1–4]. Диапазон гипергомоцистеинемии (гиперГЦ), рассматривающийся как предиктор

заболеваний, очень широк. Так, показатели ГЦ от 15 мкмоль/л до 100 мкмоль/л обозначают как умеренную, свыше 100 мкмоль/л — тяжелую гипергомоцистеинемию. Тяжелая форма гипер ГЦ, опосредуется главным образом генетическими причинами, сопряжена с дефектами фолатного цикла, связана с тяжелым поражением почек и встречается достаточно редко. Наиболее распространенной является умеренная форма гиперГЦ, нижнюю границу которой в настоящее время предлагают снизить с 15 до 10 мкмоль/л [3]. Умеренная гиперГЦ является следствием диетических, поведенческих и патологических состояний; на уровень ее развития влияют возраст, пол, прием лекарственных препаратов или БАДов [5]. Однако до сих пор не ясно, является ли умеренно повышенная концентрация гомоцистеина в плазме первопричиной или просто маркером связанного с ней патологического состояния, которое может предрасполагать к развитию сосудистых заболеваний, или их осложнений. В связи с вышеизложенным актуальными являются исследования на животных-моделях с повышенным количеством ГЦ в крови. Для этого используются подходы, связанные, например, с диетическими нарушениями [6, 7], нокаутные модели мышей и крыс [8], вариации в использовании животных разных видов с неодинаковой чувствительностью к метионину [9].

Метионин содержит асимметрический атом углерода, обладает оптической активностью и существует в виде двух энантиомеров (изомеров) — L- и D-метионина. В живых организмах встречается преимущественно в L-форме. Феномен различия в биологическом действии лекарственных препаратов, обусловленный оптической изомерией вещества, хорошо описан [10, 11]. Разнонаправленные и неоднозначные эффекты изомеров метионина, используемых в качестве пищевой добавки, добавляемой в прикорм животным, при одинаковой их биодоступности [12] отражены в ряде работ [13, 14].

Цель исследования — установить особенности биологического действия изомеров метионина, используемого в качестве биологической добавки, на биохимические и цитологические показатели крови экспериментальных животных.

Материал и методы

Эксперимент выполнен на 52 белых крысах *Rattus norvegicus Berk* обоего пола, содержащихся в виварии ФГБОУ ВО ИжГМУ, в осенне-зимний период (октябрь–декабрь). Содержание животных осуществлялось в соответствии с нормативным документом СП 2.2.1.321814 «Санитарно-эпидемиологические требования к устройству, оборудованию и содержанию экспериментально-биологических клиник (вивариев)» от 29 августа 2014 г. № 51.

Масса животных на момент введения в эксперимент составляла 220 ± 20 г. Крысы находились в одинаковых условиях содержания, получали экструдированный корм (ПК-120 ГОСТ Р 51849–2001 Р.5) при свободном доступе к воде. Все эксперименты выполнены в соответствии с Женевской конвенцией «Internetional Guiding Principals for Biomedical Involving Animals» (Geneva, 1990), а также Хельсинской декларацией Всемирной Медицинской Ассоциации о гуманном отношении к животным (редакция 2000 г.). Исследование одобрено комитетом по биомедицинской этике ФГБОУ ВО «Ижевская государственная медицинская академия» Минздрава РФ (протокол № 656 от 23.04.2019).

Животным ежедневно индивидуально скармливали шарики из сырого свиного мясного фарша весом 15,0 ± 0,5 г. У контрольных животных прикорм не содержал добавок, у крыс экспериментальной группы № 1 в фарш добавляли DL-метионин (DL), содержащий в равных количествах D и L формы (99 % DL-метионин, Бельгия), у экспериментальной группы № 2 в прикорм вводили L-метионин (L) (100 % L-метионин, Fingres Biotech inc) в количестве 0,15г/100г массы животного. Одновременно крысы получали 1 % раствора соответствующей формы метионина в воде в качестве питья. Через 25 дней у экспериментальных животных и интактных крыс под эфирным наркозом транскардиальной пункцией забирали кровь. Концентрацию гомоцистеина определяли иммуноферментным методом с использованием тест-системы Axis-Shield PLC (Великобритания); клинический цитологический

анализ белой крови и красной крови проводили на гематологическом анализаторе System XS-500i (Япония); для качественной оценки мазки крови дополнительно окрашивали с помощью гематологического красителя Лейкодиф-200 (Lachema, Чехия) и просматривали в микроскопе Carl Zeiss Primo Star (Carl Zeiss, Германия) при увеличении × 1000. Рассчитывали индекс аллергизации организма (ИАО) и нагрузочный эритроцитарный коэффициент (НЭК). ИАО определяли по формуле: Лимфоциты + 10 × (Эозинофилы + 1)/ (Нейтрофилы + Моноциты + Базофилы) [15]. Для определения НЭК использовали показатели скорости оседания эритроцитов (СОЭ) и уровень гемоглобина по формуле: СОЭ (мм/ч) × 10 / Гемоглобин,% [16].

Статистический анализ проводили с использованием методов анализа на нормальность распределения данных по Шапиро-Уилку, однофакторного дисперсионного анализа ANOVA, множественные сравнения проводились с помощью теста Тьюки. Данные считались достоверными при $p \leq 0{,}05$.

Результаты и обсуждение

Введение экспериментальным животным рацемической смеси DL (50 %/50 %) форм метионина (экспериментальная группа № 1) и 100 % L-формы аминокислоты (экспериментальная группа № 2) привело к формированию гиперГЦ с разной концентрацией гомоцистеина в крови. Так, у интактных крыс концентрация ГЦ составила 8,5 ± 0,6, в экспериментальной группе № 1–28,9 ± 2,65 (в 3,4 раза выше, p = 0,05) в экспериментальной группе № 2–79,38 ± 3,91 мкмоль/л (в 9,3 раза выше интактного контроля, $p = 0{,}005$).

Цитологический анализ крови животных с DL- и L-индуцированной формой гиперГЦ выявил существенные различия в количестве и видах гранулоцитов и агранулоцитов белой крови. Клеточный состав белой крови представлен в табл. 1.

Таблица 1 / Table 1

Показатели белой крови животных при введении DL (1 эксперимент) и L (2 эксперимент) форм метионина / White blood indices of animals with the administration of DL (1 experiment) and L (2 experiment) forms of methionine

Показатели/ Indicators	Контроль/ Control	Эксперимент 1/ Experiment 1	Эксперимент 2/ Experiment 2
Лейкоциты(10^9/л)/ Leukocytes(10^9/l /%)	10,79 ± 2,24	13,75 ± 1,76* ($p = 0{,}046$)	10,71 ± 1,12# (p = 0,001)
Лимфоциты (10^9/л /%)/ Lymphocytes(10^9/l /%)	6,51 ± 0,90/59/39 ± 5,65	5,26 ± 0,69*/ 45,57 ± 1,86 ($p = 0{,}045$)	6,04 ± 0,93/ 62,04 ± 2,7
Моноциты(10^9/л /%)/Monocytes(10^9/l /%)	0,63 ± 0,23/6,90 ± 1,76	1,92 ± 0,24*/9,32 ± 2,42 ($p = 0{,}040$)	1,4 ± 0,09* /9,86 ± 0,98 (p = 0,036)
Гранулоциты (10^9/л /%)/ Granulocytes (10^9/l /%)	3,79 ± 0,42/ 35,10 ± 4,14	5,22 ± 1,03*/ 45,10 ± 2,53 ($p = 0{,}049$)	3,33 ± 0,81#/ 28,45 ± 0,50
Нейтрофилы(10^9/л /%)/ Neutrophils(10^9/l /%)	2,62 ± 0,82/ 69,13 ± 15,2	3,33 ± 3,64*/ 63,79 ± 36,4 (p = 0,01)	0,71 ± 0,34*#/ 20,1 ± 8,6 (*p = 0,01#p = 0,001)
Эозинофилы (10^9л /%)/ Eosinophils(10^9/l /%)	0,87 ± 0,08/ 22,9 ± 9,6	1,48 ± 0,29*/28,35 (p = 0,045)	1,70 ± 0,87*#/ 50,15 ± 18,2 (*p = 0,01;#p = 0,02)
Базофилы(10^9л /%)/ Basophils(10^9l /%)	0,29 ± 0,02/ 7,65 ± 1,51	0,41 ± 0,02*/ 7,86 ± 1,51 (p = 0,03)	1,0 ± 0,08*#/ 30,03 ± 10,3 (*p = 0,024 #p = 0,043)

Примечание: * — различия достоверны в сравнении с интактным контролем, # — различия достоверны между экспериментальными группами

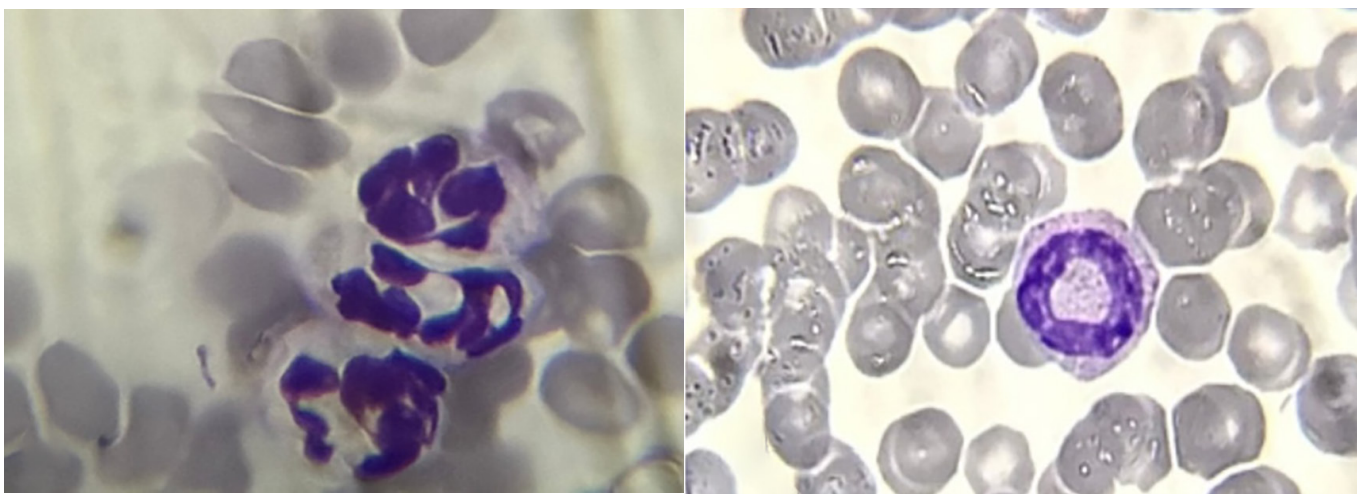
Note: * — differences are significant in comparison with the intact control, # — differences are significant between experimental groups

При оценке показателей белой и красной крови между группами наблюдалась статистически значимая разница, определяемая по однофакторному анализу ANOVA для количества лейкоцитов: $F(2,28) = 9,678$, $p = 0,001$. Согласно полученным данным, лейкоцитоз присутствовал только у животных, которым в корм добавляли DL-форму препарата: в сравнении с интактным контролем количество WBC было превышено на 27,5 % ($p = 0,046$), тогда как во 2-й группе их количество не изменилось. Повышение абсолютного числа гранулоцитарных форм лейкоцитов было выявлено только в экспериментальной группе № 1 (в 1,4 раза, $p = 0,049$), существенные различия касались их видов. Так, в 1-й экспериментальной группе количество нейтрофилов повышалось в 1,3 раза ($p = 0,049$), эозинофилов в 1,7 раз ($p = 0,045$), базофилов в 1,4 раза ($p = 0,03$). Во второй экспериментальной группе было резко снижено как абсолютное (в 3,7 раза в сравнении с контрольной группой, $p = 0,01$), так и относительное количество нейтрофилов (см. табл. 1), 80 % гранулоцитов составляли эозинофилы и базофилы (см. табл. 1). При качественной оценке мазков крови в группе с введением L-формы метионина было отмечено появление гиперсегментированных форм нейтрофилов и незрелых форм эозинофилов (рис.).

Из агранулоцитарных форм лейкоцитов в обеих экспериментальных группах было увеличено количество моноцитов: в 3,04 раза 1-й ($p = 0,040$), и в 2,22 раза ($p = 0,036$) во 2-й экспериментальной группе. Изменения не касались количества лимфоцитов в группе с введением L-формы метионина.

Учитывая дисбаланс в анализах крови по гранулоцитарным формам лейкоцитов, отражающий повышенное содержание эозинофилов и базофилов, мы рассчитали индекс аллергизации организма. ИАО во 2-й экспериментальной группе составил 10,62 ± 1,21 уе (в контроле 7,12 ± 1,02 уе), превысив, таким образом, исходный показатель на 49,2 ± 3,61 % ($p = 0,01$).

Отличались и показатели красной крови в экспериментальных группах (табл. 2).



а *б*

Мазок крови крыс в группе с ведением L-формы метионина: *а* – ядра нейтрофилов содержат более 5 сегментов; *б* – кольцевидные ядра эозинофилов. Лейкодиф-200. Ув. × 1000

Blood smear of rats in the group with the administration of L-form of methionine: *a* – Neutrophil nuclei contain more than 5 segments; *b* – Ring-shaped nuclei of eosinophils. Leukodif-200. × 1000

Таблица 2 / Table 2

Показатели красной крови животных при введении DL- (1 эксперимент) и L- (2 эксперимент) форм метионина / Red blood parameters of animals with the administration of DL (1 experiment) and L (2 experiment) forms of methionine

Показатели / Parameters	Единицы измерения / Units of measurement	Экспериментальные группы/Experimental groups		
		Контроль / Control	Эксперимент 1/ Experiment 1	Эксперимент 2 / Experiment 2
Эритроциты/Red blood cells (RBC)	10^{12}г/л10^{12} g/l	6,75 ± 0,71	6,08 ± 0,49 (p = 0,148)	5,77 ± 1,06 (p = 0,166*, p = 0,840#)
Гемоглобин/ Hemoglobin (HGB)	г/лg/l	120,62 ± 4,13	113,17 ± 4,94 (p = 0,603)	123,9 ± 3,45 (p = 0,780*, p = 0,265#)
Гематокрит/Hematocrit (HTC)	л/лl/l	0,36 ± 0,03	0,41 ± 0,003* (p = 0,001)	0,34 ± 0,04 (p = 0,798*, p = 0,001#)
Средний размер эритроцитов/ Mean Corpuscular Volume (MCV)	Фемтолитры (фл) Femtolters (fl)	54,87 ± 1,84	56,37 ± 1,52 (p = 0,993)	55,06 ± 3,31 (p = 0,912*, p = 0,943#)
Среднее содержание гемоглобина в эритроците/Mean corpuscular hemoglobin (MCH)	Пикограммы (пг) Picograms(pg)	17,72 ± 0,12	18,7 ± 0,34 (p = 0,002)	19,96 ± 0,84 (p = 0,001*, p = 0,964#)
Ширина распределе-ния эритроцитов/Red cell Distribution Width (RDW)	%	17,11 ± 0,33	17,04 ± 0,37 (p = 0,851)	14,57 ± 1,17 (p = 0,001*, p = 0,009#)

Примечания: * — различия значимы в сравнении с контрольными данными, # — различия значимы между данными экспериментальных групп.

Note: * — differences are significant in comparison with the intact control, # — differences are significant between experimental groups.

Статистически значимые изменения в показателях красной крови отмечались в группе животных с введением L-формы метионина. У крыс этой группы изменились: количество эритроцитов (было снижено на 14,5 ± 3,5 %), средняя ширина эритроцитов — показатель RDW — достоверно снижалась на 14,8 % ± 4,94, (p = 0,001); гемоглобин (HGB) не изменялся, однако его концентрация в одном эритроците (MCH) достоверно повышалась на 12,6 ± 2,88 % (p = 0,002).

Скорость оседания эритроцитов составила: 2,0 ± 0,01 мм/ч в контрольной и 2,1 ± 0,11 мм/ч (p = 0,943) в 1-й экспериментальной группе. Во 2-й группе СОЭ была повышена до 2,38 ± 0,32 (p = 0,915).

К функциональным показателям гемопоэза относится НЭК — нагрузочный эритроцитарный коэффициент. Для интактных крыс в возрасте 2-х месяцев этот показатель составил 16,6 ± 0,3 %; 18,6 ± 0,5 % (повышение составило 12,0 %, p>0,05) в 1-й, и 19,2 ± 0,3 % во 2-й экспериментальных группах (повышение составило 15,7 % (p = 0,043).

Противоположными по направлению изменений оказались показатели количества тромбоцитов в группах с введением различных изомеров метионина. Так, если в 1-й экспериментальной группе количество тромбоцитов превышало исходные показатели на 19,14 % (в интактном контроле 660,75 ± 93,18 × 10^9л *vs* в 1 эксперименте 787,25 ± 68,74 × 10^9л), тогда как во при введении L-формы метионина их количество было достоверно снижено на 37,03 % (416,08 ± 59,52 × 10^9л). Согласно однофакторному анализу ANOVA наблюдалась статистически значимая разница в количестве тромбоцитов: $F(2,28) = 8,442$, p = 0,002.

Таким образом, были выявлены отличия в показателях крови экспериментальных животных, которые содержались на «диете» с добавками различных изоформ метионина — DL- и L-вариантов.

Неодинаковый биологический эффект действия изомеров химических веществ — явление известное [10, 11]. Аминокислота метионин существует в форме правых и левых изомеров (энантиомеров,

таутомеров), отличающихся по наличию их в организме, активности действия, биологическим эффектам. В природе аминокислота представлена преимущественно левовращающей L-формой. В нашем исследовании метионин использовался как пищевая биодобавка, в результате переизбытка которого у животных формируется гиперГЦ. Однако, если в первом эксперименте прикорм, добавляемый животным, состоял из смеси в равных количествах D- (50 %) и L-форм (50 %) (кормовая добавка DL-метионин 99 %, Бельгия), то во втором — только L-форму аминокислоты (производитель Fingres Biotech INC, Китай). Эксперименты по оценке биодоступности и эффективности изомеров метионина показали одинаковую их биодоступность [12]. Классически считается, что в организме биологически активны левые L-аминокислоты. Однако в последние годы появляется все больше работ, свидетельствующих о многогранной роли правых D-изомеров аминокислот. Их рассматривают в качестве регуляторов физиологических функций организмов [13, 17], предлагают в качестве биомаркеров заболеваний [18], учитывают в прогнозировании и терапевтических вмешательствах, используя анализ хирального метаболического профилирования [19].

Важным показателем, отражающим различный биологический эффект действия изомеров различных форм метионина, является неодинаковая концентрация в крови гомоцистеина. В проведенных раннее экспериментах было показано, что при введении DL-формы метионина в течение 8–12-и недель [6] концентрация гомоцистеина плавно увеличивалась, но не превышала 28–30 мкмоль/л. В то же время, использование 100 % L-формы привело к умеренно-высоким значениям гомоцистеина (79,38 ± 3,91 мкмоль/л) уже в течение трех первых недель использования его в качестве добавления к корму.

Значимые различия в ответной реакции организма на введение либо рацемической смеси энантиомеров, либо чистого L-метионина в прикорм животным складываются из изменений в количестве, процентном состоянии, качественной характеристике клеток белой и красной крови. Основная направленность изменений — аллергическая настроенность организма характерна для обеих групп животных и проявляется в повышении числа иммунных клеток крови (эозинофилов и базофилов), увеличении ИАО, моноцитозе, но в разной степени выраженности. Лейкоцитоз с незначительным нейтрофилезом присутствовал только у животных первой группы, тогда как у крыс 2 группы наблюдалась умеренная нейтропения. Эффект сниженной функциональной активности при воздействии высоких доз гомоцистеина отмечен и в культуре клеток [20]. Появление в крови гиперсегментированных форм нейтрофилов может быть обусловлено различными причинами, включающими дефицит фолиевой кислоты и витамина B12, витамина A, дефектами липидного обмена [21]. Основной механизм, вызывающий гиперсегментацию, по-видимому, заключается как в сбоях в синтезе ДНК, так и в ее повреждении, потере структурной целостности ядра [22], что вполне согласуется с ролью метионина в организме, в частности, обеспечении метилирования ДНК.

Изменяются и показатели красной крови, что сопровождается повышением нагрузочного эритроцитарного коэффициента, отражающего функциональную нагрузку на гемопоэз. Эти изменения также наиболее выражены у животных второй экспериментальной группы с введением L-метионина, но не первой (DL-изомеры аминокислоты). Анализ морфологических особенностей эритроцитов считается прогностически значимым при сердечно-сосудистых заболеваниях [23].

Нельзя исключать возможность того, что в экспериментальных исследованиях [24], в которых использовался пищевой метионин, индуцирующий гипергомоцистеинемию, наблюдаемые поражения могли быть вызваны негативным эффектом, связанным с избыточным потреблением именно метионина, а не только и не столько повышенным уровнем гомоцистеина в крови *per se*.

Заключение

Таким образом, выраженность гипергомоцистеинемии, направленность ответных реакций белой и красной крови, коэффициентов, отражающих аллергическую настроенность организма и функциональную эритроцитарную нагрузку при избыточном введении метионина в качестве биодобавки, определяются формой изомера, входящего в ее состав.

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

ФАРМАКОЛОГИЯ
PHARMACOLOGY

DOI 10.22363/2313-0245-2025-30-3-396-403
EDN KYJZTF

ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
ORIGINAL RESEARCH

A new inhibitor binding site in bacterial RNA polymerase

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Abstract. *Relevance.* Growing resistance of bacterial pathogens to antibiotics is a fundamental threat to healthcare worldwide. Many antibiotics currently used in clinical practice were developed decades ago, and their efficiency has been declining over time. Research efforts focusing on identification and optimization of new promising antibacterial compounds and new molecular targets in bacterial cells establish the foundation for developing new potent antibiotics relevant to modern day clinical needs. The bacterial RNA polymerase is a validated target for a number of natural, semisynthetic, and synthetic antibacterial compounds. This essential enzyme maintains a high potential for developing a new generation of antibiotics. *Materials and methods.* We prepared a complex of recombinant *Escherichia coli* RNA polymerase with a new small-molecule transcription inhibitor, and studied it using cryoelectron microscopy to determine the mechanism of inhibition. *Results and discussion.* The current work establishes the molecular basis of RNA polymerase inhibition by the compound N-(9H-fluoren-9-yl)-3-(2-methylpropyl)-[1,2,4] triazolo[4,3-a]pyridine-8-carboxamide (compound A) that blocks transcription through binding to a previously unknown binding site. We describe interactions of the compound A with RNA polymerase and reveal its mechanism of action that involves steric restriction of template DNA binding in the enzyme active site. We demonstrate that the compound A targets only certain types of transcription complexes since its binding site is formed in part by a dissociable subunit of RNA polymerase. *Conclusion.* The results of the study may be used for development of conjugates of rifamycins with the compound A to obtain a new class of bacterial RNA polymerase inhibitors with a new mechanism of action.

Keywords: bacterial transcription, RNA polymerase, inhibitor, mechanism of action

Funding. This work was supported by the Russian Science Foundation grant № 25-74-30007 to V.N. Molodtsov.

Author contributions. Mironov K.S. — figure preparation, manuscript preparation; Molodtsov V.N. — experimental design, data collection and processing, figure preparation, manuscript preparation. Authors made significant contributions to the study and preparation of the article, read and approved the final version before publication.

Conflicts of interest statement. The authors declare no conflicts of interest.

Ethics approval — not applicable.

Acknowledgements.

Consent for publication — not applicable.

Received 22.12.2025. Accepted 12.01.2026.

For citation: *Mironov KS, Molodtsov VN.* A new inhibitor binding site in bacterial RNA polymerase. *RUDN Journal of Medicine*. 2026;30(3):396–403. doi: 10.22363/2313-0245-2026-30-3-396-403 EDN: KYJZTF

Introduction

Antibiotics are a pillar of modern medicine; however, existing antibacterial drugs are increasingly proving ineffective in treating infectious diseases due to the growing prevalence of drug-resistant bacterial pathogens [1]. Revolutionary changes in structural biology, particularly the increasing availability and efficiency of cryo-electron microscopy, are leading to the rapid accumulation of solved structures of biological macromolecules, facilitating the dissemination of methods for the targeted design of inhibitors of essential target molecules in bacterial cells for the development of new antibacterial drugs [2–4]. The structure of a complex of a biological macromolecule with an inhibitor allows for a detailed study of the molecular mechanism of inhibition, thereby creating the opportunity for sequential structural modification and optimization of the original inhibitor for the rational design of a new inhibitor with increased affinity and efficacy.

Bacterial RNA polymerase is an enzyme essential for gene expression, cell growth, and division [5]. During transcription, the bacterial RNA polymerase core enzyme (with the subunit formula α2ββ′ω) binds the σ factor for specific recognition and binding to the gene promoter and initiates *de novo* synthesis of RNA transcript [6]. Bacterial RNA polymerase has a shape commonly described as a "crab claw", with the active site at its base located deep within the main binding channel for DNA and RNA substrates [7]. The catalytic center of bacterial RNA polymerases is formed by a rather conserved amino acid sequence that has low homology with the catalytic centers of eukaryotic RNA polymerases, which allows bacterial RNA to be used as a target for the development of broad-spectrum antibiotics with high selectivity [8–14].

Currently, antibiotics that inhibit bacterial RNA polymerase and are approved for clinical use belong to two classes: rifamycins and fidaxomicin. Rifamycins, widely used to treat tuberculosis and other infectious diseases [15], bind RNA polymerase in a shallow pocket within the active site and sterically block elongation of the RNA transcript after it reaches a length of 2–3 nucleotides [16]. Fidaxomicin, used to treat *Clostridium difficile* infections [17], binds to RNA polymerase at switches, blocking conformational changes required for the formation of an open promoter complex [12]. A recent study analyzing components of a library of small molecules that bind to bacterial RNA polymerase revealed a novel component, N-(9H-fluoren-9-yl)-3-(2-methylpropyl)-[1,2,4]triazolo[4,3-a] pyridine-8-carboxamide (compound A), with antibacterial activity [18]. This molecule was found to inhibit transcription in rifamycin-resistant *E. coli* strains, and mutations in RNA polymerase associated with acquired resistance to compound A were located outside the known binding sites of bacterial RNA polymerase inhibitors. **The aim of this work** was to investigate the

binding site of the inhibitor compound A in *E. coli* RNA polymerase and to establish the molecular mechanism of transcription inhibition.

Materials and methods

E. coli RNA polymerase

E. coli RNA polymerase σ70 holoenzyme was prepared as described previously [9].

Sample preparation for cryo-electron microscopy

An aliquot of *E. coli* RNA polymerase σ70 holoenzyme was thawed on ice, and 10 μL of 20 μM enzyme were mixed with 1 μL of 1 mM N-(9H-fluoren-9-yl)-3-(2-methylpropyl)-[1,2,4]triazolo[4,3-a] pyridine-8-carboxamide (Merck; CAS No. 2095432-38-3) diluted in DMSO (Sigma-Aldrich), 2 μL of buffer (10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 5 mM dithiothreitol), and 7 μL of distilled water. After 20 min of incubation at 25 °C, the complex was immediately applied to cryo-electron microscopy substrates. Substrates with cryo-electron microscopy samples were prepared on a Vitrobot Mark IV autoplunger (FEI/ThermoFisher) at 22 °C and 100% humidity in the blotting chamber. Samples (3 μL) were applied to 2/1 Quantifoil Cu 300 holey-carbon grids (Quantifoil), treated for 60 s in a PELCO surface charge remover (Ted Pella) immediately before application. Grids with samples were blotted with #595 filter paper disks for 7 s at 22 °C, after which they were flash-frozen by plunging to liquid ethane cooled with liquid nitrogen. The prepared grids with the sample were stored frozen in liquid nitrogen until the beginning of experimental data collection.

Cryo-electron microscopy: data acquisition and processing

Experimental data were collected at the Rutgers University Cryo-Electron Microscopy and Nanoimaging Facility (USA) on a Talos Arctica 200 keV electron microscope (FEI/ThermoFisher) equipped with a GIF Quantum K2 direct electron detector (Gatan). Data were collected automatically using the EPU software (FEI/ThermoFisher) at a nominal resolution of 130,000x, a calibrated pixel size of 1.038 Å/pixel, and a irradiance of 4.8 electrons/pixel/sec. Video images were recorded at 200 milliseconds/frame for 6 sec (30 frames), resulting in a total cumulative irradiance dose of 26.7 electrons/Å2. The defocus distance ranged from –1.25 μm to –2 μm. 2000 micrographs were recorded from one sample substrate over 2 days.

Micrographs were normalized and corrected for defects. Data processing was performed on a Tensor TS4 Linux GPU workstation equipped with four GTX 1080 Ti video cards (NVIDIA). Dose-dependent correction of particle motion (3 × 3 tiles; b-factor = 150) was performed using Motioncor2 [19]. Contrast transfer function (CTF) estimation was performed using CTFFIND-4.1[20]. Post-processing of images was performed using Relion 3.0 [21]. Automatic particle extraction using a Laplace-Gaussian filter yielded a set of 364,630 particles. Particles were extracted using a 250 × 250 pixel extraction square and subjected to reference-free 2D classification, removing sparsely populated and misshapen classes. This yielded a set of 107,112 selected particles. This set was then subjected to 3D classification using C1 symmetry and a *de novo* 3D model obtained using 3D_initial_model in Relion 3.0. Classes with clear density corresponding to RNA polymerase were combined and subjected to automatic 3D refinement. The resulting particle set was further refined using the "solvent flattening" setting and postprocessed, with a final reconstruction of the complex at a resolution of 3.9 Å, determined according to the Fourier distribution correlation (FSC) standard. The initial atomic model was constructed based on the σ70 structure of the *E. coli* RNA polymerase holoenzyme obtained by X-ray crystallography (PDB 4KN7) [9]. The structure of compound A was drawn in an online graphics editor, converted into a SMILES formula in an online converter, and processed in the eLBOW program [22] in PHENIX 1.20.1–4487 [23]. The resulting structure of compound A was manually fitted into the additional electron density present in the active site of the initial RNA polymerase model, after which the model was subjected to several rounds of manual refinement in Coot [24] and automated refinement using real-space refinement in PHENIX 1.20.1–4487 [23]. Visualization of the resulting structure was performed using Coot and PyMOL (Schrödinger).

Results and discussion

Binding site and mechanism of transcription inhibition of compound A

In the study identifying compound A as a bacterial transcription inhibitor [18], mutations leading to resistance to compound A were clustered in a small segment of RNA polymerase comprising the β' and ω subunits (Figure 1*a*). For this reason, initial analysis of the electron density map of the resulting RNA polymerase complex with compound A expected to see additional electron density at this site, corresponding to the bound inhibitor. However, additional electron density, with a size and geometry consistent with compound A, was found away from the putative binding site, within the main RNA polymerase channel near the catalytic center and the rifamycin-binding site (Figure 1*a*). compound A was inserted into this additional electron density, and its interactions with RNA polymerase were analyzed (Figure 1 *b, c*). The binding site of compound A is a shallow pocket formed by amino acid residues of the β' and σ70 subunits. The β' subunit interacts with compound A via β' amino acid residues L255, D256, and R259, which are part of the conserved structural element, the β' lid, which contacts the active site RNA during transcription and promotes the separation of RNA from DNA in the RNA-DNA hybrid [25]. The σ70 subunit interacts with the inhibitor via σ70 amino acid residues I505, I511, and F522, which are part of the conserved domain 3.2 of the basic σ-factor (Figure 1 *b, c*). Domain 3.2 of the σ-factor interacts with RNA at the early stages of transcription initiation, preventing its elongation after reaching a length of 4–6 nucleotides [26] and blocking the RNA exit channel from the transcription complex, which triggers σ-factor dissociation during the transition from transcription initiation to elongation [27]. Thus, since the binding site of compound A is present only in the free RNA polymerase holoenzyme, the inhibitor presumably blocks transcription at the stage of promoter complex formation and is unable to affect transcription at other stages. Modeling of the DNA open promoter complex (PDB 4YLP) [28] into the derived structure of the RNA polymerase complex with compound A shows that binding of the template DNA strand leads to a steric clash with one end of compound A (Figure 2), prevents the formation of contacts between the template DNA strand and the amino acid residues of the β' cap, and does not allow the template DNA strand to assume a position in the catalytic center that promotes optimal placement of the first two ribonucleotides during the formation of the first phosphodiester bond during transcription initiation. The combination of these factors explains the mechanism of bacterial transcription inhibition by compound A. However, the obtained structure leaves open the question of the allosteric mechanism of resistance to the inhibitor of RNA polymerase variants with mutations in the β' and ω subunits, discovered in a previous study [18].

Potential of a new binding site for the development of antibiotics

Given the low affinity of compound A for bacterial RNA polymerase (Kd ~ 3 μM) [18], the potential of the inhibitor as a platform for the development of a new stand-alone antibiotic seems low. However, in recent years, studies of the activity of drug conjugates based on drugs used in clinical practice have become increasingly common in the field of medicinal chemistry. A recent study provides an example of the successful development of a new type of antibiotic by chemical conjugation of rifabutin with metronidazole, which made it possible to achieve a synergistic effect and high efficacy in the treatment of infectious diseases caused by *H. pylori*, including drug-resistant strains [14]. We note that in RNA polymerase, compound A and rifamycin antibiotics such as 25-O-benzoyl-rifabutin [13] and TNP-2198 [14] are separated by a distance of 9–12.9 Å (Figure 3 *a, b*). This observation suggests that compound A and rifamycin antibiotics can be conjugated using a linker to create novel antibacterial compounds with enhanced affinity for bacterial RNA polymerase. Such conjugates may have a different mechanism of transcription inhibition than both parent molecules, based on complete steric blocking of the template-strand DNA loading to the active site of the RNA polymerase holoenzyme by a linker connecting the two pharmacophores. The synthesis and testing of such compounds will be the subject of future research.

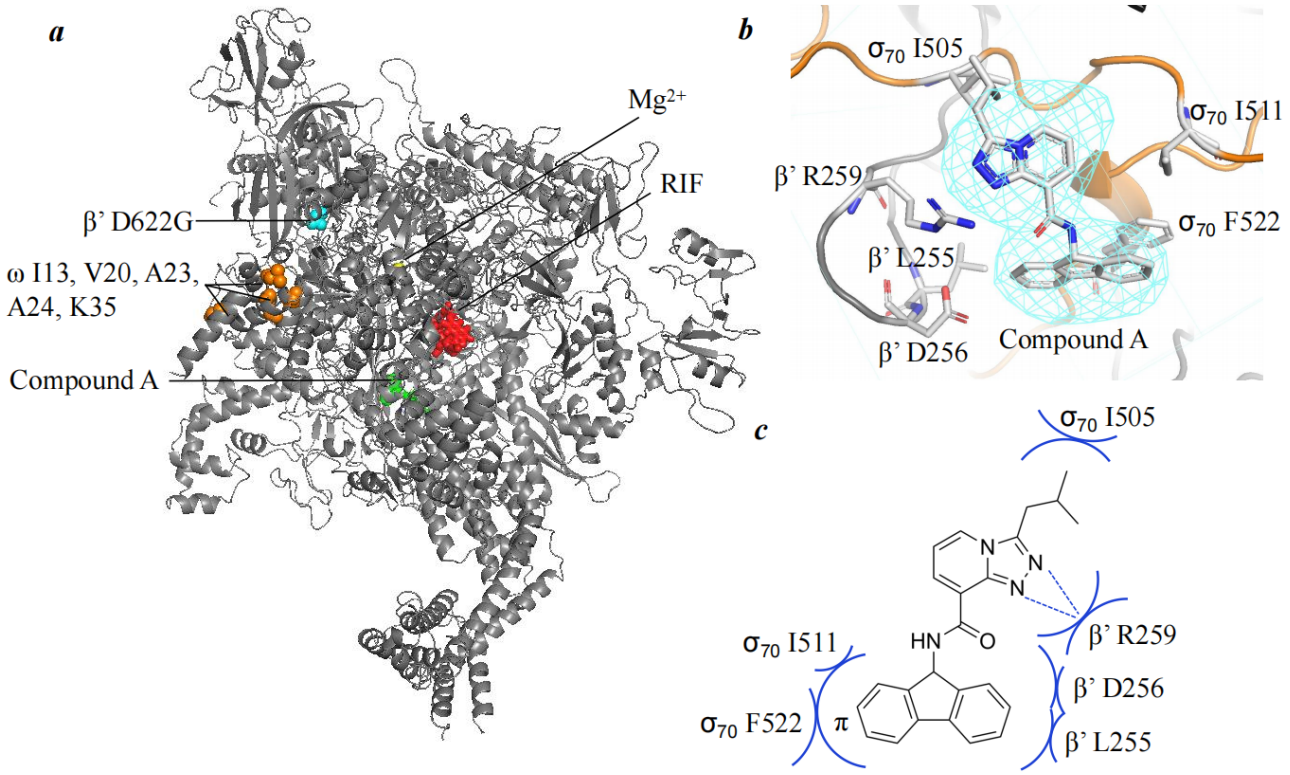

Figure 1. Structural basis of bacterial RNA polymerase inhibition. (*a*) Model of the transcription initiation complex of E. coli RNA polymerase. RNA polymerase, rifampin, and compound A are shown in gray, red, and green, respectively. The Mg^{2+} ion in the active site is shown as a yellow sphere. Amino acid residues in the β′ and ω subunits, mutations in which lead to resistance to the inhibitory action of compound A, are shown in the enzyme structure as blue and orange spheres. (*b*) The binding site of compound A. Some structural elements of the binding site that obscure the view have been omitted for clarity. The experimental three-dimensional electron density of compound A is shown as a blue grid with an embedded atomic model of compound A colored according to atomic composition. The β′ and σ70 subunits are shown in gray and orange, respectively. The side chains of the amino acid residues of the β′ and σ70 subunits interacting with compound A are colored according to their atomic composition. (c) Schematic representation of the interactions of compound A with the amino acid residues in the binding site. Van der Waals interactions are shown as blue arcs, hydrogen bonds are indicated by blue dashed lines, and the π-stacking interaction is marked with the letter π.

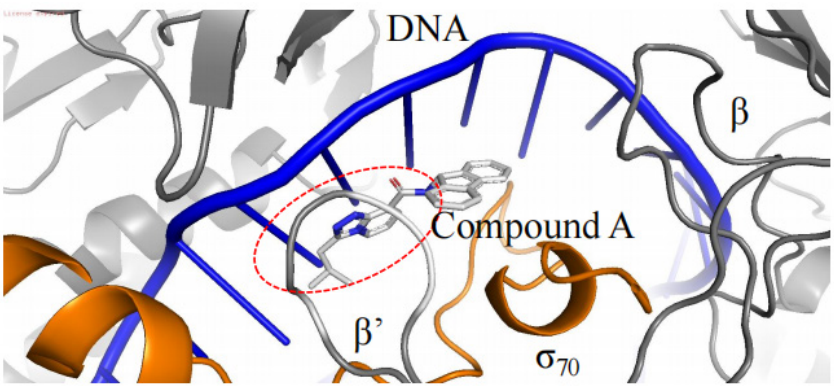

Figure 2. Mechanism of bacterial transcription inhibition by compound A. Some structural elements of RNA polymerase that obscure the overview have been omitted for clarity. compound A, β, β', and σ70 subunits are shown as in Figure 1*a-b*. The DNA model is shown in blue. The collision of the template-strand DNA with compound A in the open promoter complex of *E. coli* RNA polymerase is shown by the red dotted oval

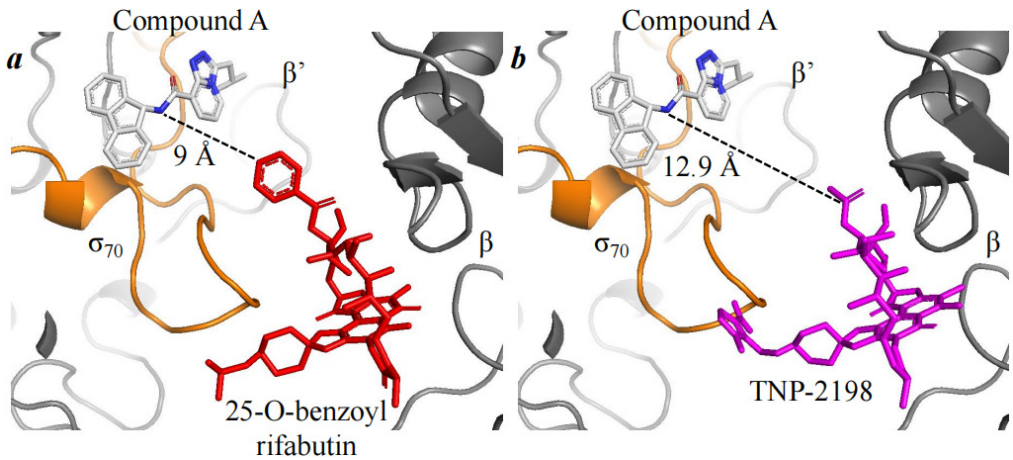

Figure 3. Relative positioning of compound A and rifamycin antibiotics in the binding sites of bacterial RNA polymerase. Some structural elements of RNA polymerase that obscure the overview have been omitted for clarity. Compound A, β, β', and σ70 subunits are shown as in Figure 1*b*. (*a*) Modeling of compound A and 25-O-benzoyl-ribofabutin in the active site of *E. coli* RNA polymerase. The atomic model of 25-O-benzoyl-ribofabutin is shown in red. The minimum distance between compound A and 25-O-benzoyl-ribofabutin is shown as a dotted line. (*b*) Modeling of compound A and TNP-2198 in the active site of *E. coli* RNA polymerase. The atomic model of TNP-2198 is shown in magenta. The minimum distance between compound A and TNP-2198 is shown as a dotted line.

Conclusion

The obtained results expand the existing list of inhibitor binding sites in bacterial RNA polymerase and provide an opportunity for the rational design of new antibacterial drugs that inhibit bacterial transcription by binding to this site. Further research in this direction can be aimed at creating dual inhibitors based on rifamycins and compound A-like conjugates that bind to bacterial RNA polymerase at both binding sites simultaneously.

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Новый сайт связывания ингибитора в бактериальной РНК полимеразе

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Аннотация. *Актуальность.* Растущая устойчивость бактериальных патогенов к антибиотикам представляет собой фундаментальную угрозу для здравоохранения во всем мире. Многие антибиотики, используемые в настоящее время в клинической практике, были разработаны десятилетия назад, и их эффективность со временем снижается. Исследования, направленные на идентификацию и оптимизацию новых перспективных антибактериальных соединений и новых молекулярных мишеней в бактериальных клетках, закладывают основу для разработки новых сильнодействующих антибиотиков, отвечающих современным клиническим потребностям. Бактериальная РНК-полимераза является подтвержденной мишенью для ряда природных, полусинтетических и синтетических антибактериальных соединений. Этот важный фермент обладает высоким потенциалом для разработки нового поколения антибиотиков. *Материалы и методы.* Мы получили комплекс рекомбинантной РНК-полимеразы *Escherichia coli* с новым низкомолекулярным ингибитором транскрипции и изучили его с помощью криоэлектронной микроскопии для определения механизма ингибирования. *Результаты и обсуждение.* В данной работе установлена молекулярная основа ингибирования РНК-полимеразы соединением N-(9H-флуорен-9-ил)-3-(2-метилпропил)-[1,2,4]триазоло[4,3-a]пиридин-8-карбоксамидом (соединение А), которое блокирует транскрипцию путем связывания с ранее неизвестным сайтом связывания. Мы описываем взаимодействие соединения А с РНК-полимеразой и раскрываем его механизм действия, который

включает стерическое ограничение связывания матричной ДНК в активном центре фермента. Мы демонстрируем, что соединение А нацелено только на определенные типы транскрипционных комплексов, поскольку его сайт связывания частично образован диссоциируемой субъединицей РНК-полимеразы. *Выводы*. Результаты исследования могут быть использованы для разработки конъюгатов рифамицинов с соединением А с целью получения нового класса ингибиторов бактериальной РНК-полимеразы с новым механизмом действия.

Ключевые слова: Бактериальная транскрипция, РНК полимераза, ингибитор, механизм действия

Информация о финансировании. Исследование выполнено при финансовой поддержке Российского научного фонда (грант РНФ 25-74-30007).

Вклад авторов. Молодцов В.Н. — экспериментальный дизайн, сбор и обработка экспериментальных данных, анализ полученных данных, написание статьи; Миронов К.С. — анализ полученных данных, написание статьи. Все авторы внесли существенный вклад в подготовку рукописи, прочли и одобрили итоговую версию перед публикацией.

Информация о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение — неприменимо.

Благодарности — неприменимо.

Информированное согласие на публикацию — неприменимо.

Поступила 22.12.2025. Принята 12.01.2026.

Для цитирования: *Mironov K.S., Molodtsov V.N.* A new inhibitor binding site in bacterial RNA polymerase // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 396–403. doi: 10.22363/2313-0245-2026-30-3-396-403 EDN: KYJZTF

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Вестник РУДН. Серия: МЕДИЦИНА
RUDN Journal of MEDICINE. ISSN 2313-0245 (Print). ISSN 2313-0261 (Online)
2026;30(3)
http://journals.rudn.ru/medicine

DOI 10.22363/2313-0245-2025-30-3-404–416
EDN KZVBIU

REVIEW
ОБЗОРНАЯ СТАТЬЯ

Silver nanoparticles: known and unknown facts in a review of properties, mechanisms of action, and medical applications

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Abstract. *Relevance.* The review focuses on the current state of the art in the use of nano-objects in medicine and pharmacy. The relevance of this topic stems from the unique properties of nanomaterials, which exhibit various size-dependent properties in the range of 1–100 nm, where quantum phenomena come into play. The *aim* of this work is to outline the main strategies of modern science for the development and successful stabilization of nanoparticles intended for both the diagnosis and treatment of pathological diseases. Nanostructured heterogeneous matrices containing highly dispersed particles with sizes corresponding to the de Broglie wavelength open up promising opportunities for their use in various scientific and clinical studies. The diversity of the nature, morphology, sizes, and properties of nanoparticles used in medicine for the diagnosis and treatment of diseases of bacterial, viral, and malignant neoplasm stimulates interest in particles exhibiting quantum size effects (QZE). The QZE of nanoobjects are characterized by unique optical and magnetic properties that differ from the properties of physical structures in which particles are larger than 100 nm. Fluorophores based on quantum heterogeneous structures are actively used in medicine and biology, for example, in systems for labelling and visualizing biological data, including in the detection and control of tumor cells. *Conclusion.* This review describes modern approaches to the synthesis and functionalization of nanoparticles based on top-down and bottom-up principles, as well as the potential mechanisms of action of nanoparticles depending on their morphology, size, and ionic (atomic) form. The unique physicochemical properties of nanoparticles, which are due to quantum size effects, are discussed. The article describes various forms of nanoparticles, mechanisms of action, molecular targets, as well as methods of synthesis and application. These aspects are crucial for understanding the role of nanoparticles in combating infections and developing new therapeutic approaches, ensuring their continued relevance in modern science and medicine.

Keywords: silver nanoparticles, biocidal properties, antimicrobial surfaces, quantum size effect, oligodynamic effect, green chemistry

Funding. This publication has been supported by the RUDN University Scientific Projects Grant System, project No. 033322-2-000.

Author contributions. Antonov D.V. — research design, data processing, writing; Uspenskaya E.V. — research design, data processing, writing; Levitskaya O.V. — editing. All authors contributed significantly to the development of the concept and preparation of the article, read and approved the final version prior to publication.

Conflict of interest statement. The authors declare no conflict of interest.

Ethics approval — not applicable.

Acknowledgments. The authors are thankful to the leadership of the RUDN Medical Institute for equipping the department with high-tech equipment for conducting scientific research.

Consent for publication — not applicable.

Received 14.06.2025. Accepted 14.07.2025.

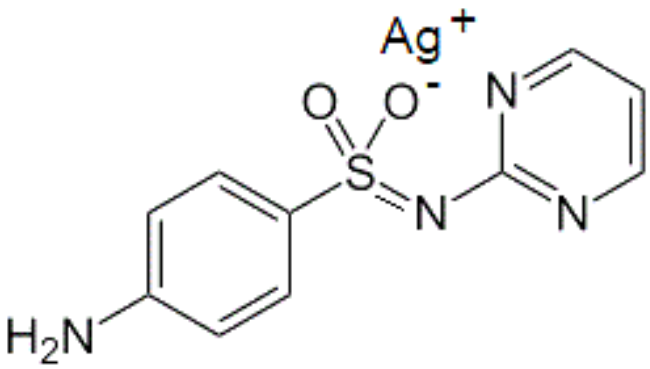
For citation: Antonov DV, Levitskaya OV, Uspenskaya EV. Silver nanoparticles: known and unknown facts in a review of properties, mechanisms of action, and medical applications. *RUDN Journal of Medicine.* 2026;30(3):404–416. doi: 10.22363/2313-0245-2026-30-3-404-416 EDN: KZVBIU

Introduction

For a long time, silver and its compounds have been widely used in various fields of human activity, including medicine and everyday life, due to their pronounced biocidal properties due to the high reactivity of the metal [1]. The use of silver for the treatment of various diseases or to prevent the transmission of infection dates back to 4,000 years BC. In the manuscripts, there were mentions of a dangerous biogenic agent that threatens human health in enclosed spaces — mold associated with the so-called “leprosy” [2]. This disease served as a generalized term for a group of infectious dermatological diseases caused by bacteria or fungal spores, which were treated with metals and their derivatives. Silver and its compounds have been used to fight various microorganisms since the time of ancient civilizations. For example, the ancient Greeks and Egyptians used silver vessels to store drinking water and other liquids, which significantly reduced the risk of infection. In the Middle Ages, the well-to-do sections of the population ate from silverware, which is believed to have prevented the reproduction of pathogenic microbes. According to some theories, such dishes could protect nobles from the devastating effects of the bubonic plague pandemic [3].

The active introduction of silver compounds into medical practice began with the use of silver nitrate, which was originally used as a solid mass, known as "lapis lazuli" (from lat. *lapis* — stone and *lazur* — blue): mineral from the class of silicates with a framework structure of crystal grid, chemical composition $Ca_2Na_6[AlSiO_4]_6[SO_4][S_2]$. Silver nitrate was used throughout the 1800s to treat burns, ulcers, and infected wounds [4]. The use of silver, according to official European pharmaceutical practice, dates back to 1881 with the experience of the German obstetrician-gynecologist Karl Kred, who proposed a method for the prevention and treatment of neonatal inclusion blennorrhea in newborns by instilling 1–2% silver nitrate solution into the eyes [5]. Since the beginning of the 19th century, silver nitrate solutions have become even more widespread in medicine: at the beginning of the 19th century, a dosage form was proposed that included a 3.4% solution of silver nitrate with the addition of linseed oil, which was used to treat pathologies associated with excessive overgrowth of connective tissues due to inflammation. This formulation has proven itself to be the standard for preoperative preparation, although its use declined after World War II and the advent of antibiotics. This discovery stimulated doctors, especially surgeons, to search for new effective ways to use silver nitrate. One of the key areas was the treatment of wound injuries and related infectious complications. Biochemist Charles Lewis Fox (USA) revived the use of silver compounds

by developing in 1966 an ointment composition based on a sulfonamide agent, sulfadiazine 1% in combination with silver (*Sulfarginum or Sulfadiazine Silver* $C_{10}H_9AgN_4O_2S$) [6, 7] (Figure 1).



4-amino-N-pyrimidin-2-yl-benzenesulfonamide silver salt

Figure 1. Structure of sulfadiazine silver solt (lactam form)

Therefore, in addition to the biological properties of API, the clinical effect also depends on the method of administration, which is achieved by creating the appropriate form of the drug. Despite the apparent biocidal effect of the use of silver compounds, the description of the mechanisms of biological activity was first announced in the 1890s with the introduction of the term "oligodynamic effect", thanks to the work of Swiss-German scientist Carl Wilhelm von Negeli [8]. This means the toxic (from greek *oligos* — small, *dynamis* — force, "acting in small doses") effect of metal ions (Ag^+, Cu^{+2}, Au^{+3}) on prokaryotes and eukaryotes in relatively small amounts [9]. Later, when using radiolabeled sulfadiazine, it was shown that silver in the form of nitrates and salts with sulfadiazine forms a complex with DNA in vitro and has the highest degree of bacterial binding. The interaction between silver and DNA can be described as a process: there is a weak, reversible intercalative interaction between DNA and the sulfadiazine moiety, during which sodium sulfadiazine competes with Ag for binding to DNA and then a stronger, tightly bound complex is formed that involves coordination of the silver atom [10]. Additional studies have shown that resistant bacteria are those that are unable to bind Ag+ ions more strongly to release AgCl [11]. The antimicrobial activity of silver is based on the ability of Ag^+ ions to penetrate bacterial cell walls through pinocytosis, which leads to increased cellular oxidative stress followed by protein denaturation and growth inhibition [12]. Ionic silver also has the ability to bind to the microbial genome (DNA or RNA), which inhibits the replication of nucleic acids and prevents the reproduction of microorganisms.

At the beginning of the 20th century, innovative forms appeared in clinical practice, such as silver foil with silver nanoparticles (AgNPs), which proved their effectiveness in the treatment of burns and wound defects, including skin grafts. The use of silver foil not only reduced pain in patients, but also demonstrated pronounced bactericidal properties, which accelerated healing processes by stimulating the formation of granulation tissue. The emergence of new medical applications for silver is associated with the development of its unique forms, such as nanoparticles (AgNPs), and their integration into various materials, such as polymers and biocompatible matrices. One of the most important advantages of antimicrobial coatings is their ability to continuously disinfect a surface as long as it remains undamaged. Although this does not eliminate the need for regular sanitary measures, such coatings provide additional protection between hygiene procedures. For example, it has been found that pre-treated silver surfaces are able to effectively destroy fungi, mold and bacteria even after ten years of continuous use both at home and in medical institutions. Table 1 shows the forms and compounds of silver [13].

A surge of publications on nanoparticles, formed dispersions, and medical applications can be traced in international databases since 1995 [14]. During this period, the first publications appeared in the Russian-language scientific literature, including patents for inventions of methods for the treatment of purulent wounds using a pharmaceutical substance based on silver (d~nm) nanoparticles stabilized with polyvinylpyrrolidone (PVP) — poviargolum (Figure 2).

Table 1

Properties of silver atoms, ions and compounds

Form (ionic/neutral) of silver	Electronic formula (outer energy levels)	Compounds
Ag^0	$d^{10}s^1$	Ag nanodispersions
Ag^+	d^{10}	Ag_2O, $[Ag(OH)_2]^-$, $[Ag(H_2O)_4]^+$, AgF, AgCl, $AgNO_3$, Ag_2SO_4, Ag_2S, $Ag(CN)_2^-$
Ag^{+2}	d^9	AgF_2, $[Ag(C_5H_5N)_2]^+$, $AgSO_4$
Ag^{+3}	d^8	$[AgF_4]$–, $[AgF_6]^{3-}$, rare

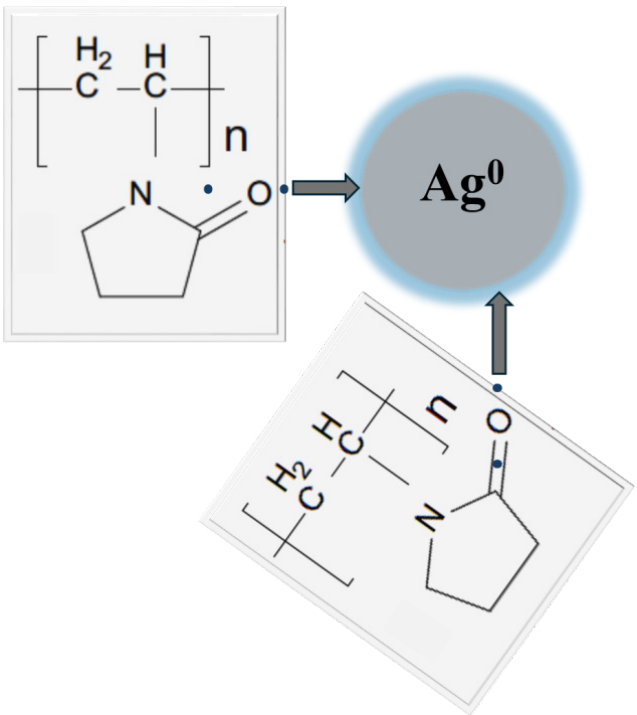


Figure 2. Binding of polyvinylpyrrolidone (PVP) with Ag nanoparticles

The synergistic effect of silver and PVP is directed against Gram-positive bacteria: *K. pneumoniae*, *P. aeruginosa* and *A. baumannii*, resistant to carbapenems, aminoglycosides, polymyxins and tigecycline [15].

Structure and properties of silver nanoparticles

Silver nanoparticles (AgNPs) stand out among metallic nanoparticles for their unique physicochemical properties and biological effects, which makes them the subject of close study in the field of nanobiotechnology [16]. AgNPs particles exhibit various size-dependent properties in the range of 1–100 nm, where quantum phenomena are involved. A decrease in the size of nanoparticles contributes to a more pronounced manifestation of the quantum size effects (QZE), which manifests itself in the quantization of the energy of the excited state of charge carriers — the electron gas in the nanoparticle system and in the shift of the conduction band upward in energy levels (Figure 3).

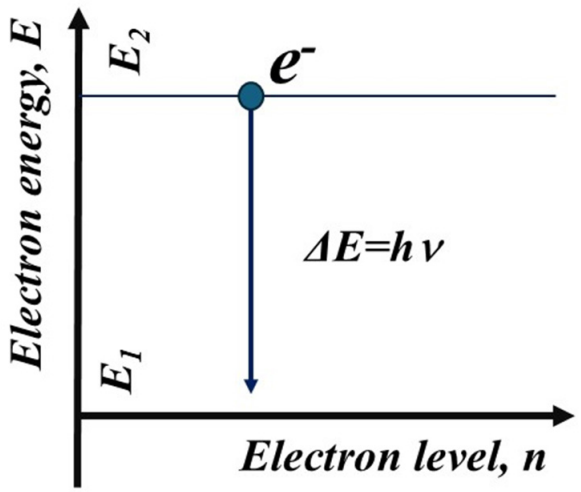


Figure 3. Discreteness of electron energy

The transition of an electron from one stationary state (E_2) to another (E_1) is accompanied by the emission of a quantum of light with a frequency:

$$\nu = \Delta E / h, \quad (1)$$

where ΔE is the energy difference between the initial and final states of the electron, and h is Planck's constant.

The quantum structures of the material are characterized by unique optical and magnetic properties that differ from those of physical structures in which particles have a size exceeding the de Broglie wavelength (macroscopic bodies). Fluorophores based on quantum heterostructures are actively used in medicine and biology, for example, in tagging and imaging systems for biological objects, including in the detection and control of tumor cells [17]. These properties are significantly influenced by factors such as surface area, surface charge, morphological diversity (for example, rods, triangles, color-like structures, spheres), as well as size variations from 1 to 100 nm. For example, spherical AgNPs with smaller dimensions (d~1–20 nm) exhibit excellent antibacterial activity due to their increased reactivity. In contrast, larger spherical particles or particles with similar sizes but different shapes, such as triangular nanoparticles with sharp edges, exhibit different efficiencies, with triangular shapes often outperforming spherical ones due to their reactive edge structures (Figure 4).

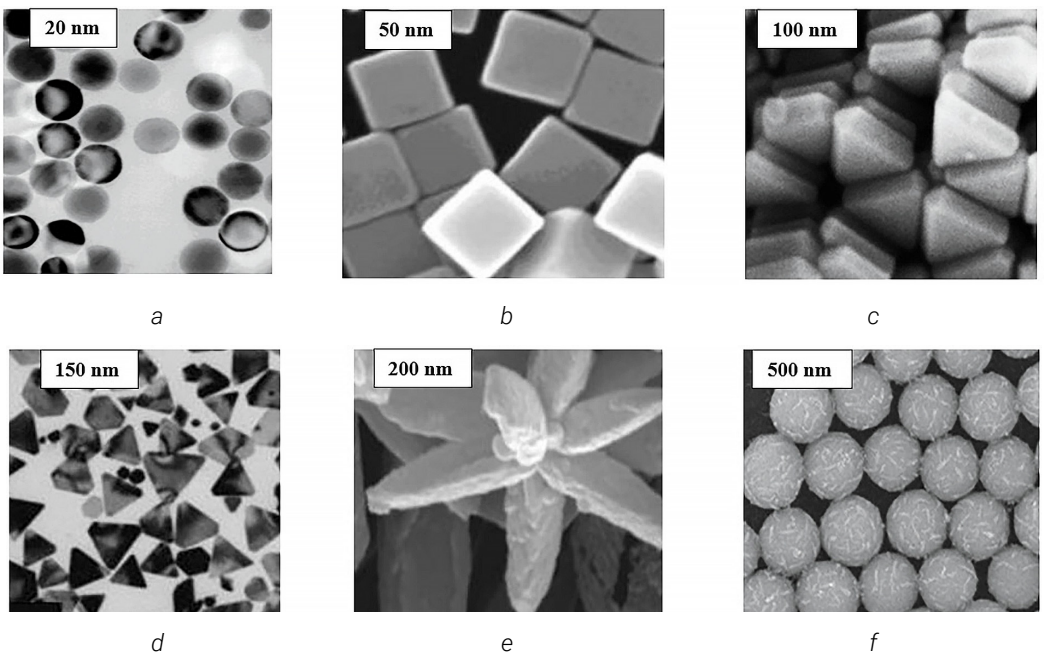


Figure 4. Different shapes of Ag nanoparticles depending on size:
(*a*) sphere, (*b*) a nanocubes, (*c*) pyramids, (*d*) prisms, (*e*) flower, (*f*) ellipse [21]

The stated differences in the antibacterial effectiveness of nanoobjects can be explained by the anisotropic shape of some silver nanoparticles, which differ in the dense arrangement of atoms on the surface, which enhances the effect on their surface, enhancing the biocidal effect [18]. Experimental data show that quasi-spherical AgNPs (~20 nm in diameter) and spherical particles with a smaller diameter (~9 nm) exhibit pronounced antimicrobial effects. In addition, AgNPs synthesized in a phosphate-salt buffer in the size range of 5–20 nm exhibit significant antiviral activity [19]. Thus, the functional versatility of AgNPs in medicine is closely related to their size, shape, and surface morphology. This diversity highlights the need for ongoing research to optimize their design and application in this dynamic field of research [20].

The growth of nanoparticles during solution-phase synthesis proceeds in stages: nucleation — seeding — growth. One can control the shape of the synthesized nanoparticles by tuning the thermodynamics and kinetics at each stage of their solution-phase synthesis [21]. Thus, the shape and size of the resulting silver nanoparticles depend on experimental conditions such as: synthesis temperature, concentration of the starting materials and stabilizer, pH of the solution, as well as the approach used ("bottom-up" or "top-down").

Mechanism of action and targets

Silver nanoparticles (AgNPs) exhibit exceptional quantum dimensional properties due to their pronounced surface area, ability to interact with microbial biomolecules, penetrate cells, generate reactive oxygen species (ROS) and free radicals, and interact with microbial signaling molecules [22, 23]. Modification of the surface of AgNPs can enhance their oligodynamic effects (in small doses), improving stability and physico-chemical characteristics. Metallic silver ions become biologically active as a result of reduction under the action of oxidizing agents — sodium citrate, glucose, etc. [24]. The restored form binds to the membranes of bacterial cells, causing structural damage and morphological changes. Unlike other metal nanoparticles, AgNPs minimize toxic effects. The authors [25] cite research results confirming significant changes in antibiotic sensitivity and the ability to form bacteria biofilms (multicellular-like behaviors or forms) observed during prolonged exposure of pathogens causing diseases of the genitourinary system to combinations of chemotherapeutic antibacterial agents and AgNPs.

For example, copper nanoparticles (CuNPs) exhibit a more pronounced antibacterial effect, but their cellular toxicity limits their practical use. Particles CuNPs destroy bacterial membranes and interfere with electron transfer during photocatalysis, but their harsh effect contrasts with the balanced effectiveness of silver. The paper [26] presents the results of a study of the wound-healing activity of methylcellulose-based ointments with different concentrations of copper nanoparticles measuring 103 nm (71% crystalline copper). It was found that an ointment containing copper nanoparticles at a concentration of 0.002% achieves the optimal effect on wound healing.

The proposed mechanisms of action of AgNPs include adhesion to cell walls/membranes, intracellular penetration; disruption of organelles and induction of oxidative stress and modulation of signaling pathways (Figure 5).

Silver nanoparticles are able to firmly attach and accumulate on the surface of gram-negative bacterial cells, as well as penetrate into microbial cells through pores, which are water-filled channels located in the outer membrane of gram-negative bacteria [28]. Porins play a key role in the passive movement of hydrophilic molecules of various sizes and charges through the membrane. On the contrary, the thicker cell wall of Gram-positive bacteria can prevent the penetration of silver ions into the cytoplasm [29]. Lipopolysaccharides present in the cell walls of gram-negative bacteria probably help maintain their structural integrity, but also make them more vulnerable to silver nanoparticles. The negative charge of lipopolysaccharides further promotes the adhesion of nanoparticles. Some studies show that silver nanoparticles can adhere to the surface of bacteria, altering the properties of membranes and potentially causing damage to bacterial DNA [30].

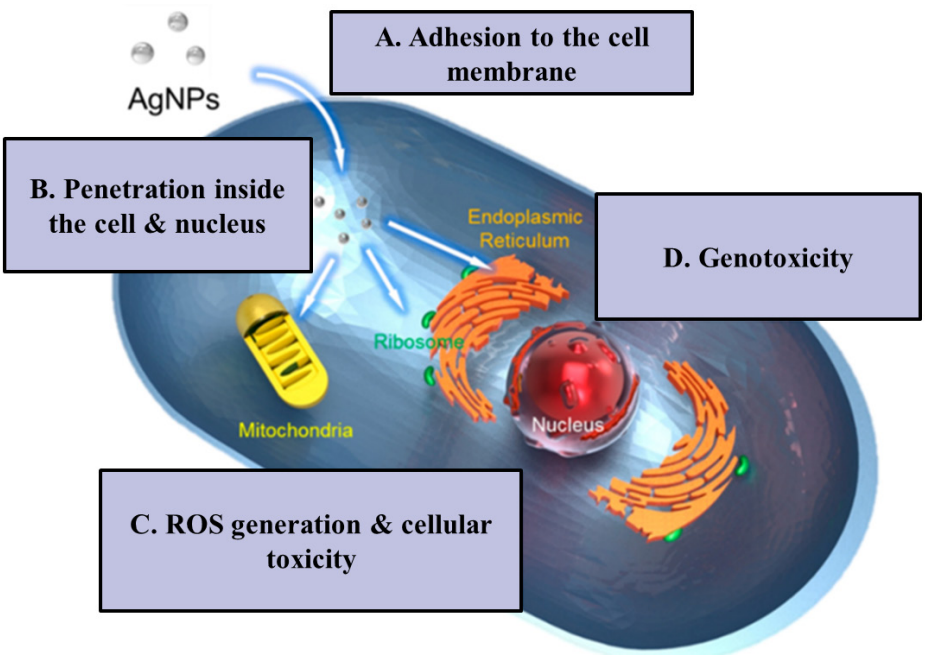


Figure 5. Ways of biocidal action of AgNPs: (A) alters membrane structure & permeability, leakage of cellular content & ATP, (B) destabilize and denature proteins, interact with DNA, (C) oxidize proteins & lipids, mitochondrial dysfunction, ribosome destabilization, (D) DNA base damages, inhibition of replication and transcription [27]

Since the attachment of bacteria to the surface is the first step in the development of biofilms (polycellular forms), silver nanoparticles binding to the cell surface can hinder this process. This aspect is of great practical importance in the fight against pathogens, indicating that silver nanoparticles can be useful not only for preventing infections, but also for combating antibiotic-resistant strains [31, 32].

Synthesis methods

To obtain nanoparticles with the claimed properties, a carefully controlled synthesis process is necessary. Since the advent of nanotechnology, many methods of producing silver nanoparticles have been developed. In general, these approaches are divided into two main categories: “top-down” and “bottom-up” (Figure 6).

The top-down method involves crushing massive materials into metal nanoparticles using various physical methods such as mechanical crushing, grinding, as well as electrical methods such as electric discharge and laser ablation. Thermal methods, including vapor condensation, also play an important role. Physical methods usually produce nanoparticles of high purity and uniform size. Although such processes do not require potentially dangerous chemicals or stabilizers, they require complex and expensive equipment, as well as significant energy consumption.

In contrast, the bottom-up method is based on the assembly of nanoparticles from molecular building blocks through the processes of growth and nucleation. Chemical and biological synthesis are widely used in this approach. Chemical synthesis can be accelerated and optimized using various energy sources — visible or ultraviolet light, electricity, microwaves, or ultrasound — which makes it possible to quickly obtain nanoparticles of various shapes [34]. However, chemical synthesis can be accompanied by risks associated with toxic by-products, which limits its use for medical and pharmaceutical purposes.

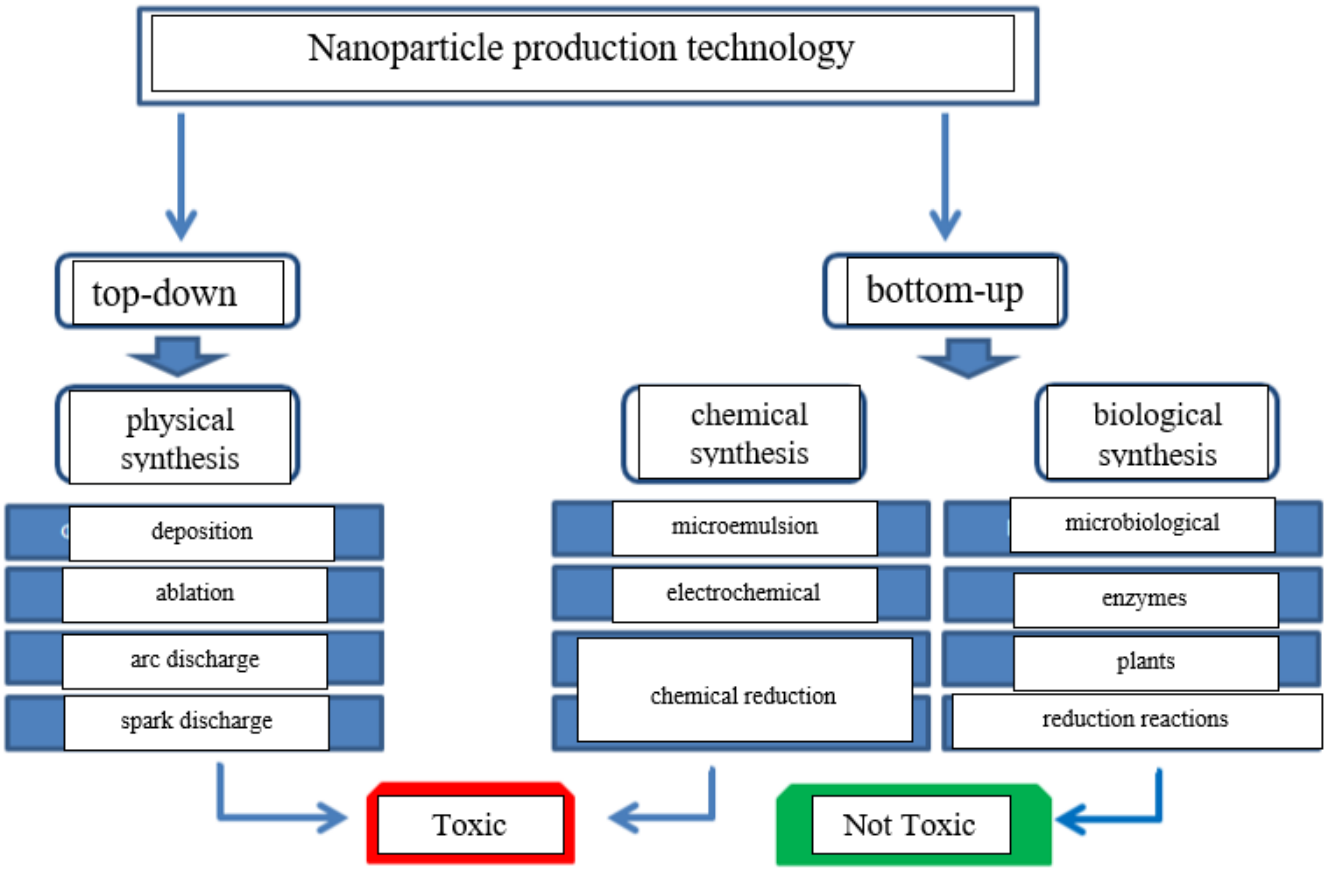


Figure 6. The main approaches to obtaining nanoparticles [33]

Biological synthesis is attracting increasing attention due to its environmental safety and sustainability. It uses biologically active compounds such as alcohols, flavonoids, and phenolic compounds as reducing agents [35].

Traditional methods of nanoparticle synthesis can be expensive and pose risks to human health and the environment. To overcome these disadvantages, researchers are actively exploring so-called “green” methods using natural materials and their derivatives. Biological synthesis can be divided into methods using fungi, yeasts (eukaryotes), bacteria, and actinomycetes (prokaryotes), as well as approaches based on plants, plant extracts, and even viruses as templates [36, 37].

The chemical synthesis of silver nanoparticles consists in the reduction of silver salts in water or organic solvents. This process is based on three key components: reducing agents, metal precursors, and stabilizing or coating organic polymers. The choice of reducing agents and coatings is extremely important, since they can significantly affect the physico-chemical properties of the released nanoparticles [38, 39]. Despite these disadvantages, chemical methods provide high yield, excellent reproducibility, and lower cost compared to physical methods.

Physical synthesis, as a top-down method, uses physical effects such as electromagnetic radiation, plasma, and thermal effects. This method allows nanoparticles to be evenly distributed on thin films without contamination with solvents [40]. Vapor condensation and laser ablation are among the most common physico-chemical methods. Vapor condensation is a two — stage process involving evaporation and condensation in a specialized tubular

furnace at normal atmospheric pressure; the size of the resulting nanoparticles varies from 3 to 50 nm [41]. However, physical synthesis is energy-intensive and time-consuming, which can lead to an increase in ambient temperature.

Nanodispersions from 10 to 30 nm with a given morphology and properties can be obtained by laser ablation in a liquid using an environmentally friendly and affordable approach [42].

The choice of a method for synthesizing silver nanoparticles is a critically important decision that depends on the required characteristics of the final product and its field of application. Each top-down or bottom-up approach has its advantages and limitations, which requires careful analysis in terms of environmental safety, economic efficiency, and technological feasibility.

The latest application

More and more studies indicate the occurrence and stable retention of pathogens (bacteria d>5 mcm, viruses d~20–200 nm, protein infectious particles of prions d<5 nm, fungi, yeast and protozoa d>200 mcm) in patient care in clinical settings, which can cause infection of hospital patients and cross-contamination. The infections between them are nosocomial infections [43]. Widespread disinfectants alone can stimulate the development of resistance. For example, quaternary ammonium compounds (QACs) have long been considered an effective class of disinfectants that are immune to bacterial resistance. However, methicillin-resistant staphylococcus aureus (MRSA) isolates show an increase of about 30% in their resistance genes. Disinfection of rooms by exposure to high-intensity UV light can be effective in destroying most microorganisms. However, this measure requires the complete emptying of the room by both staff and patients during the process in accordance with safety requirements [44]. As a result, the development of new materials with surface antimicrobial properties based on metallic silver nanoparticles is extremely promising for medical applications.

Silver has been used in medicine for many decades, and its antimicrobial activity is recognized as one of the first effective disinfection technologies [45]. Silver nanoparticles are widely used in dressings — numerous studies confirm that they significantly accelerate wound healing and at the same time prevent the development of bacterial infections. As already noted, the ability to bind to various molecular targets in microbial cells provides silver nanoparticles with a prolonged antibacterial effect. For example, AgNPs incorporated into the bacterial cellulose matrix exhibit pronounced bactericidal activity against gram-negative bacteria such as *Escherichia coli*, while not having a toxic effect on epidermal cells, which makes them ideal for medical use [46].

In modern experimental medicine, the possibility of synergetic use of silver nanoparticles as powerful antibacterial agents in combination with various antibiotics is actively being investigated [47]. However, it is worth noting that this synergy is not observed for all classes of antibiotics, which requires further study of the mechanisms of their interaction.

Despite all the attractive advantages of silver-based drugs for medicine, it should be remembered their possible toxic effects and contraindications to use. Considered [48], that the toxicity of silver is a rare diagnosis, usually manifested by skin manifestations in the form of argiria disease (greek. ἄργυρος silver). Long-term deposition of silver compounds (silver dust, colloidal silver or silver nanoparticles) in a dose of 70 to 1500 mg Ag/kg body weight leads to irreversible strong skin pigmentation. An *in vivo* study using radiolabelled silver showed the onset of accumulation effect 2–8 hours after exposure, with a half-life period of 2 weeks. Oral absorption through the gastrointestinal tract may vary depending on other food components that potentially affect the bioavailability of silver; absorption is up to 10% [49]. Metal silver presents minimal health risk — according to the American Conference of Governmental Industrial Hygienists (ACGIH), the threshold limit for metallic silver is 0.1 mg/m^3, while soluble silver compounds — an order of magnitude less than 0.01 mg/m^3. A single lethal dose of silver nitrate is estimated to be about 10 g, an intravenous dose of about 50 mg of silver causes necrosis of bone marrow, kidneys

and liver, bleeding and swelling of the lungs [50, 51]. However, such cases are known only among suicidal or other preventive actions.

In addition to medical purposes, silver nanoparticles are used as effective heterogeneous catalysts that are actively used to reduce halogenated organic pollutants. Their unique catalytic properties also enhance the bleaching ability of organic dyes. Tubular silver nanoparticles have high catalytic activity, which expands their use as effective catalysts for the tasks of green chemistry and environmental restoration [52–54]. The particles AgNPs are widely used not only in medicine, but also in other scientific fields due to their unique physico-chemical properties — high conductivity and photocatalytic activity. One of the key areas of their use is electronics, where AgNPs are used as conductive materials to create innovative flexible electronics, sensors and solar panels, opening up new opportunities for energy-saving technologies.

Conclusion

The metal and oxide nanoparticles have proven to be an original potential approach for applications in a wide variety of fields, from medicine to physics and chemistry. This was made possible by their pronounced antimicrobial properties and high biocompatibility. Silver, known to mankind for centuries, has become an important element in various fields of culture and medicine, primarily due to its ability to effectively resist microorganisms. Numerous studies confirm that silver nanoparticles significantly accelerate wound healing and prevent bacterial infection due to the high reactivity of the surface and the ability to interact with cellular structures of pathogens. The introduction of silver nanoparticles into medical devices and technologies is a breakthrough area that requires special attention and further study. The combination of silver nanoparticles with traditional antibiotics opens up new horizons in the treatment of infectious diseases, although additional research is needed to optimize such synergistic interactions. Given the growing interest from the scientific community and industry, silver nanoparticles remain an important research object, confirming their significant potential for developing effective solutions that promote sustainable development in a wide variety of industries.

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Серебряные наночастицы: известные и неизвестные факты в обзоре свойств, механизмов действия и применения в медицине

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Аннотация. *Актуальность.* Обзор посвящен современному состоянию дел в области использования нанообъектов в медицине и фармации. Актуальность этой темы обусловлена уникальными свойствами наноматериалов, которые демонстрируют различные размерные зависимости в диапазоне 1–100 нм, где вступают в игру квантовые явления. *Цель* данной работы — обозначить основные стратегии современной науки по разработке и успешной стабилизации наночастиц, предназначенных как для диагностики, так и для лечения патологических заболеваний. Наноструктурировнаные гетерогенные матрицы, содержащие высокодисперсные частицы с размером, соответствующим длине волны де Бройля, открывают многообещающие возможности для их использования в различных не только в научных, но клинических исследованиях. Разнообразие природы, морфологии, размеров и свойств наночастиц, применяемых в медицине для диагностики и терапии заболеваний бактериальной, вирусной и злокачественной этиологии, стимулирует интерес к частицам, проявляющим квантово-размерный эффект. Квантовые структуры нанообъектов характеризуются уникальными оптическими и магнитными свойствами, отличными от свойств физических структур, в которых частицы имеют размер, превышающий 100 нм (макроскопические тела). Флуорофоры на основе квантовых гетероструктур активно применяются в медицине, биологии например, в системах мечения и визуализации биологических объектов в том числе при обнаружении и борьбе с опухолевыми клетками. *Выводы.* В данном обзоре описаны современные подходы к синтезу и функционализации наночастиц на основе принципов «сверху вниз» и «снизу вверх», а также потенциальные механизмы действия наночастиц в зависимости от их морфологии, размера и ионной (атомной) формы. Обсуждаются уникальные физико-химические свойства наночастиц, обусловленные квантовыми эффектами размера. В статье описываются различные формы наночастиц, механизмы действия, молекулярные мишени, а также методы синтеза и применения. Эти аспекты имеют решающее значение для понимания роли наночастиц в борьбе с инфекциями и разработке новых терапевтических подходов, обеспечивая их постоянную актуальность в современной науке и медицине.

Ключевые слова: наночастицы серебра, биоцидные свойства, антимикробные поверхности, квантово-размерный эффект, олигодинамический эффект, «зеленая» химия

Информация о финансировании. Публикация выполнена в рамках проекта № 033322–2–000 Системы грантовой поддержки научных проектов РУДН.

Вклад авторов. Антонов Д.В. – дизайн исследования, обработка материала, написание работы; Успенская Е.В. – дизайн исследования, обработка материала, написание работы; Левицкая О.В. – редактирование работы. Все авторы внесли значительный вклад в разработку концепции и подготовку статьи, прочитали и одобрили окончательный вариант перед публикацией.

Информация о конфликте интересов. Авторы заявляют об отсутствии конфликта интересов.

Этическое утверждение — неприменимо.

Благодарность. Авторы выражают благодарность руководству Медицинского института РУДН за оснащение отделения высокотехнологичным оборудованием для проведения научных исследований.

Информированное согласие на публикацию — неприменимо.

Поступила 14.06.2025. Принята 14.07.2025.

Для цитирования: *Antonov D.V., Levitskaya O.V., Uspenskaya E.V.* Silver nanoparticles: known and unknown facts in a review of properties, mechanisms of action, and medical applications // Вестник Российского университета дружбы народов. Серия: Медицина. 2026. Т. 30. № 3. С. 404–416. doi: 10.22363/2313-0245-2026-30-3-404-416 EDN: KZVBIU

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