National Research University Higher School of Economics
Faculty of Biology and Biotechnology

as a manuscript

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CD44 Receptor and Laminins in Colorectal Cancer Resistance and Metastasis

Specialty 1.5.3 – Molecular Biology

Dissertation Summary
for the purpose of obtaining
academic degree Doctor of Science in Biology

Moscow – 2025

The dissertation was carried out at the Faculty of Biology and Biotechnology of the National Research University Higher School of Economics.

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Doctor of Biological Sciences,
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GENERAL CHARACTERISTICS OF THE WORK

Significance of the Study

Over the past few years, the effectiveness of colorectal cancer (CRC) therapy has significantly improved. Nevertheless, CRC remains the third most common malignancy and the second leading cause of cancer-related mortality worldwide [1,2]. More than 70% of CRC-related deaths are attributed to liver metastases, which are present in approximately 25% of patients at the time of initial diagnosis. Despite advances in systemic therapies, the five-year survival rate for patients with distant organ metastases does not exceed 15% [3]. Acquired resistance to therapy, observed in 90% of patients with metastatic CRC, is considered the primary cause of treatment failure [4].

Currently, the prediction of treatment outcomes and therapy selection for CRC patients is primarily based on the TNM classification system [5,6]. However, TNM staging is insufficient for accurately predicting disease progression in early-stage CRC. Furthermore, clinical outcomes and patient responses to therapy vary widely [6,7]. Therefore, the development of methods to predict disease progression rates, assess the efficacy of specific therapeutic approaches, and identify novel treatment strategies remains a highly relevant and critical challenge.

Epithelial tumors at early stages are typically surrounded by a basement membrane (BM), a sheet-like structure of the extracellular matrix located in close proximity to the cell surface [8,9]. Cellular contact with the BM is essential for tumor growth [10]. Laminins (LMs) are the principal adhesive components of the epithelial BM [11]. By interacting with cell surface receptors, LMs can initiate intracellular signaling cascades that influence various cellular processes, including the balance between survival and apoptotic signals [10,11]. This balance can significantly affect the efficacy of chemotherapy. However, the investigation of these mechanisms has been hindered by the unavailability of most LM isoforms in purified form. Laminins cannot be extracted from tissues due to their strong covalent interactions with other extracellular matrix components *in vivo*. Additionally, LMs cannot be produced in bacterial cells, as they undergo extensive post-translational modifications *in vivo* that are essential for their proper function. At the time our research was initiated, the technology for the production and purification of recombinant LM isoforms via ectopic expression in mammalian cells had only recently been described.

In this context, the first part of the study focuses on investigating the effects of laminins on the properties of CRC cells and their sensitivity to chemotherapeutic agents. Additionally, the prognostic significance of laminin chain expression in CRC was evaluated. The analysis highlighted the importance of the α5 LM chain (encoded by the *LAMA5* gene), the role of which was further examined through shRNA-mediated knockdown of *LAMA5* in the HT-29 colorectal adenocarcinoma cell line.

The second part of this study is devoted to investigating the role of CD44 protein isoforms in CRC metastasis. CD44 is a transmembrane glycoprotein that plays an essential role in numerous cellular processes in normal tissues due to its signaling functions [12]. At the same time, CD44 is involved in tumor biology [13], including proliferation, differentiation, invasion, cell migration, epithelial–to–mesenchymal transition (EMT), and is recognized as a well-established marker of colorectal cancer stem cells (CSCs) [14]. Aberrantly elevated CD44 expression has been observed in various cancer types, including CRC [15]. These findings underscore the relevance of CD44 as a promising candidate for diagnostic and prognostic applications in CRC, as well as a potential therapeutic target.

The functional diversity of CD44 is largely attributed to the existence of multiple isoforms. In humans, the CD44 gene consists of 19 exons, and alternative splicing can generate numerous CD44 isoforms, eight of which have been experimentally confirmed [16]. The shortest isoform, composed solely of constitutive exons, is expressed in most tissue types and is referred to as the standard isoform (CD44s, isoform 4). Inclusion of variant exons v2–v10 gives rise to higher molecular weight isoforms (CD44v), which are expressed only in specific epithelial tissues and tumor types [12]. For example, isoform 1—the longest CD44 variant—contains all variant exons from v2 to v10 in addition to the constitutive ones; isoform 2 includes v3–v10; and isoform 3 contains v8–v10. An increasing body of evidence suggests that CD44 isoforms play distinct functional roles in tumor development and progression. For instance, isoform 4 (CD44s) has been associated with a more mesenchymal tumor cell phenotype, whereas isoforms containing variant exons (such as isoform 3) are linked to a more epithelial phenotype [17,18]. During EMT, a switch in expression between different isoforms is observed. However, the specific expression patterns and functions of individual isoforms remain poorly characterized in many tumor types, including CRC. Prior studies have shown the expression of both the standard isoform 4 and variant isoforms containing exons v2, v3, v6, and v9 in CRC cells [15].

The HT-29 cell line was selected as a model to study the role of the CD44 receptor in CRC, as it predominantly expresses clinically relevant CD44 isoforms, specifically isoforms 3 and 4. An shRNA-mediated knockdown of the *CD44* gene was achieved via lentiviral transduction. The effect of CD44 knockdown on CRC progression *in vivo* was assessed using a spontaneous metastasis model following subcutaneous xenotransplantation of cells into immunodeficient mice.

Aim and Main Objectives of the Study

The aim of this study was to elucidate the role of laminins and the CD44 receptor in the pathogenesis of colorectal cancer.

To achieve this goal, the following objectives were formulated and addressed:

1. To assess the prognostic significance of individual laminin chains and their combinations in colorectal cancer.
2. To determine the impact of the laminin α5 chain on the properties of colorectal cancer cells.
3. To investigate the association between CD44 isoform expression and overall survival in patients with colorectal cancer.

4. To evaluate the effect of CD44 expression on the phenotype and dissemination of HT-29 colorectal cancer cells using a spontaneous metastasis model in immunodeficient mice.
5. To examine the impact of CD44 knockdown on the expression of miRNAs and their targets in xenograft tumors.

Scientific Novelty, Theoretical and Practical Significance of the Study

This study is the first to evaluate the prognostic significance not only of individual laminin chains, but also of their various combinations in CRC. It was demonstrated that the classifier based on the combined expression of all laminin chains possesses the highest prognostic value. However, the prognostic power of co-expression of the *LAMA5*, *LAMC1*, and *LAMC2* genes is comparable to that of the classifier constructed from the expression of all laminin chains.

A systematic investigation of the chemoprotective effects of different LM isoforms on CRC cell lines in response to standard chemotherapeutic agents—5-fluorouracil, regorafenib, and sorafenib—revealed that laminins can modulate cellular drug sensitivity. The most pronounced effect was observed for LM-521. Nevertheless, LMs are not universal regulators of chemotherapy response in tumor cells.

The study also confirmed the role of LMs in modulating EMT in CRC cells. It was established that the direction of this effect depends on the intrinsic properties of the cells, the expression profile of laminin receptors, and the culture conditions (2D or 3D).

For the first time, it was shown that knockdown of the *LAMA5* gene, which encodes the laminin α5 chain, is associated with partial dedifferentiation and increased sensitivity of HT-29 cells to 5-fluorouracil. Collectively, the results of this work expand the current understanding of the role of laminins in the biology and progression of CRC.

It was established that CD44 isoform 3 is predominantly expressed in tumors of CRC patients; however, high expression of isoform 3 is associated with improved overall survival in patients with left-sided CRC. In contrast, high expression of CD44 isoform 4 correlates with decreased patient survival. An analysis of the potential molecular mechanisms underlying the distinct prognostic impacts of isoforms 3 and 4 revealed that both isoforms regulate largely overlapping gene sets, with over 98% of these genes being regulated in opposite directions by the two isoforms.

Using a xenograft model of the HT-29 cell line in immunodeficient mice, it was demonstrated that CD44 knockdown prevents metastatic spread of CRC tumors to distant organs. The mechanism of metastasis suppression following CD44 knockdown involves stimulation of angiogenesis and reduction of hypoxia levels in primary tumors, as well as a partial decrease in EMT and stem-like properties of tumor cells. These findings challenge the conventional view that increased microvessel density promotes metastasis, showing instead that enhanced angiogenesis can improve tumor oxygenation, thereby reducing metastatic potential. Furthermore, for the first time, it was shown that while hypoxia and HIF-1α activate CD44 expression, suppression of CD44 expression can, in turn, lead to a decrease in tumor hypoxia.

CD44 knockdown was also found to alter the expression of miRNAs and their targets involved in EMT and the Notch signaling pathway. These miRNAs may participate in the molecular regulation of the anti-metastatic effects associated with CD44 suppression in CRC.

Taken together, the findings suggest that CD44 isoform 4 may represent a promising therapeutic target for the development of clinically relevant inhibitors of hematogenous metastasis in CRC. The results obtained in this study may be applied in research practices within the fields of molecular biology and oncology.

Key Findings to Be Defended

1. The prognostic significance of the co-expression of *LAMA5*, *LAMC1*, and *LAMC2* genes is comparable to that of the classifier based on the expression of all laminin chains.
2. Endogenous expression of the α5 laminin chain influences the degree of differentiation and sensitivity of colorectal cancer cells to 5-fluorouracil.
3. The expression levels of CD44 isoforms 3 and 4 are associated with overall survival in colorectal cancer patients. Isoforms 3 and 4 of CD44 regulate similar gene sets, with more than 98% of these genes being regulated in opposite directions by the two isoforms.

4. CD44 knockdown suppresses metastasis to distant organs by altering the properties of primary tumor cells. The anti-metastatic effect of total CD44 knockdown appears to be primarily mediated by suppression of isoform 4, the second most highly expressed CD44 isoform in colorectal cancer tumors.
5. CD44 knockdown is accompanied by enhanced angiogenesis, reduced hypoxia, partial inhibition of EMT and stem-like properties, and increased expression of mitochondrial proteins and oxidative phosphorylation-related genes in primary xenograft tumors. Thus, increased microvessel density in colorectal tumors improves oxygenation, which in turn reduces the metastatic potential of tumor cells.
6. Changes in the expression of miRNAs and their targets involved in EMT and the Notch signaling pathway are associated with CD44 knockdown in xenograft tumors.

MAIN CONTENT OF THE STUDY

I. Laminins in Resistance and Progression of Colorectal Cancer

Prognostic Value of Laminin Chain Expression in Colorectal Cancer

Laminins are heterotrimeric proteins composed of one α, one β, and one γ chain [11]. In human cells, five different α, four β, and three γ chains can be expressed, allowing the formation of 16 distinct heterotrimeric complexes. This study assessed the prognostic significance not only of individual laminin chains but also of their various combinations in CRC. It was demonstrated that the highest prognostic power was achieved with a classifier based on the combined expression of all laminin chains (Figure 1). Among the components of this classifier, the genes *LAMA4*, *LAMC1*, and *LAMC2*—encoding the α4, γ1, and γ2 chains, respectively—contributed the most. Increased expression of each of these genes was associated with a higher risk of disease recurrence. Analysis of the prognostic significance of pairs and triplets of LM chains revealed that the prognostic power of the co-expression of the gene sets (*LAMA4, LAMC1, LAMC2*) and (*LAMA5, LAMC1, LAMC2*) is comparable to that of the classifier based on the expression of all laminin chains. Notably, *LAMA4* and *LAMA5* genes entered their respective classifiers with coefficients of opposite signs (high risk of recurrence was associated with high *LAMA4* expression and low *LAMA5* expression), indicating the importance of the expression ratio of *LAMA4* to *LAMA5* (α4 and α5 chains).

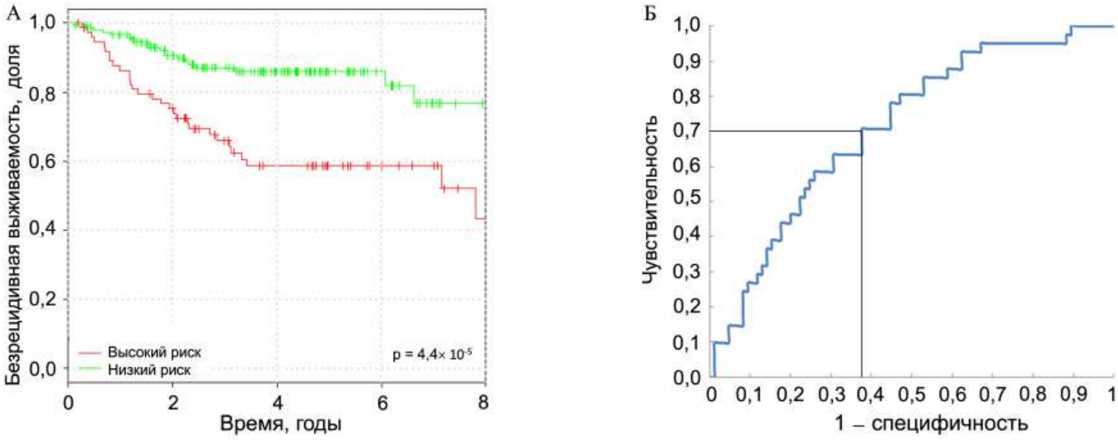

Figure 1 – Prognostic significance of the combined expression of all laminin chains in colorectal cancer. Classifier evaluation on the test cohort: (A) Kaplan–Meier survival curves; (B) ROC curve

It is worth noting that changes in the ratio of these chains within the BM affect its physical properties and can modulate cellular characteristics. Specifically, increased α5 chain content contributes to the formation of a denser and stiffer polymeric “network” as well as tighter cell-cell contacts [10]. Such alterations in laminin chain expression are also observed during EMT [10]. Furthermore, previous studies have demonstrated that the α5 chain participates in the morphogenesis and differentiation of the mouse intestinal epithelium [19]. Therefore, investigating the impact of endogenous α5 LM chain expression on the properties of CRC cells was of particular interest.

Effect of Laminins on the Sensitivity of Colorectal Cancer Cells to Chemotherapeutic Agents

To induce intracellular survival signaling, the binding of LMs to cell surface receptors is required. Therefore, the adhesion capacity of CRC cell lines HT-29, HCT-116, RKO, and SW480 to LM isoforms they express was initially investigated (Figure 2). Preliminary analysis using qPCR and publicly available microarray data revealed that the studied cell lines differ in the expression profiles of genes encoding α chains, which confer signaling properties to LMs.

The results demonstrated that the most effective substrates for CRC cell adhesion were LMs containing the α5 chain (LM-521 and LM-511); in the case of the SW480 cell line, LM-332 also facilitated adhesion (Figure 2). Consequently, the effects of LM-511, LM-521, and LM-332 on the survival of SW480 cells were further examined under treatment with chemotherapeutic agents 5-fluorouracil (5-FU) and regorafenib, which differ significantly in their mechanisms of cytotoxic action.

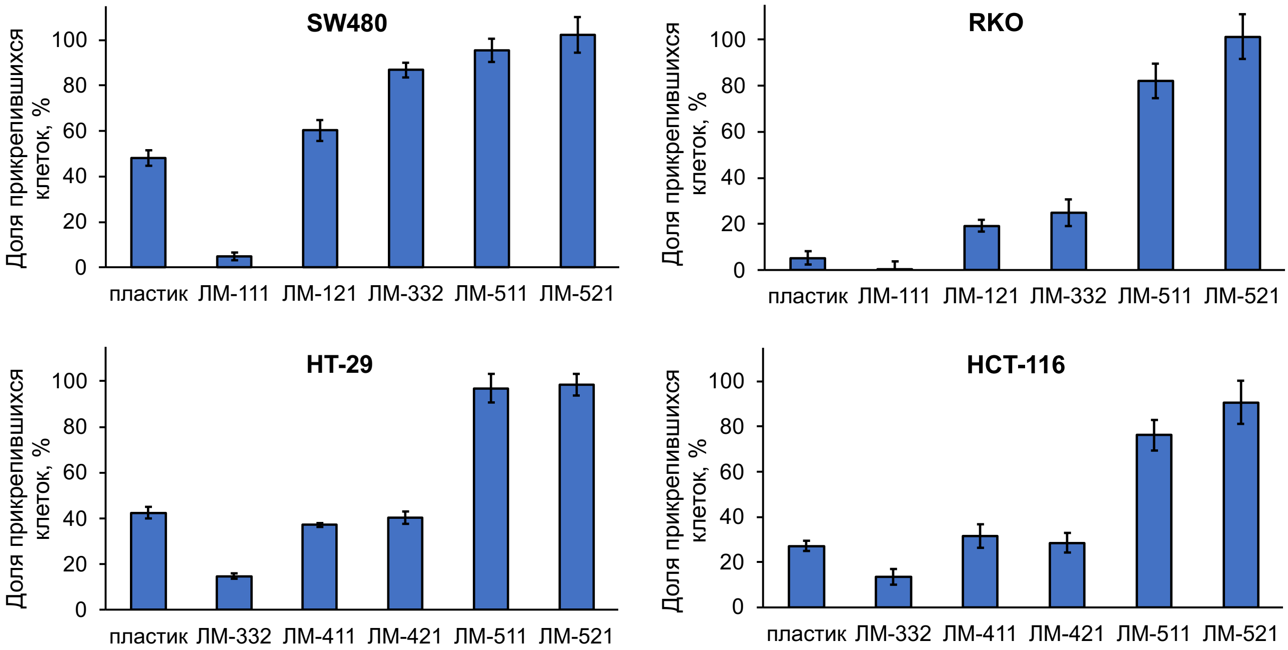

Figure 2 — Adhesion of RKO, HT-29, HCT-116, and SW480 cell lines to plastic coated with various LMs or left uncoated

As can be seen from the obtained dose–response curves for cell viability (Figure 3), in most cases, the presence of laminins had little to no effect on the cytotoxicity of the tested compounds. The most pronounced effect—a 1.6-fold increase in the IC_{50} value—was observed for LM-521 in combination with regorafenib. Therefore, LM-521 was selected for further investigation of cytotoxic effects.

For the RKO and HCT-116 cell lines, a statistically significant chemoprotective effect of LM-521 was observed only upon treatment with 5-FU, resulting in a 1.9- and 1.6-fold increase in IC_{50}, respectively. In contrast, in HT-29 cells, the presence of LM-521 led to a 2.1-fold increase in sensitivity to 5-FU.

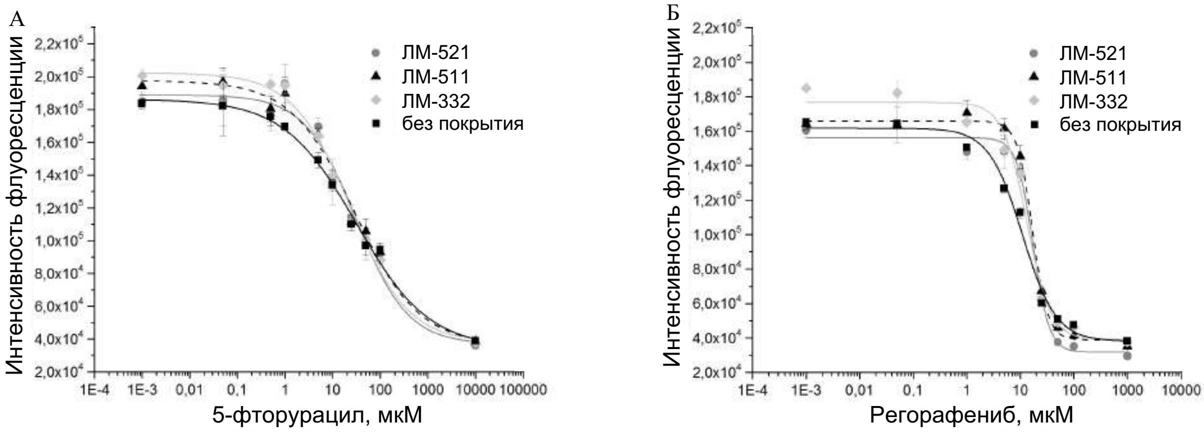

Figure 3 – Effect of laminins on the cytotoxic activity of 5-FU (A) and regorafenib (B) in SW480 cell line

These findings suggest that, in the context of CRC cell lines, LM-521 can indeed modulate cellular sensitivity to cytotoxic agents. However, its effects are not universal and depend on the specific cell line and drug used. Moreover, the influence of LM-521 is not always chemoprotective.

Effect of Laminins on Epithelial–to–Mesenchymal Transition in Colorectal Cancer Cells

Based on gene expression profiling of EMT-associated markers using microarray transcriptome analysis and qPCR, the investigated CRC cell lines can be ranked by the degree of epithelial phenotype expression as follows: HT-29 > HCT-116 > RKO. HT-29 cells exhibited the most pronounced epithelial characteristics, HCT-116 cells represented a hybrid epithelial state, and RKO cells exhibited a hybrid mesenchymal state.

To investigate the role of LMs in modulating epithelial–mesenchymal (E/M) properties of CRC cells, LM-332 and LM-411 were selected. LM-332 is a potent inducer of intracellular signaling, predominates in epithelial tissues, and is a key component of hemidesmosomes. Interestingly, during EMT, expression level of the LM α3 chain gene (*LAMA3*) is reported to either decrease or increase, depending on the study [10]. LM-411 expression is more typical of mesenchymal cells and tends to increase during EMT [10]. The influence of LMs on the E/M properties of CRC cell lines was assessed under both two-dimensional (2D) conditions—by culturing cells on plastic surfaces coated with a specific LM or uncoated—and three-dimensional (3D) conditions, using the hanging drop method, in standard culture medium with or without the addition of individual LMs.

Based on qPCR analysis of EMT-associated gene expression, it was found that under two-dimensional (2D) culture conditions, cultivation of RKO cells—characterized by the most mesenchymal phenotype among the studied lines—on LM-332 and LM-411 further enhanced the mesenchymal phenotype. In contrast, adhesion of HT-29 cells to LM-332 and LM-411 promoted the development of a more epithelial phenotype. Notably, the data indicate that LM-332 exerted a stronger effect compared to LM-411. For the HCT-116 cell line, LM-411 had a more pronounced impact on modulating the EMT phenotype, specifically leading to a reduction in the expression of mesenchymal marker genes.

Transitioning from 2D to three-dimensional (3D) culture conditions did not reveal any consistent trends in EMT gene expression profiles across the three cell lines. Moreover, the phenotypic differences between the cell lines were not diminished under 3D conditions. In 3D culture the epithelial phenotype was pronounced consistent with that observed in 2D: HT-29 > HCT-116 > RKO.

Among the studied cell lines, HCT-116 was the most responsive to LM treatment under 3D culture conditions. The presence of LM-332 in the culture medium led to a decrease in the expression of mesenchymal markers (with the exception of *SNAI1*) expressed by this line, whereas LM-411, in contrast, stimulated their expression. The expression of epithelial markers in the presence of LM-332 changed in a non-uniform manner, while LM-411 had little to no effect on their expression.

In HT-29 cells, which exhibit the most epithelial-like phenotype, LM treatment in 3D conditions resulted in a significant increase in the expression of only two genes—*SNAI1* and *DSP*—compared to 2D conditions.

The effects of both LMs on gene expression in RKO cells were less pronounced than in the other two lines. When compared to 2D conditions, a significant difference was detected only for the mesenchymal marker vimentin, with a slight increase also observed for *SNAI1* expression.

Thus, the direction and magnitude of LM-induced effects varied among the CRC cell lines studied (Table 1), likely due to differences not only in their intrinsic phenotypic states and culture conditions but also in LM receptor expression patterns. Interestingly, *SNAI1* was the only gene whose expression consistently increased in all three cell lines under 3D conditions following treatment with both LM-332 and LM-411.

Table 1 – Effects of LM-332 and LM-411 on E/M properties of CRC cell lines

<table>
<tr><th rowspan="2">Cell line</th><th rowspan="2">Laminin</th><th colspan="2">Culture conditions</th></tr>
<tr><th>2D</th><th>3D</th></tr>
<tr><td rowspan="2">RKO</td><td>LM-332</td><td>Augmented expression of mesenchymal markers.</td><td>Increased SNAI1 expression.</td></tr>
<tr><td>LM-411</td><td>Augmented expression of mesenchymal markers.</td><td>Increased SNAI1 expression.</td></tr>
<tr><td rowspan="2">HT-29</td><td>LM-332</td><td>Augmented expression of epithelial markers.</td><td>Increased SNAI1 expression.</td></tr>
<tr><td>LM-411</td><td>Augmented expression of epithelial markers.</td><td>Increased SNAI1 expression.</td></tr>
<tr><td rowspan="2">HCT-116</td><td>LM-332</td><td>Без значимых изменений.</td><td>Heterogeneous alterations. A modest upregulation of SNAI1.</td></tr>
<tr><td>LM-411</td><td>Decreased expression of mesenchymal markers.</td><td>Increased expression of mesenchymal markers including SNAI1.</td></tr>
</table>

* Bold font indicates that the effect of the corresponding LM was more pronounced.

Effect of Laminin α5 Chain Knockdown on the Properties of HT-29 Cells

The HT-29 cell line was selected as a model system to study the impact of endogenous LM expression on CRC cell behavior. A distinctive feature of HT-29 cells is their capacity for reversible differentiation, indicating a high degree of plasticity—a trait known to facilitate metastatic dissemination of tumor cells.

HT-29 cells were transduced with lentiviral particles encoding shRNA targeting the mRNA of the *LAMA5* gene. Among the three shRNA constructs tested, only one (shLAMA5#3) achieved statistically significant suppression of *LAMA5* expression—1.7-fold at the mRNA level and 3.8-fold at the protein level ($p < 0.001$) (Figure 4).

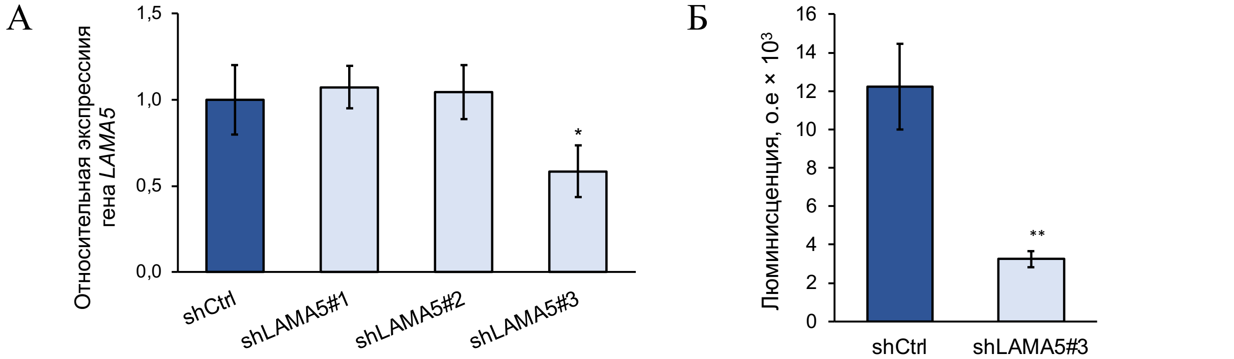

Figure 4 – Evaluation of *LAMA5* gene knockdown efficiency by RT-qPCR (A) and In-Cell ELISA (B). * p = 0,012, ** p < 0,001

The knockdown of *LAMA5* (LAMA5 KD) resulted in a 1.6-fold reduction in HT-29 cell proliferation, both on uncoated plastic and on plastic coated with various LMs (Figure 5). In addition, HT-29 shLAMA5#3 cells exhibited significantly reduced migration on LM-332 and LM-511 compared to control shRNA-transduced HT-29 cells (HT-29 shCtrl) (Figure 6). A similar trend was observed on LM-411, although the differences were not statistically significant.

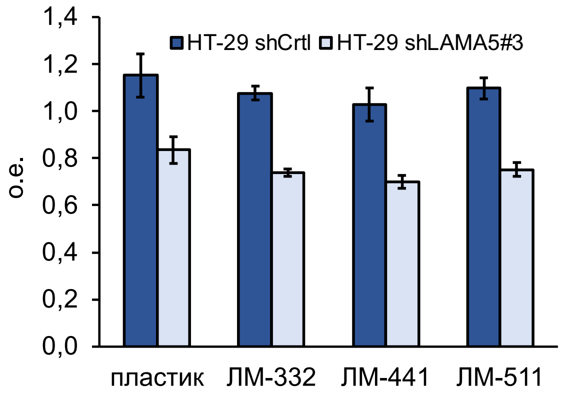

Figure 5 – LAMA5 KD reduces HT-29 cell proliferation on both uncoated plastic and plastic coated with LMs (p < 0.001)

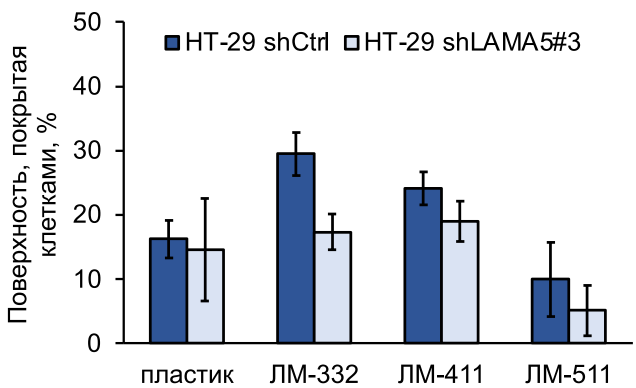

Рисунок 6 – LAMA5 KD reduces HT-29 cell migration on plastic coated with LMs. p < 0,001 (for LM-332), p <0,05 (for LM511)

LAMA5 Knockdown Is Associated with ER Stress Activation and Partial Dedifferentiation of HT-29 Cells

Comparative transcriptome analysis of HT-29 shLAMA5#3 and HT-29 shCtrl cells using Affymetrix microarrays revealed that LAMA5 KD had a relatively moderate impact on the transcriptome of HT-29 cells. A ≥1.5-fold change in gene expression was detected for 308 protein-coding genes, with 178 genes downregulated and 130 upregulated. Functional enrichment analysis of the differentially expressed genes (DEGs) identified several overrepresented pathways, including membrane transport processes, cellular stress responses, and mechanisms of adaptation to external stimuli. The expression changes of selected DEGs were validated by qPCR.

In addition to transcriptome profiling, mass spectrometry-based proteome analysis was performed, revealing 87 proteins with expression changes of two-fold or greater between HT-29 shLAMA5#3 and HT-29 shCtrl cells.

Detailed analysis indicated that LAMA5 KD led to reduced expression of intestinal epithelial differentiation markers such as *KRT20*, *ANXA13*, *MUC13*, and *SI*. Among these, decreased expression of KRT20 and ANXA13 was also confirmed at the protein level, with even more pronounced downregulation—11.8-fold for ANXA13 and 3.4-fold for KRT20.

A decrease in the expression of such important genes involved in stem cell differentiation as *NDRG1* and *BGN*, was also detected. *NDRG1* is known as a negative regulator of EMT in CRC cells. The *BGN* gene encodes biglycan, an extracellular matrix component that interacts with LRP6, a co-receptor of Wnt receptors, thereby activating the canonical Wnt signaling pathway. Importantly, Wnt signaling is essential for maintaining the undifferentiated state of intestinal stem cells [20]. In addition, upregulation of the intestinal epithelial stem cell marker *LGR5* was observed. *LGR5* encodes the receptor for R-spondin and is likewise involved in the activation of canonical Wnt signaling.

Transcriptome profiling further revealed increased expression of a number of genes associated with endoplasmic reticulum (ER) stress, indicating activation of ER stress-related signaling pathways. These included *DDIT3*, *DDIT4*, *SESN2*, and *DEPTOR*—

genes encoding proteins that inhibit the activity of the mTORC1 complex. It is noteworthy that suppression of mTORC1 activity has been linked to the acquisition of pluripotent characteristics, whereas its activation is required for proper cellular differentiation.

According to proteome analysis, LAMA5 KD was associated with an increase (1.8-fold) in the expression of the α6 integrin subunit and a decrease (1.8-fold) in the expression of the β4 integrin subunit—components of the α6β4 and α6β1 laminin receptors. As the expression of the β1 integrin subunit remained unchanged, it can be assumed that these expression shifts may lead to an increased presence of the α6β1 integrin heterodimer on the cell surface. Notably, α6β1 plays an important role in promoting stem-like properties of intestinal epithelial cells [26].

Taken together, these findings indicate that LAMA5 KD induces partial dedifferentiation of HT-29 cells. Importantly, the results obtained for the HT-29 cell line are consistent with previous observations showing that *Lama5* knockout in mice impairs intestinal epithelial differentiation through disruption of Wnt signaling [21].

Altered Sensitivity of HT-29 Cells to 5-FU Following *LAMA5* Knockdown

It is well established that ER stress plays a dual role in cellular responses to adverse conditions [22]. On the one hand, ER stress may act as part of an adaptive mechanism, promoting the restoration of homeostasis and cell survival. Indeed, in HT-29 cells with LAMA5 KD, increased expression of *SESN2* and *ANKRD1* was observed, both of which are known to inhibit ER stress-induced apoptosis. On the other hand, when cellular homeostasis is severely disrupted, ER stress can trigger apoptotic pathways. Indeed, experimental data demonstrated that LAMA5 KD resulted in a 2.2-fold increase ($p < 0.05$) in HT-29 cell sensitivity to 5-FU, with IC_{50} values decreasing from 224 μM in control cells to 102 μM in KD cells (Figure 7A).

Given that LM-521 modulates the sensitivity of CRC cells to chemotherapeutic agents, the next stage of the study examined the effect of this LM on the cytotoxic activity of 5-FU in HT-29 shCtrl and HT-29 shLAMA5#3 cell lines (Figure 7B and 7C).

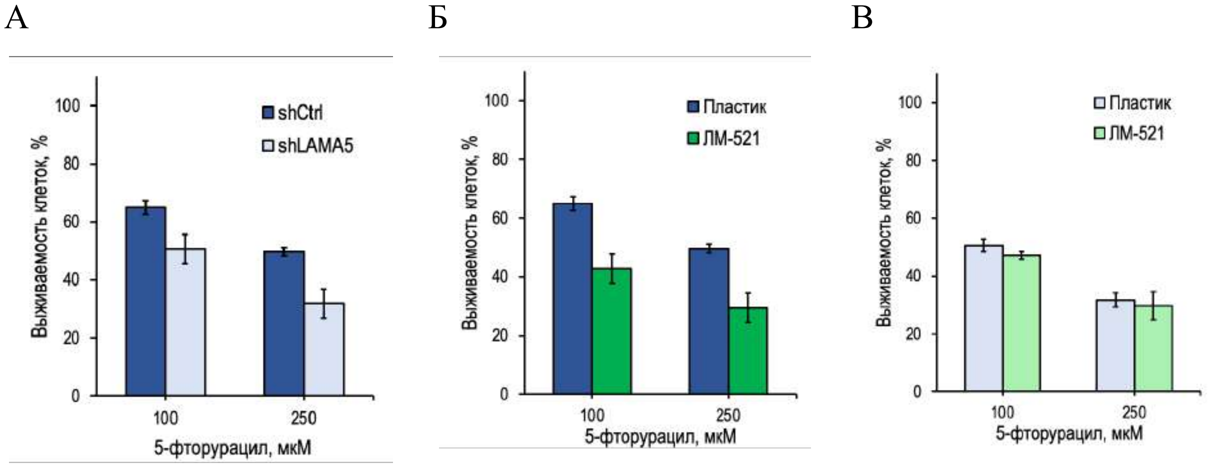

Figure 7 – *LAMA5* knockdown increases HT-29 cell sensitivity to 5-FU (A) and results in loss of sensitivity to the presence of LM-521. Effect of LM-521 on viability of HT-29 shCtrl cells (B) and HT-29 shLAMA5#3 cells (C)

Consistent with the data obtained for the parental HT-29 cell line (see above), HT-29 shCtrl cells cultured on LM-521 exhibited a 2.4-fold increase in sensitivity to 5-FU (IC_{50} = 92 μM) compared to cells cultured on uncoated plastic. In contrast, HT-29 shLAMA5#3 cells showed no substrate-dependent differences in sensitivity to 5-FU.

Transcriptome and proteome analyses allowed for hypothesizing potential mechanisms underlying both the increased sensitivity of HT-29 and HT-29 shCtrl cells to 5-FU when cultured on LM-521, and the loss of sensitivity following LAMA5 KD. The proposed mechanism for the enhanced sensitivity of HT-29 cells to 5-FU in the presence of LM-521 may involve the following cascade: binding of α3β1 integrin to LM-521 activates the FAK/PI3K/Akt signaling pathway, leading to phosphorylation of YAP and the formation of the 14-3-3σ/p-YAP complex, which prevents YAP nuclear translocation and thereby inhibits its anti-apoptotic function. LAMA5 KD led to suppressed expression of the *SNF* gene, encoding the 14-3-3σ subunit, and may have altered distribution of the β1 integrin subunit between integrin heterodimers, potentially reducing α3β1 integrin levels. This, in turn, could promote nuclear translocation of YAP and activation of its target genes. Indirect evidence of increased YAP activity in HT-29 shLAMA5#3 cells includes the upregulation of its target genes, *ANKRD1* and *PTGS2*, despite the fact that ER stress is generally expected to suppress *ANKRD1* expression.

Summary of Observed Changes and Their Potential Molecular Mechanisms in HT-29 Cells Following LAMA5 Knockdown

A summary of the observed changes and their potential molecular mechanisms in HT-29 cells as a result of LAMA5 KD is presented in Figure 8.

In the adult human small intestine, a gradient of α5 laminin chain expression exists along the crypt-villus axis, with predominant localization of the α5 chain in villi, whereas its expression is absent in the crypt regions, which represent intestinal stem cell niches (Figure 9) [23,24].

As mentioned above, integrins are considered the main receptors of LMs on the cell surface [10]. According to proteome analysis, LAMA5 KD was accompanied by increased expression of the α6 integrin subunit and decreased expression of the β4 integrin subunit. Since the expression of the β1 subunit remained unchanged, it is plausible that redistribution of integrin heterodimers occurs at the cell surface, leading to an increase in α6β1 integrin dimers.

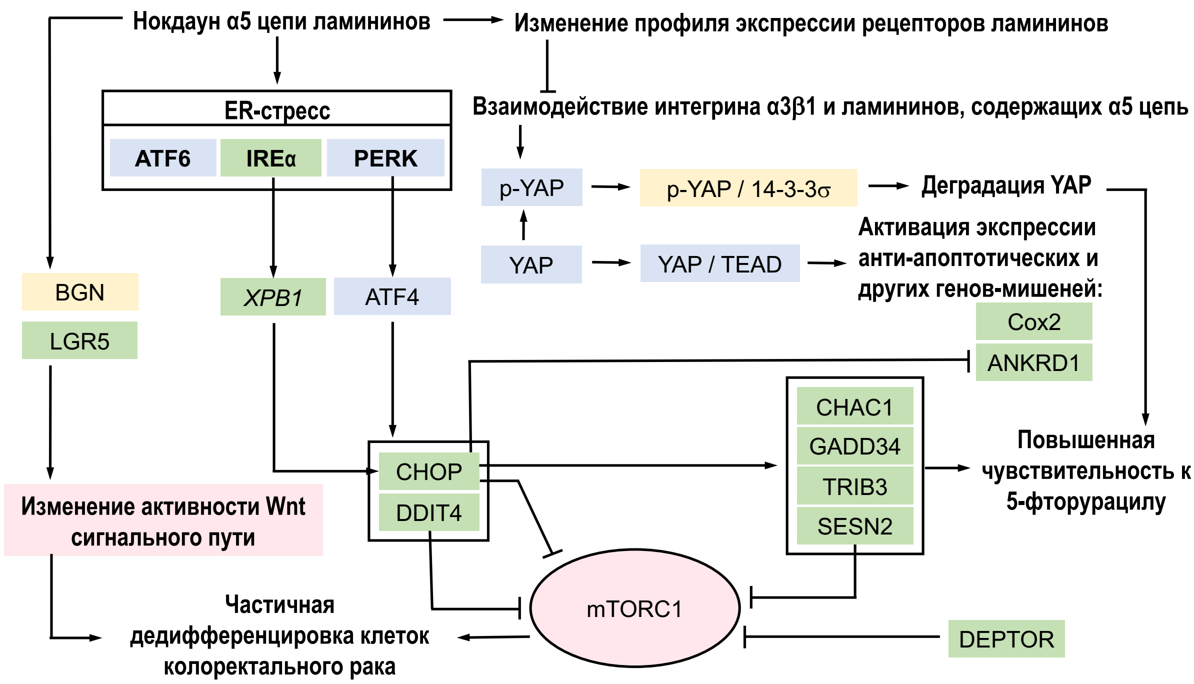

Figure 8 – Schematic representation of possible molecular mechanisms of partial dedifferentiation of HT-29 cells upon LAMA5 KD. Proteins for which gene expression was downregulated are marked in yellow, those with upregulated expression in green, and signaling pathways potentially contributing to the observed partial dedifferentiation are highlighted in pink

Interestingly, the α6 and β1 integrin subunits exhibit a uniform expression pattern along the crypt–villus axis in the human intestine. However, the isoforms of the α6 subunit differ between the crypt and villus regions. Specifically, undifferentiated crypt cells predominantly express the α6A isoform, whereas differentiated enterocytes in the villus region express the α6B variant. For the β4 subunit, its distribution has been reported either as being restricted to the villus region or extending along the entire crypt–villus axis. However, at the protein level, the β4 subunit in the crypt region is expressed in a functionally inactive form [25]. Since the β4 subunit can form a heterodimeric complex exclusively with the α6 subunit, functional α6β4 integrin complexes are present only in the villus region (Figure 9) [25]. Consequently, in the crypt region, the α6 subunit primarily exists as the α6β1 heterodimer, where it plays a crucial role in maintaining stem cell properties [26]. Thus, in the small intestine, the expression level of α6β1 integrin is expected to inversely correlate with the expression of the α5 laminin chain (Figure 9), which is consistent with our observed changes in integrin subunit expression in response to LAMA5 KD.

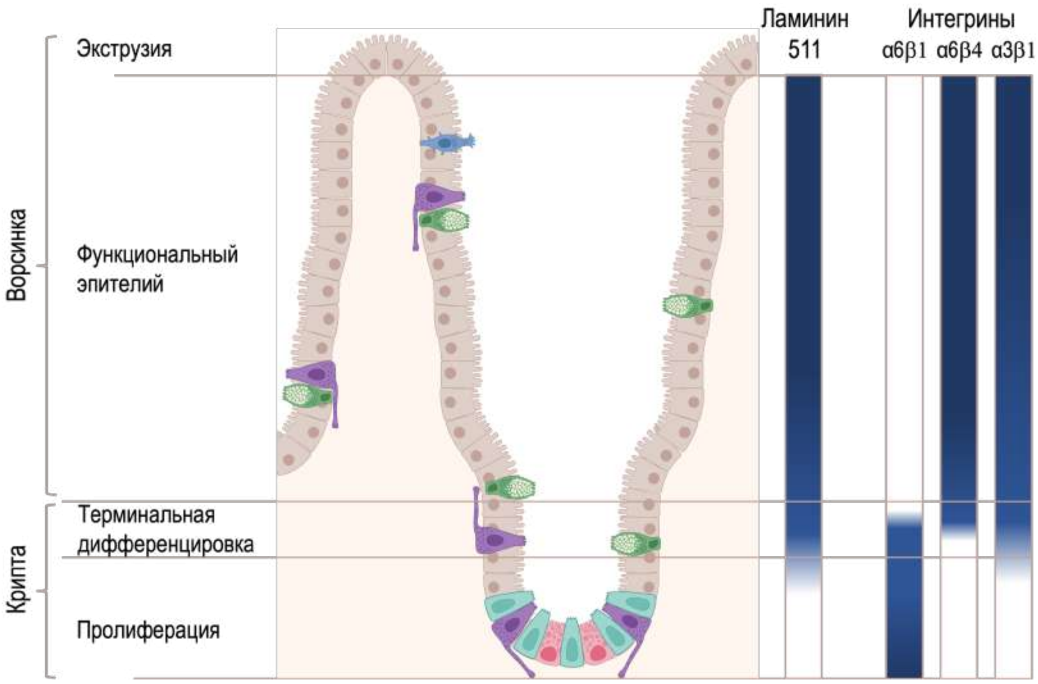

Figure 9 – Distribution of LM-511 and integrin expression along the crypt–villus axis in the intestine. The figure was created using BioRender

In summary, the knockdown of *LAMA5* leads to partial dedifferentiation of HT-29 cells, presumably through the activation of ER stress and the Wnt signaling pathway, along with suppression of mTORC1 complex activity. This is consistent with the observation that high *LAMA5* expression is associated with a favorable prognosis in patients. Furthermore, the activation of ER stress signaling increases cellular susceptibility to apoptotic stimuli, thereby enhancing sensitivity to 5-FU.

II. The Role of CD44 Receptor in Colorectal Cancer Metastasis

Prognostic Significance of CD44 Isoforms in Colorectal Cancer

According to the NCBI database, alternative splicing of *CD44* mRNA gives rise to eight distinct isoforms of this receptor (Figure 10). In the initial phase of investigating the role of CD44 in CRC progression, the expression profiles of *CD44* isoforms were analyzed in primary tumor samples and adjacent normal tissues from CRC patients using publicly available mRNA sequencing data from the TCGA-COAD database.

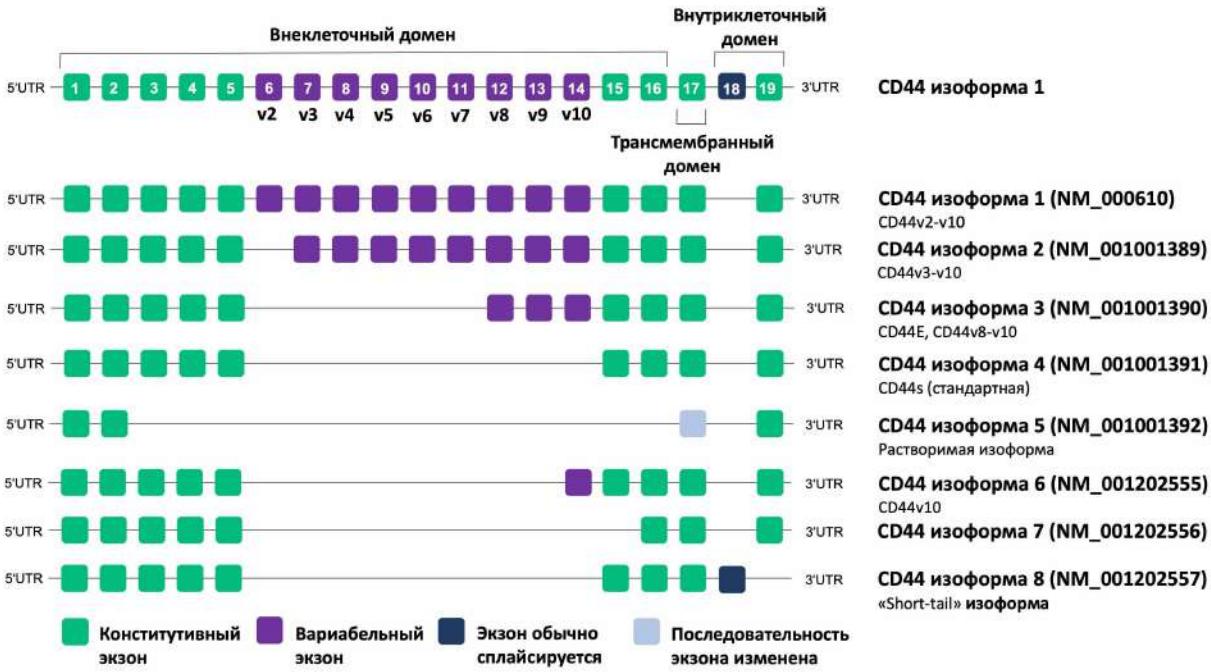

Figure 10 – Alternative splicing of *CD44* mRNA according to the NCBI database

The results revealed distinct differences in *CD44* mRNA isoform expression profiles between normal and tumor tissues (Figure 11). Normal tissue samples predominantly expressed isoforms 3 and 4. In contrast, most tumor samples showed a strong predominance of isoform 3, with expression levels approximately seven times higher than those in adjacent normal tissues. Conversely, the expression of isoform 4 (CD44s) was approximately two-fold lower in tumor tissues compared to normal counterparts (Figure 11). Isoforms 1, 2, and 5–7 were either expressed at low levels or nearly undetectable. Expression data for isoform 8 were not available in the TCGA-COAD dataset.

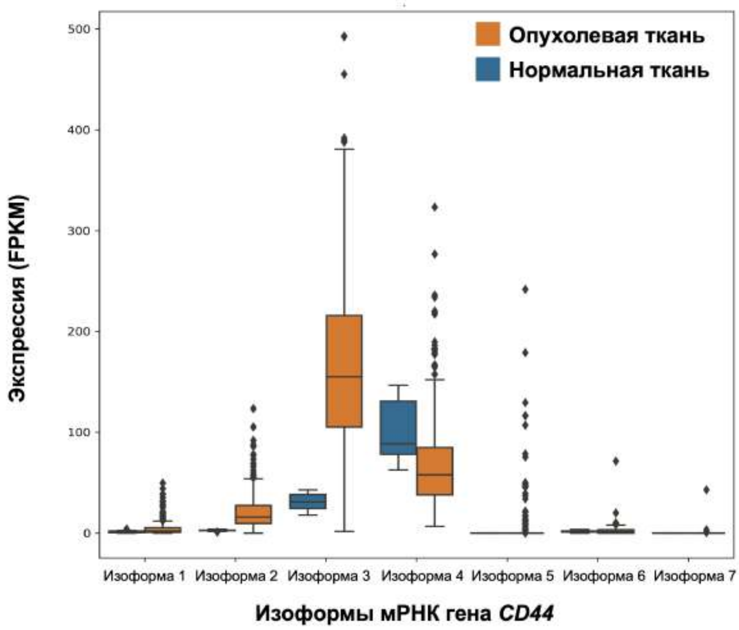

Figure 11 – Expression of *CD44* mRNA isoforms in tumor samples and adjacent normal tissues from CRC patients (based on TCGA-COAD data)

Subsequently, the expression patterns of *CD44* isoforms were analyzed separately in tumor samples derived from the left and right sides of the colon, in light of accumulating evidence suggesting biological differences between these regions. No statistically significant differences in *CD44* isoform expression were found between left- and right-sided colorectal cancers. However, Cox proportional hazards regression analysis for left-sided colon cancer samples revealed that patients with higher expression levels of isoform 3 had improved overall survival (OAS), despite the fact that this isoform is predominantly expressed in tumors (Figure 12). In contrast, higher expression of isoform 4 (CD44s) was associated with poorer OAS. Moreover, a higher isoform 4:3 expression ratio was linked to an even more unfavorable prognosis. Notably, for right-sided colon cancer samples, no statistically significant association was found between the expression of *CD44* isoforms and OAS. In addition, the total expression of all *CD44* isoforms combined did not demonstrate prognostic significance in either left- or right-sided colorectal cancer.

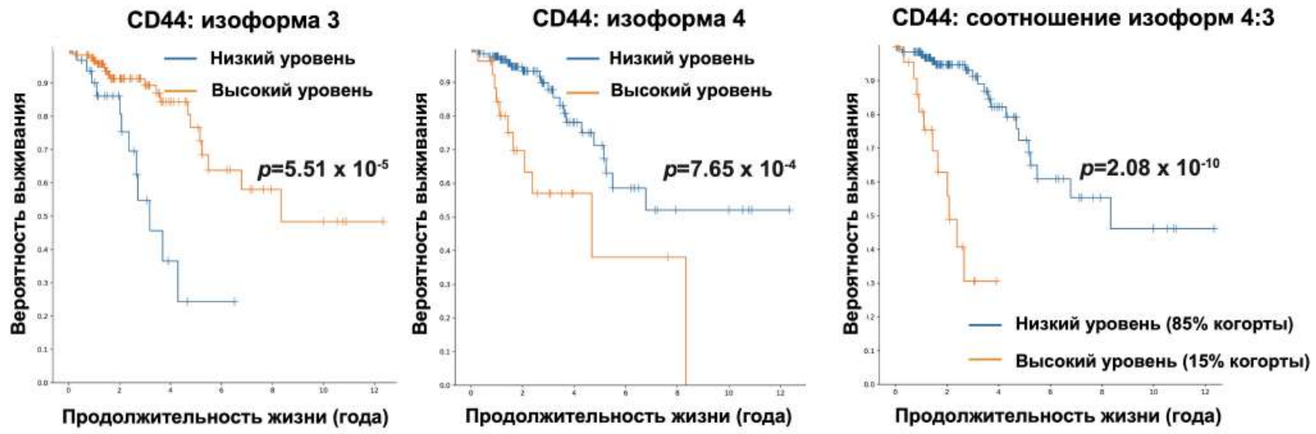

Figure 12 – OAS of CRC patients with high vs. low expression levels of *CD44* isoform 3, isoform 4, or the isoform 4:3 expression ratio (based on TCGA-COAD data)

These findings were further validated at the protein level using immunohistochemical analysis with antibodies specific to the variant exon CD44v9, which is included in isoform 3. It was demonstrated that progression-free survival (PFS)—defined as the time from surgical resection of the primary tumor to disease progression—was shorter in CRC patients lacking CD44v9 expression compared to those with positive CD44v9 staining (Figure 13).

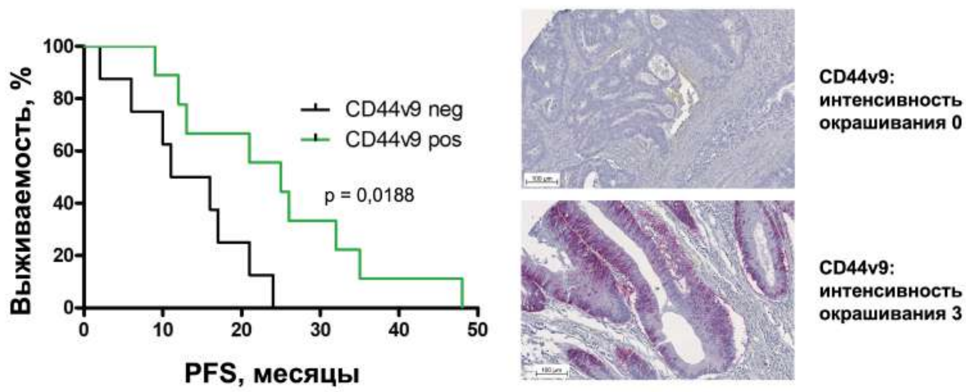

Figure 13 –PFS of CRC patients with negative (staining intensity = 0) versus positive (staining intensity = 3) CD44v9 expression in primary tumors

To elucidate potential molecular mechanisms underlying the distinct prognostic effects of CD44 isoforms 3 and 4, differential mRNA expression profiling was conducted in tumor samples stratified by high and low expression levels of these isoforms. A total of 1,903 differentially expressed genes (DEGs) were identified between the high and low isoform 3 groups, and 3,371 DEGs were identified between the high and low isoform 4 groups. Interestingly, the majority of genes (n = 1,504) overlapped between both comparisons, with 1,483 of them exhibiting changes in opposite directions (Figure 14).

Figure 14 – Venn diagram illustrating the substantial overlap of DEGs identified by comparing patient tumors with high vs. low expression of CD44 isoform 3 and those with high vs. low expression of CD44 isoform 4

Gene set enrichment analysis (GSEA) of the DEGs revealed several statistically significant enriched pathways (n = 23 for isoform 4 and n = 10 for isoform 3). Notably, based on the directionality of changes in overlapping genes, six pathways exhibited opposite activity patterns between the two isoform groups. Among them, EMT was downregulated in tumors with high isoform 3 expression and upregulated in those with high isoform 4 expression; conversely, oxidative phosphorylation (OxPhos) was upregulated in the high isoform 3 group and downregulated in the high isoform 4 group (Figure 15).

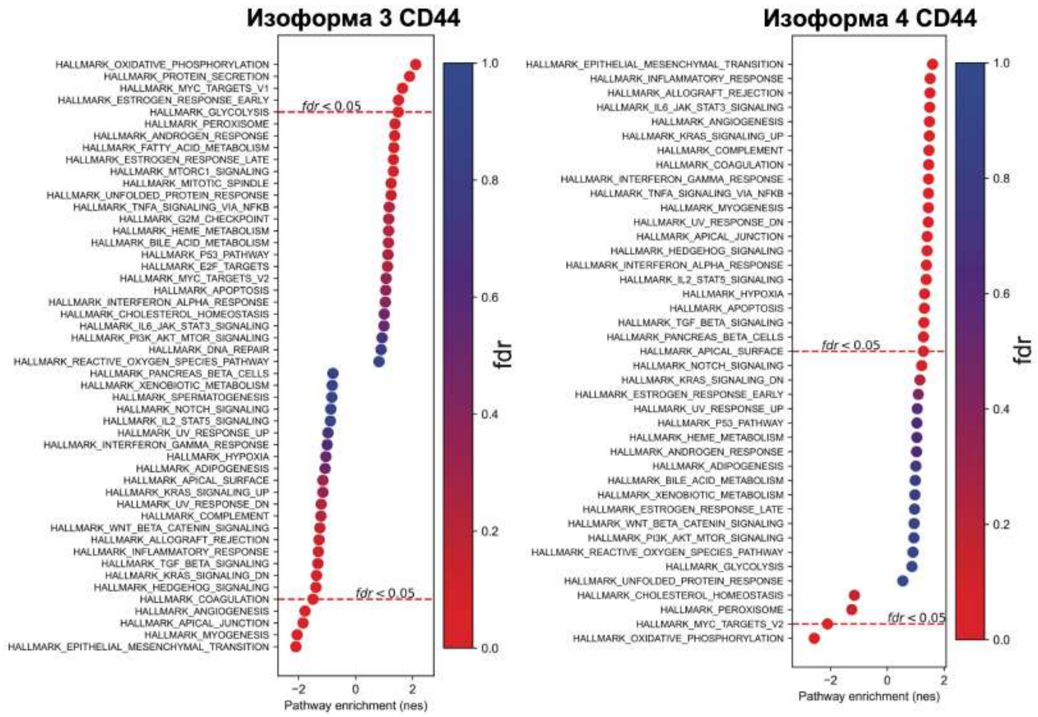

Figure 15 – Gene set enrichment analysis (GSEA) of DEGs identified by comparing tumors from patients with high vs. low expression of CD44 isoform 3 or isoform 4

Thus, although CD44 isoform 3 is the predominant transcript in CRC patient tumors, the data indicate that isoform 4 is more strongly associated with a more aggressive tumor phenotype.

CD44 Knockdown Suppresses Metastasis of HT-29 Xenograft Tumors in Immunodeficient Mice

To further investigate the role of the CD44 receptor in CRC progression, the HT-29 cell line was selected, as it predominantly expresses CD44 mRNA isoforms 3 and 4, with isoform 3 being the most abundant—similar to the pattern observed in patient tumor samples (Figure 16).

A stable HT-29 cell line with shRNA-mediated knockdown of the *CD44* gene (HT-29 shCD44) was generated using lentiviral transduction. Cells transduced with lentiviral particles encoding shRNA against firefly luciferase (HT-29 shLuc) were used as a control. The efficiency of the knockdown was confirmed by flow cytometry, which demonstrated a reduction in CD44 protein expression by more than 95% (Figure 17).

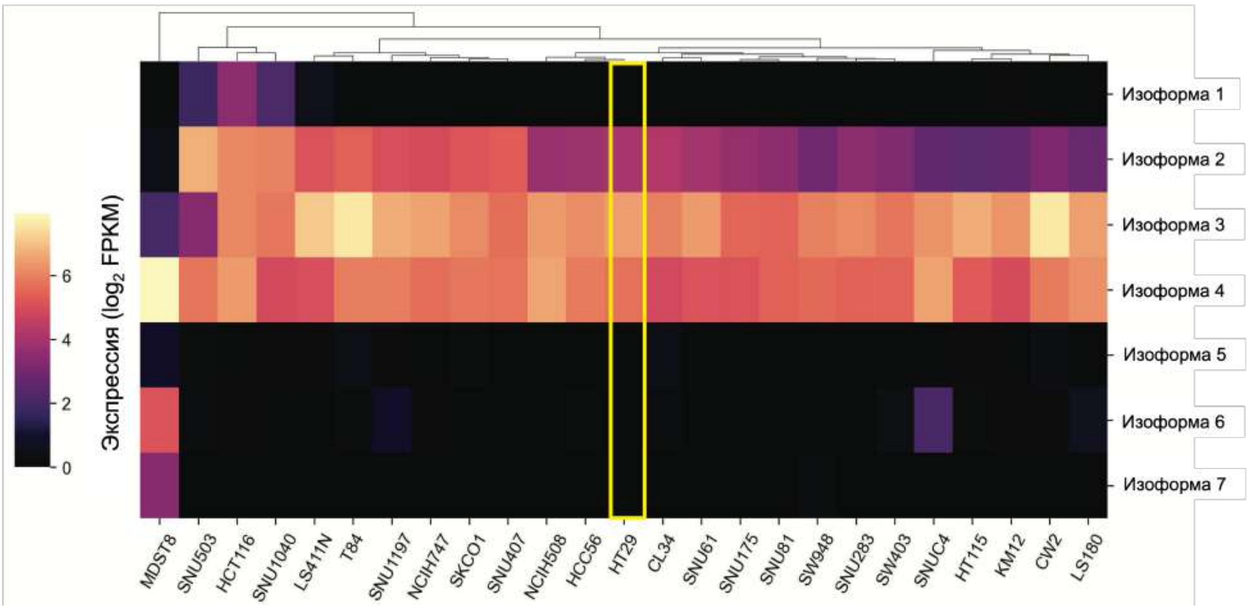

Figure 16 – Expression of *CD44* mRNA isoforms in colorectal cancer cell lines (based on CCLE database data)

No significant differences in morphology were observed between HT-29 shLuc and HT-29 shCD44 cells. *In vitro* assays assessing the impact of CD44 knockdown (CD44 KD) on HT-29 cell behavior yielded mixed results. Specifically, an increase in proliferation rate and enhanced migration were observed, while invasive capacity was reduced. Moreover, the level of sLe[a]—a classical carbohydrate ligand of E-selectin—on the cell surface, as well as E-selectin binding and adhesion to vascular endothelium, were significantly higher in HT-29 shCD44 cells compared to HT-29 shLuc controls.

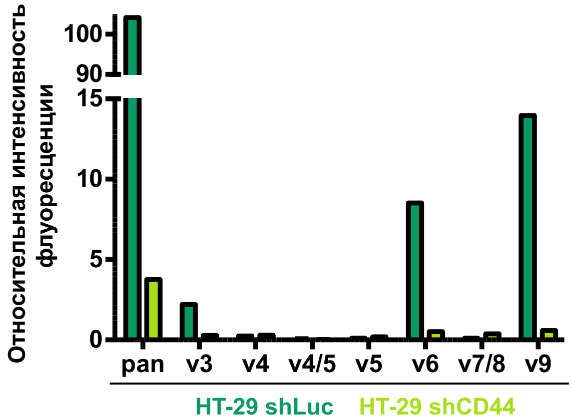

Figure 17 – Downregulation of total CD44 protein and CD44 variant isoforms in HT-29 cells following KD, as assessed by flow cytometry using antibodies specific to different protein domains

Subsequently, subcutaneous transplantation of HT-29 shCD44 and HT-29 shLuc cells was performed in immunodeficient scid mice, with injections administered into the interscapular region. The absence of CD44 protein expression in primary tumors derived from CD44 KD cells was confirmed by immunohistochemical staining (Figure 18).

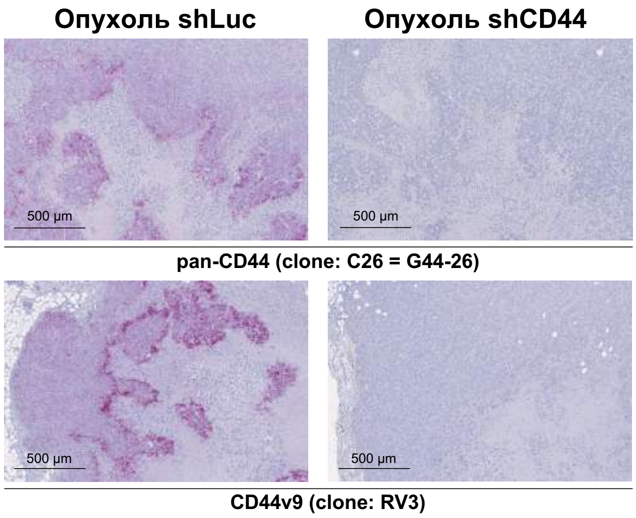

Figure 18 – Confirmation of CD44 knockdown in primary xenograft tumors in animals

The mass of primary tumors in the CD44 KD group was 1.4-fold lower than in the control group (Figure 19A). Given this observation, tumor mass was taken into account in subsequent analyses and statistical assessments to control for potential confounding effects. In addition, the CD44 KD group showed a trend toward increased overall survival, and the number of lung metastases, as detected by immunohistochemistry, was significantly reduced (Figure 19).

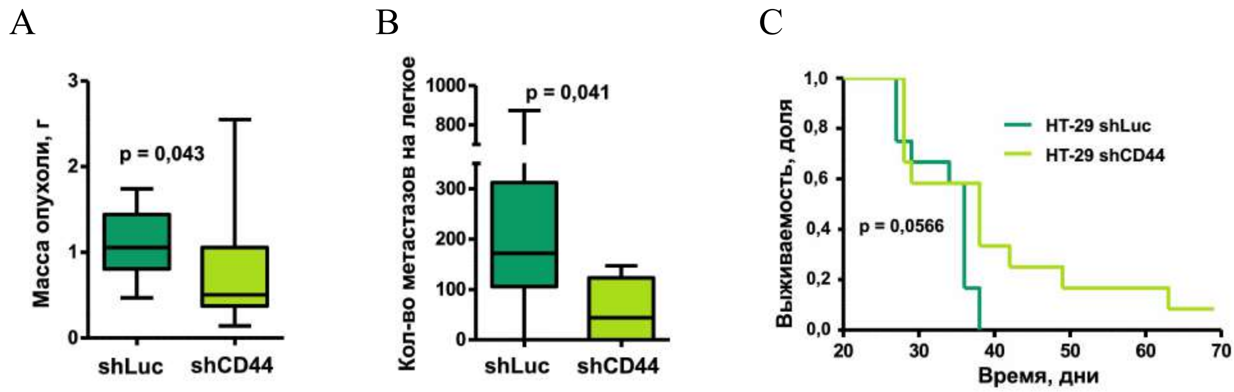

Figure 19 – Effect of CD44 knockdown on primary tumor mass (A), the number of lung metastases (B) detected by immunohistochemical staining, and overall animal survival (C)

QPCR targeting human-specific Alu repeat sequences revealed a reduction in the presence of HT-29 tumor cells in the liver and bone marrow of animals (but not in peripheral blood), and further confirmed the decrease in the number of lung metastases following CD44 KD (Figure 20).

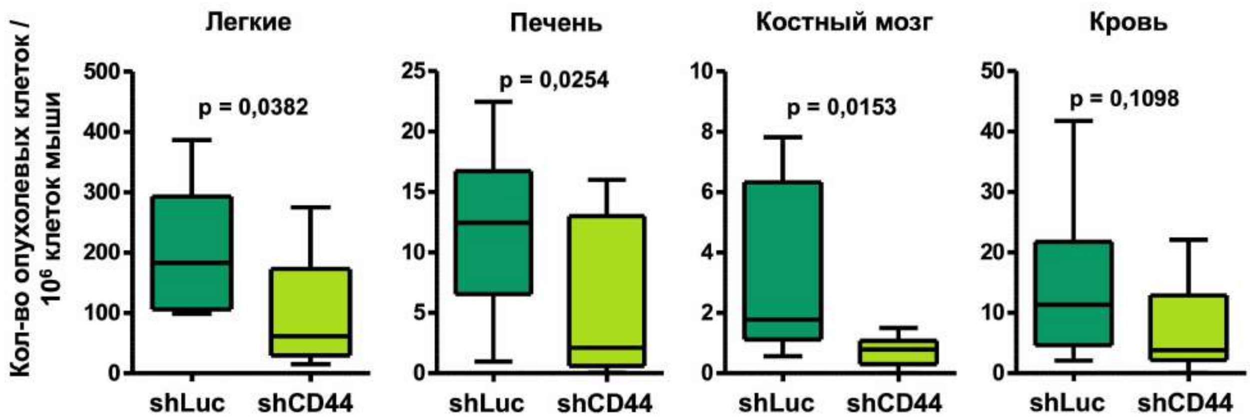

Figure 20 – Effect of CD44 knockdown on the number of disseminated HT-29 tumor cells in various organs and tissues of experimental animals

Thus, CD44 KD in HT-29 cells reduces the metastatic potential of primary tumors *in vivo*. The absence of specific staining with pan-CD44 and CD44v9 antibodies in lung metastases (data not shown) suggests that the anti-metastatic effect of CD44 KD is attributable to altered properties of the primary tumor cells.

CD44 knockdown alters EMT marker expression and contributes to reduced stemness in HT-29 tumors

To elucidate the potential mechanisms underlying the observed decrease in metastatic potential of HT-29 tumors following CD44 KD, comparative transcriptome and proteome analyses were performed. These included mRNA sequencing and mass spectrometry-based proteome profiling of tumors derived from HT-29 shCD44 and HT-29 shLuc cells. In addition, mRNA sequencing provided insights into the isoform composition of CD44 transcripts in control versus CD44 KD tumors (Figure 21). As expected, none of the CD44 mRNA isoforms were detected in HT-29 shCD44 tumors. In control tumors, as in the parental HT-29 cell line, isoforms 3 and 4 predominated.

Transcriptome analysis identified 4,214 protein-coding genes whose expression levels were significantly altered (≥1.5-fold change) in response to CD44 KD. Subsequent enrichment analysis of these DEGs revealed dysregulation of more than 40 biological pathways and processes, including cell–cell adhesion, extracellular matrix organization, cell migration, hypoxia, glycolysis, oxidative phosphorylation (OxPhos), angiogenesis, and signaling via NIK/NF-κB and Wnt pathways, as well as EMT (Figure 22).

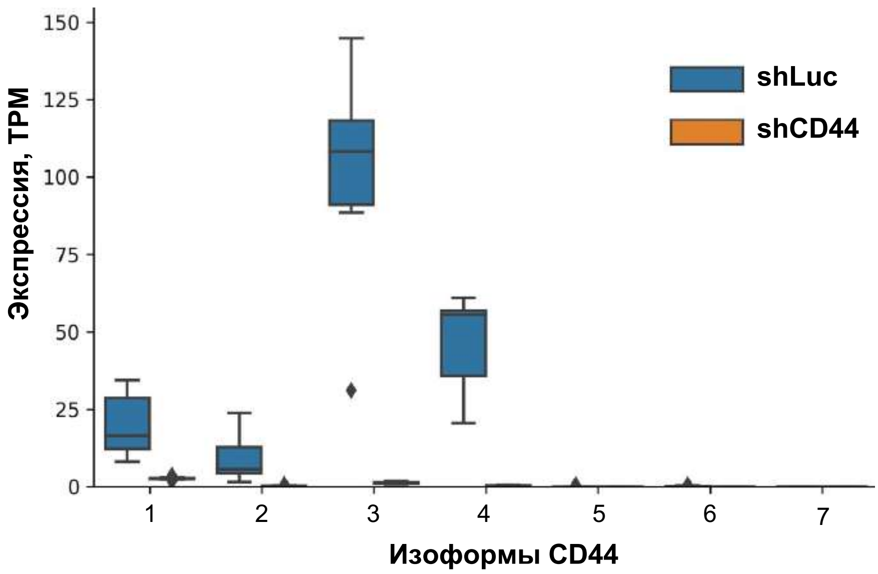

Figure 21 – Expression of *CD44* mRNA isoforms in primary xenograft tumors derived from HT-29 shLuc and HT-29 shCD44 cells

A more detailed analysis revealed that CD44 KD resulted in downregulation of both epithelial and mesenchymal genes. Among the repressed epithelial genes were *CDH1*, *JUP*, *DSP*, and the laminin-332 chains (*LAMA3*, *LAMB3*, *LAMC2*). Repressed mesenchymal genes included *MMP14*, *MMP2*, *SPARC*, *SERPINE1*, *TGFBI*, *TIMP1*, *IL1B*, *ITGAV*, and *ZEB2*. The reduced mRNA levels of some of these genes, as well as others mentioned later, were validated by qPCR. According to RNA-Seq data, classical EMT-associated transcription factors (*SNAI1*, *SNAI2*, *ZEB1*, *TWIST*) were expressed at very low levels in HT-29 xenograft tumors, consistent with previous *in vitro* findings for the HT-29 cell line. Only *ZEB2* was expressed at near-threshold levels, and its expression was significantly reduced (1.5-fold) upon CD44 KD.

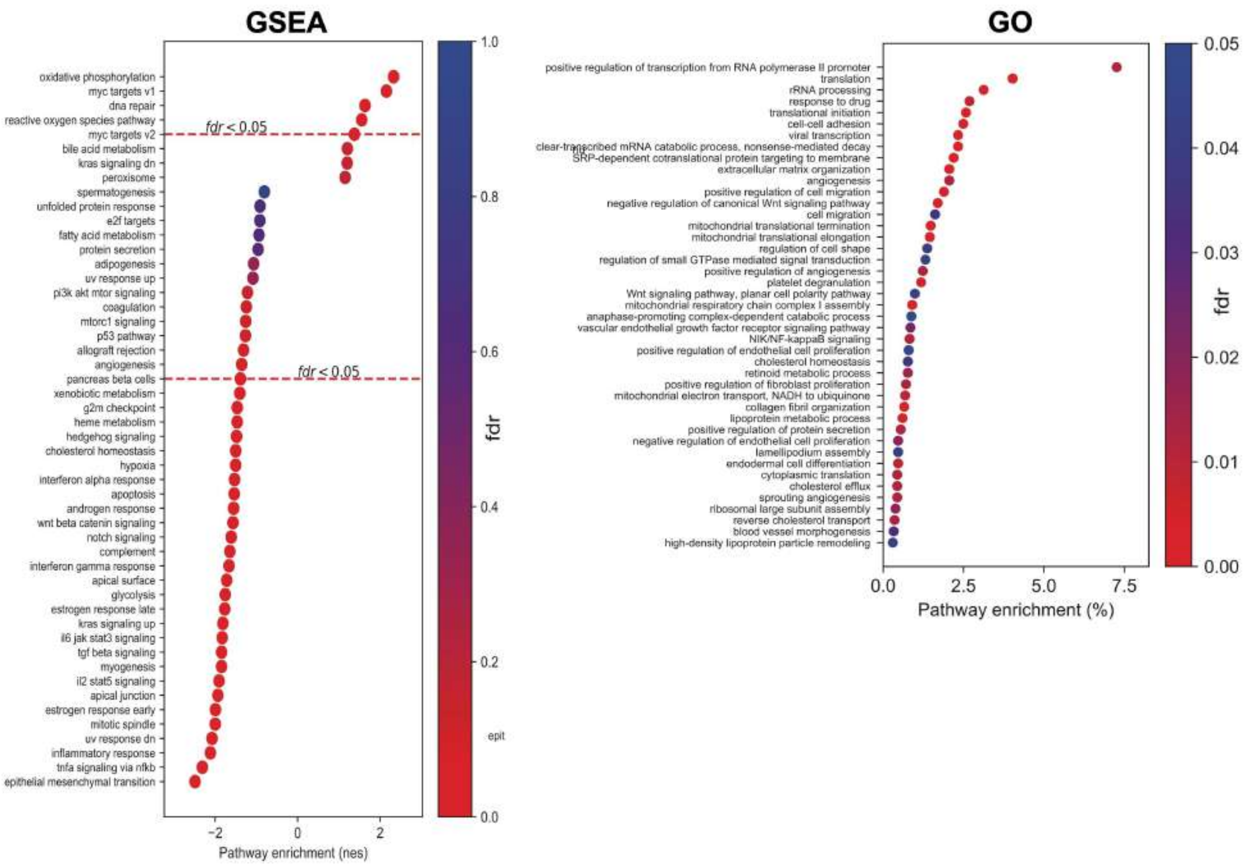

Figure 22 – Gene set enrichment analysis (GSEA) of DEGs in HT-29 tumors with CD44 KD

Importantly, in the case of E-cadherin, encoded by *CDH1*, protein-level analysis by immunohistochemical staining revealed an increase in its expression in the paranecrotic regions of primary tumors with CD44 KD (Figure 23). Notably, these same regions in control tumors exhibited more intense CD44 staining (see Figure 18). The elevated E-cadherin levels suggest a more epithelial-like phenotype in tumors lacking CD44 expression.

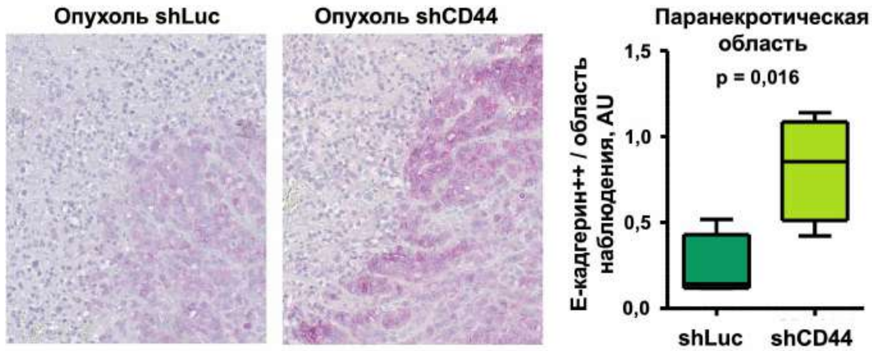

Figure 23 – Comparison of E-cadherin expression in primary xenograft tumors of the control group (shLuc) versus the CD44 KD group (shCD44)

Proteome analysis of HT-29 shCD44 tumors identified statistically significant changes (≥1.5-fold) in the expression of 415 proteins, out of a total of 2,403 detected proteins. As expected, the tumors with CD44 KD showed a 13.9-fold reduction in the level of human CD44 protein, whereas the level of murine CD44 protein remained unchanged. GSEA analysis of the differentially expressed proteins revealed many of the same pathways previously identified in the transcriptomic analysis, including EMT and OxPhos.

Notably, there was a high correlation between mRNA and protein expression changes in HT-29 shCD44 tumors compared to controls, as determined by transcriptome and proteome data (Figure 24). The overlap between differentially expressed proteins and genes identified 130 shared entries.

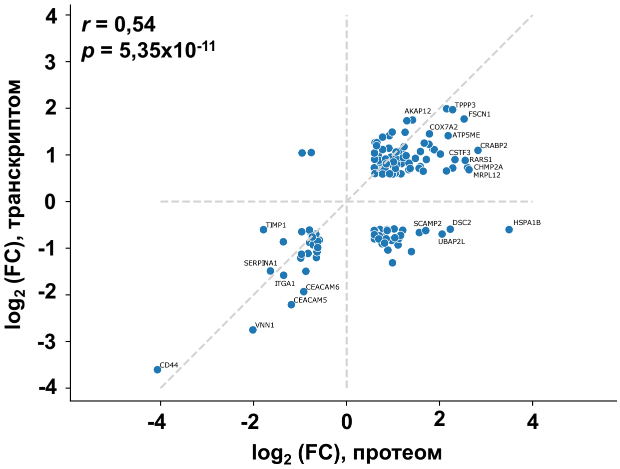

Figure 24 – Correlation of changes in mRNA and protein expression in HT-29 shCD44 tumors compared to HT-29 shLuc tumors (based on transcriptome and proteome data)

It is well established that EMT can contribute to the acquisition of stem-like properties by tumor cells. Notably, in tumors with CD44 KD, a decrease in the expression of several intestinal stem cell (ISC) and CSC markers was detected, including *LRG5*, *PROM1*, *ALCAM*, *KLF4*, *ITGA6*, *CEACAM5*, and *CEACAM6*. Among these, *KLF4*, *CEACAM5*, and *CEACAM6* are also known to be associated with EMT. The expression of CEACAM5, CEACAM6, and ITGA6 was also reduced at the protein level, as confirmed by the proteomic analysis. A decrease in CEACAM5 expression in the paranecrotic regions of xenograft tumors following CD44 KD was further confirmed by immunohistochemistry (Figure 25).

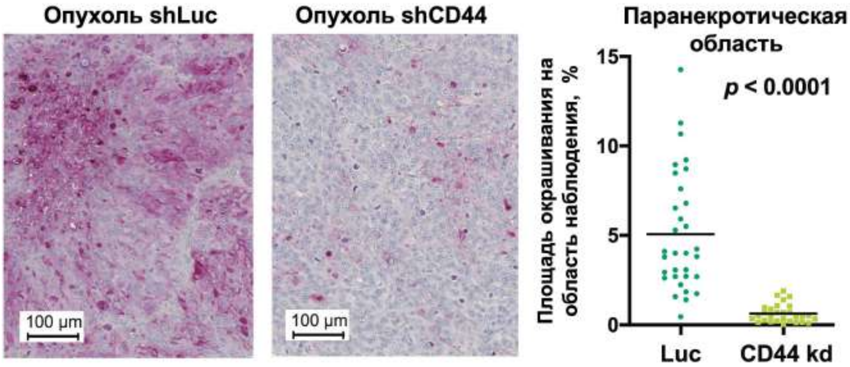

Figure 25 – Decreased expression of CEACAM5 in the paranecrotic regions of HT-29 tumors following CD44 knockdown

In addition, reduced expression of the *NOTCH1* and *NOTCH2* receptors, as well as their ligand *JAG1*, indicated a suppression of Notch signaling activity in tumors with CD44 KD. This conclusion was further supported by a reduction in the expression of the metalloproteases *ADAM17* and *ADAM10*, which are responsible for proteolytic cleavage of Notch receptors, leading to the release of the Notch intracellular domain (NICD). The NICD then translocates to the nucleus, where it regulates the transcription of genes involved in the maintenance of intestinal stem cells.

Of particular interest was the OxPhos process, which was enriched among both differentially expressed genes and proteins. The expression of almost all genes from the OxPhos gene set (52 out of 60 genes) was upregulated in tumors with CD44 KD. Previous studies have shown that the suppression of mitochondrial gene expression correlates with EMT-associated gene expression patterns across various cancer types. Moreover, EMT has been suggested to enhance glycolysis and confer CSC-like features to tumor cells. Accordingly, the upregulation of OxPhos-related genes observed here likely reflects a reduced mesenchymal phenotype and stemness in HT-29 tumors with CD44 KD. This was also indicative of decreased hypoxia in HT-29 shCD44 tumors, which is consistent with the increased microvessel density observed in these tumors compared to the HT-29 shLuc control group (see Figure 29). The stimulation of the OxPhos pathway following CD44 KD could also imply an increased mitochondrial content in HT-29 shCD44 tumor cells. This is supported by the enrichment of mitochondrial-related pathways, as revealed by transcriptome analysis (Figure 22). Immunohistochemical analysis further confirmed an elevated level of the mitochondrial marker HSP60 in the paranecrotic regions of HT-29 shCD44 primary tumors (Figure 26).

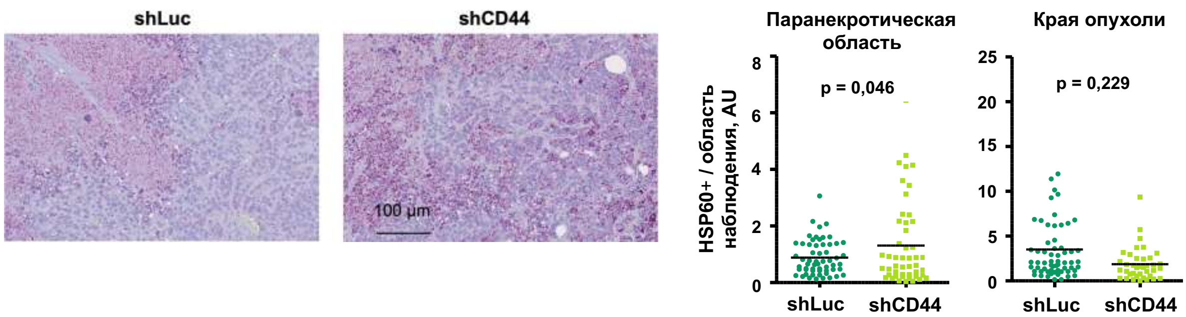

Figure 26 – Increased expression of the mitochondrial marker HSP60 in the paranecrotic region of primary HT-29 shCD44 tumors

Subsequent immunohistochemical staining of primary tumors from CRC patients demonstrated that high HSP60 expression was associated with longer PFS compared to patients whose tumors exhibited low HSP60 expression (Figure 27).

Figure 27 – PFS of CRC patients with high and low HSP60 expression levels in primary tumors

Thus, transcriptomic and proteomic data collectively indicate that CD44 KD is associated with a less pronounced mesenchymal phenotype and reduced stem-like properties in primary HT-29 tumor cells.

Reduction of Metastasis Upon CD44 Knockdown Is Associated with Enhanced KDR Receptor Activity, Increased Microvessel Density, and Decreased Hypoxia in Paranecrotic Tumor Regions

At the next stage, the impact of CD44 KD on tyrosine kinase activity was assessed using the high-throughput PamStation microarray platform. The results demonstrated an increase in the specific activity of the receptor tyrosine kinase KDR (VEGFR-2) in the less metastatic HT-29 shCD44 tumors (Figure 28).

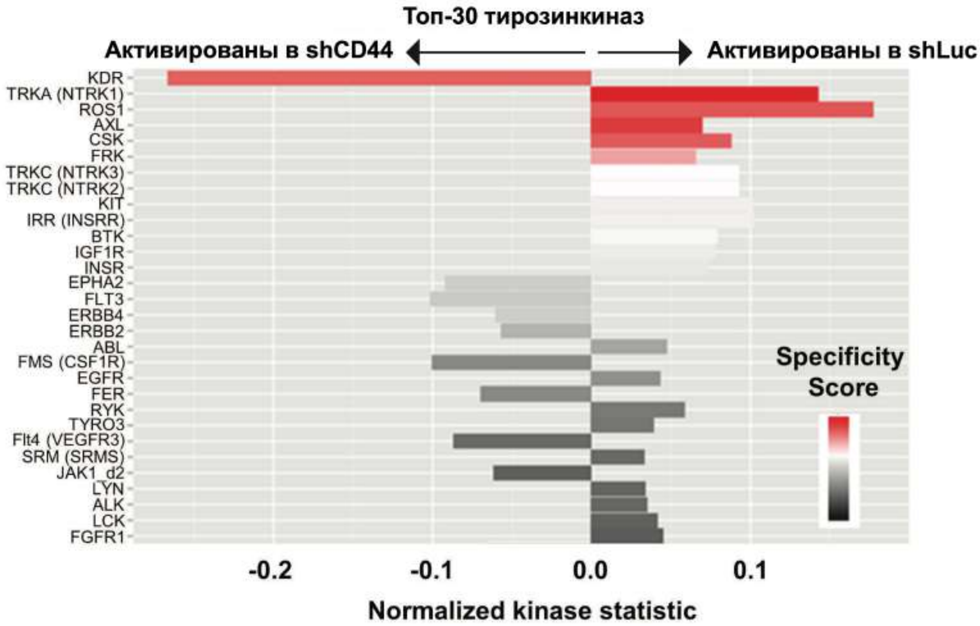

Figure 28 – Profiling of protein tyrosine kinase activity in HT-29 tumors

The observed upregulation of KDR activity was consistent with changes in angiogenesis and related processes, as identified through the analysis of enriched pathways among DEG (Figure 22). Therefore, a comparative analysis of microvessel density was subsequently performed in xenograft tumors from control animals and those bearing tumors with CD44 KD. Immunohistochemical staining of primary tumors with antibodies specific to CD31 (a marker of murine vascular endothelium) revealed an increased density of microvessels in the paranecrotic regions of HT-29 shCD44 tumors (but not in peripheral areas), compared to HT-29 shLuc control tumors (Figure 29).

This observation suggests a reduction in intratumoral hypoxia as a consequence of CD44 KD. Indeed, immunohistochemical analysis revealed a decrease in the expression of the transcription factor HIF-1α, a key regulator of the cellular response to hypoxia, in tumors following CD44 knockdown, both in paranecrotic and peripheral tumor regions (Figure 30).

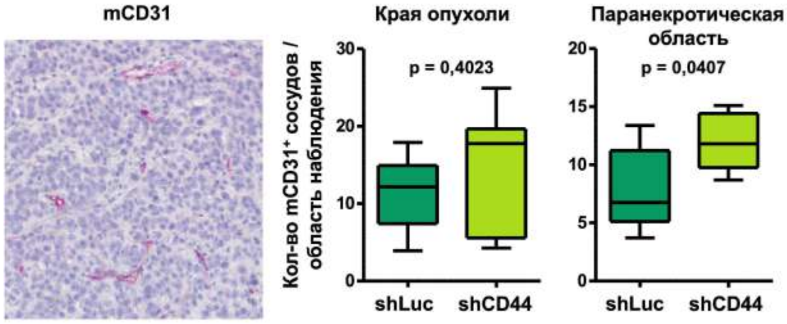

Figure 29 – Detection of vascular endothelial marker CD31 in primary HT-29 tumors

To further investigate the relationship between CD44, hypoxia, and angiogenesis, HT-29 cells were cultured under 3D conditions in a matrix containing Matrigel and hyaluronic acid. Under normoxic conditions, 3D colonies of HT-29 shCD44 released significantly less VEGF per colony area compared to 3D colonies of HT-29 shLuc (Figure 31, left panel). However, hypoxic conditions dramatically increased VEGF secretion by HT-29 shCD44 3D colonies—by 16.1-fold—whereas control HT-29 shLuc 3D colonies showed only a 2.45-fold increase in VEGF secretion (Figure 31, right panel).

To determine whether the amount of VEGF secreted by HT-29 shCD44 cells was sufficient to stimulate angiogenesis, we compared the ability of endothelial cells to form tube-like structures when cultured in conditioned media derived from 3D colonies of either HT-29 shCD44 or HT-29 shLuc cells, grown under normoxic or hypoxic conditions (Figure 32).

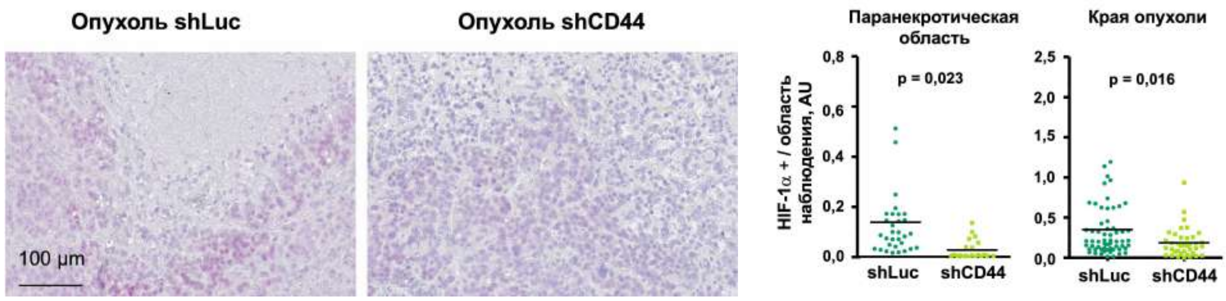

Figure 30 – HIF-1α expression in HT-29 tumors following CD44 knockdown

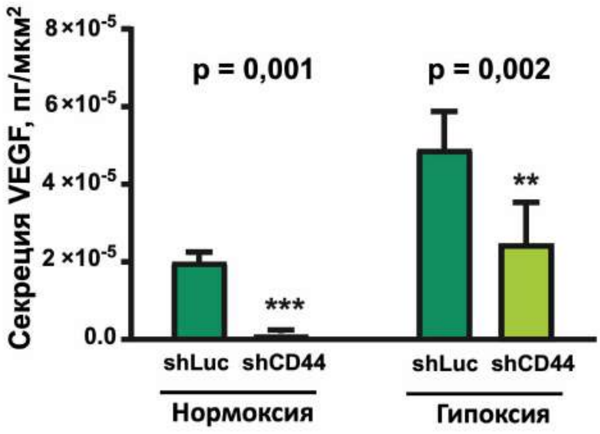

Figure 31 – VEGF secretion by 3D colonies of HT-29 shCD44 and HT-29 shLuc cells under normoxic and hypoxic conditions

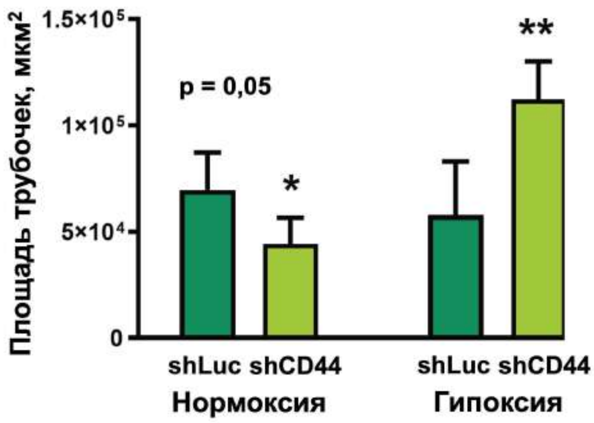

Figure 32 – Endothelial tube formation during cultivation on Matrigel in response to conditioned media from 3D colonies of HT-29 shLuc or HT-29 shCD44 cells under normoxic and hypoxic conditions

The ability of endothelial cells to form tube-like structures was slightly reduced in the conditioned media derived from HT-29 shCD44 3D colonies cultured under normoxic conditions. However, conditioned media from HT-29 shCD44 3D colonies following hypoxic cultivation significantly enhanced tube formation.

Taken together, the accumulated data suggest that one of the primary mechanisms underlying the observed changes in HT-29 tumor cell properties and the reduction in metastasis following CD44 KD is the alleviation of intratumoral hypoxia and the associated decrease in HIF-1α expression.

Transcriptomes of Patient Samples with Low Expression of CD44 Isoform 4 Overlap with Transcriptomes of HT-29 Tumors with CD44 Knockdown

Our previous analysis of TCGA data revealed that expression levels of *CD44* mRNA isoforms 3 and 4 are oppositely correlated with OAS in patients (Figure 12). Of these two isoforms, only isoform 4 was consistent with the phenotype observed in HT-29 tumors with CD44 KD; specifically, low expression of isoform 4 was associated with improved patient prognosis. Comparison of transcriptome changes in HT-29 tumors after CD44 KD with transcriptome differences observed in CRC patient tumors with high versus low expression of CD44 isoform 4 demonstrated a significant overlap in DEGs between these two datasets (Figure 33). In contrast, a similar comparison with patient tumors stratified by expression levels of CD44 isoform 3 revealed no statistically significant DEG overlap (Figure 33).

A similar trend was observed in the analysis of enriched biological pathways: low isoform 4 expression (but not isoform 3) in patients was associated with reduced EMT and increased OxPhos activity (Figure 15), which mirrors the phenotype of HT-29 xenograft tumors with total CD44 KD. These findings suggest that the *in vivo* functional effects observed following total CD44 KD are primarily driven by reduced expression of the second most abundant isoform, namely isoform 4.

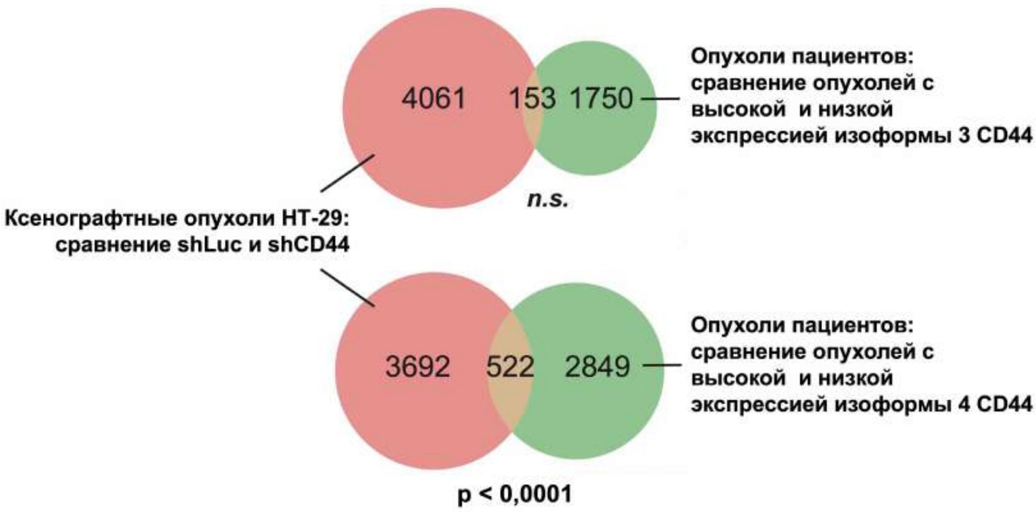

Figure 33 – Comparison of transcriptomes of HT-29 tumors and primary CRC patient tumors (TCGA)

CD44 Knockdown Alters miRNA Expression and Their Target Genes in HT-29 Tumors

To assess the potential involvement of microRNAs (miRNAs) in the regulation of molecular mechanisms contributing to the suppression of metastasis in HT-29 tumors following CD44 KD, small RNA sequencing was performed on xenograft tumor samples. Studies suggest that not only canonical miRNAs, but also their 5′-isomiRs—generated through alternative processing of miRNA precursors (pri-miRNAs and pre-miRNAs) by Drosha and Dicer nucleases—may play important biological roles. Therefore, in the present study, attention was given to both canonical miRNAs and their 5′-isomiRs. The full set of canonical and non-canonical 5′ variants is hereafter referred to as isomiRs.

Among the highly expressed isomiRs in HT-29 tumor cells, the expression of 13 isomiRs was upregulated and 8 were downregulated following CD44 KD. Notably, the majority of differentially expressed isomiRs (18 out of 21) were canonical miRNAs. Based on enrichment analysis of validated (where applicable) and predicted mRNA targets among protein-coding DEGs, seven canonical miRNAs (let-7a-5p, let-7b-5p, let-7c-5p, miR-203a-3p, miR-101-3p, miR-185-5p, and miR-125a-5p) and two 5′-isomiRs (miR-203a-3p|+1 and miR-200b-3p|+1) were identified as potential regulators of molecular pathways underlying the observed suppression of metastasis in HT-29 tumors upon CD44 KD.

Integrated analysis of potential upstream regulators of mRNA expression changes and the identified isomiRs revealed that HIF-1α, STAT3, and NF-κB may serve as key transcriptional regulators. A substantial body of literature supports a close interrelationship among the signaling and regulatory mechanisms controlled by these transcription factors.

Enrichment analysis of biological pathways associated with differentially expressed target genes of the key canonical miRNAs revealed critical processes related to tumor metastasis, including EMT and the Notch signaling pathway.

Summary of Findings on the Role of CD44 in CRC Metastasis

A summary of the findings from this study on the role of CD44 in CRC metastasis is presented in Figure 34.

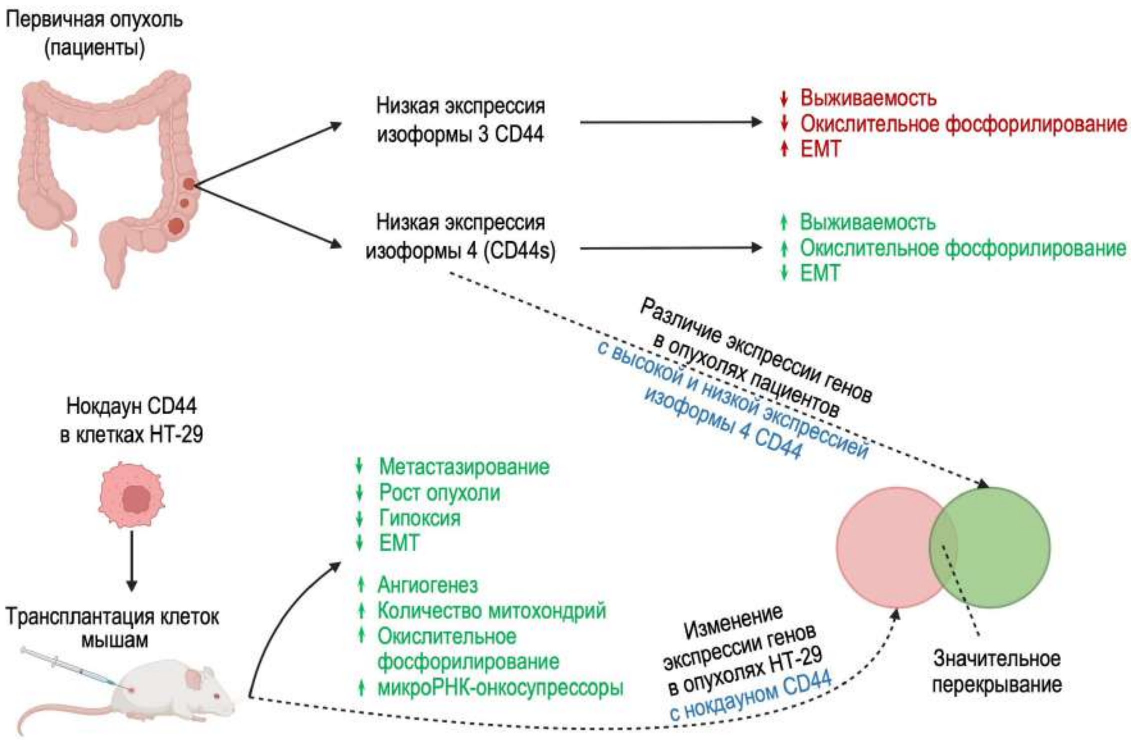

Figure 34 – Schematic Representation of the Key Findings on the Role of CD44 in CRC Metastasis

Direct evidence has been obtained showing that CD44 promotes spontaneous metastasis to distant organs by modulating the properties of cells within the primary tumor. Although CD44 isoform 3 exhibits the highest expression level in CRC tumors, transcriptomic alterations in HT-29 tumors following CD44 KD specifically overlap with gene expression changes observed in tumors from patients with high versus low expression of CD44 isoform 4, but not isoform 3. Consequently, the prognostic value of CD44 isoform 4 in CRC patients directly reflects the pro-metastatic role of CD44 observed in the *in vivo* model of spontaneous metastasis.

The data demonstrate a critical role for CD44 isoform 4 in metastasis through the regulation of tumor angiogenesis under hypoxic conditions. Reduced hypoxia, EMT, stemness, and improved mitochondrial metabolism collectively limit the metastatic potential of HT-29 xenografts. Thus, CD44 isoform 4 emerges as a potential therapeutic target for the development of clinically relevant inhibitors aimed at preventing hematogenous metastasis in colorectal cancer.

Conclusions

1. Laminin expression demonstrates prognostic significance in colorectal cancer patients. The combined prognostic value of all laminin chains exceeds that of any single chain. However, a classifier based on the combined expression of *LAMA5*, *LAMC1*, and *LAMC2* genes provides comparable prognostic performance to one constructed from the expression of all laminin chains.
2. Knockdown of the α5 laminin chain induces partial dedifferentiation of HT-29 colorectal cancer cells. The dedifferentiation process may involve activation of ER stress signaling and the Wnt pathway, along with suppression of mTORC1 complex activity. ER stress activation also increases the susceptibility of HT-29 cells to apoptotic signals and may underlie enhanced sensitivity to 5-fluorouracil.
3. High expression of CD44 isoform 3 is associated with improved overall survival in CRC patients, whereas high expression of CD44 isoform 4 correlates with worse prognosis. These two isoforms regulate largely overlapping gene sets, with over 98% of these genes being regulated in opposite directions by isoforms 3 and 4.
4. CD44 knockdown suppresses metastasis to distant organs by altering the properties of primary tumor cells. The transcriptomic profile of HT-29 xenografts with CD44 knockdown significantly overlaps with genes regulated by CD44 isoform 4—but not isoform 3—in patient tumors. Thus, the observed functional effects of total CD44 knockdown are likely mediated by suppression of isoform 4, the second most highly expressed CD44 isoform in CRC tumors.
5. CD44 knockdown improves angiogenesis, reduces hypoxia, partially suppresses EMT and stemness features, and enhances mitochondrial protein and OxPhos gene expression in primary HT-29 xenografts. These findings suggest that increased microvessel density does not necessarily promote metastasis; rather, improved vascularization may enhance tumor oxygenation, which in turn reduces metastatic potential. The results also imply a bidirectional relationship, wherein not only do hypoxia and HIF-1α induce CD44 expression, but CD44 suppression also contributes to reduced hypoxia levels.

6. CD44 knockdown alters the expression of miRNAs and their targets involved in EMT and the Notch signaling pathway. These miRNAs may play key roles in regulating the molecular mechanisms underlying the anti-metastatic effects of CD44 knockdown in colorectal tumors.

List of Publications Related to the Dissertation

The results of this dissertation have been published in 30 peer-reviewed scientific articles indexed in the Web of Science and Scopus databases. Of these, 16 articles have been published in journals included in List A of the Higher School of Economics (HSE).

1) **Maltseva D.V.**, Khaustova N.A., Fedotov N.N., Matveeva E.O., Lebedev A.E., Shkurnikov M.U., Galatenko V.V., Schumacher U., Tonevitsky A.G. High-throughput identification of reference genes for research and clinical RT-qPCR analysis of breast cancer samples // Journal of clinical bioinformatics. 2013. Т. 3. № 1. С. 13.
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