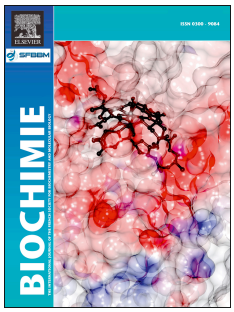

Knockdown of the α5 laminin chain affects differentiation of colorectal cancer cells and their sensitivity to chemotherapy

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PII: S0300-9084(20)30086-9

DOI: https://doi.org/10.1016/j.biochi.2020.04.016

Reference: BIOCHI 5878

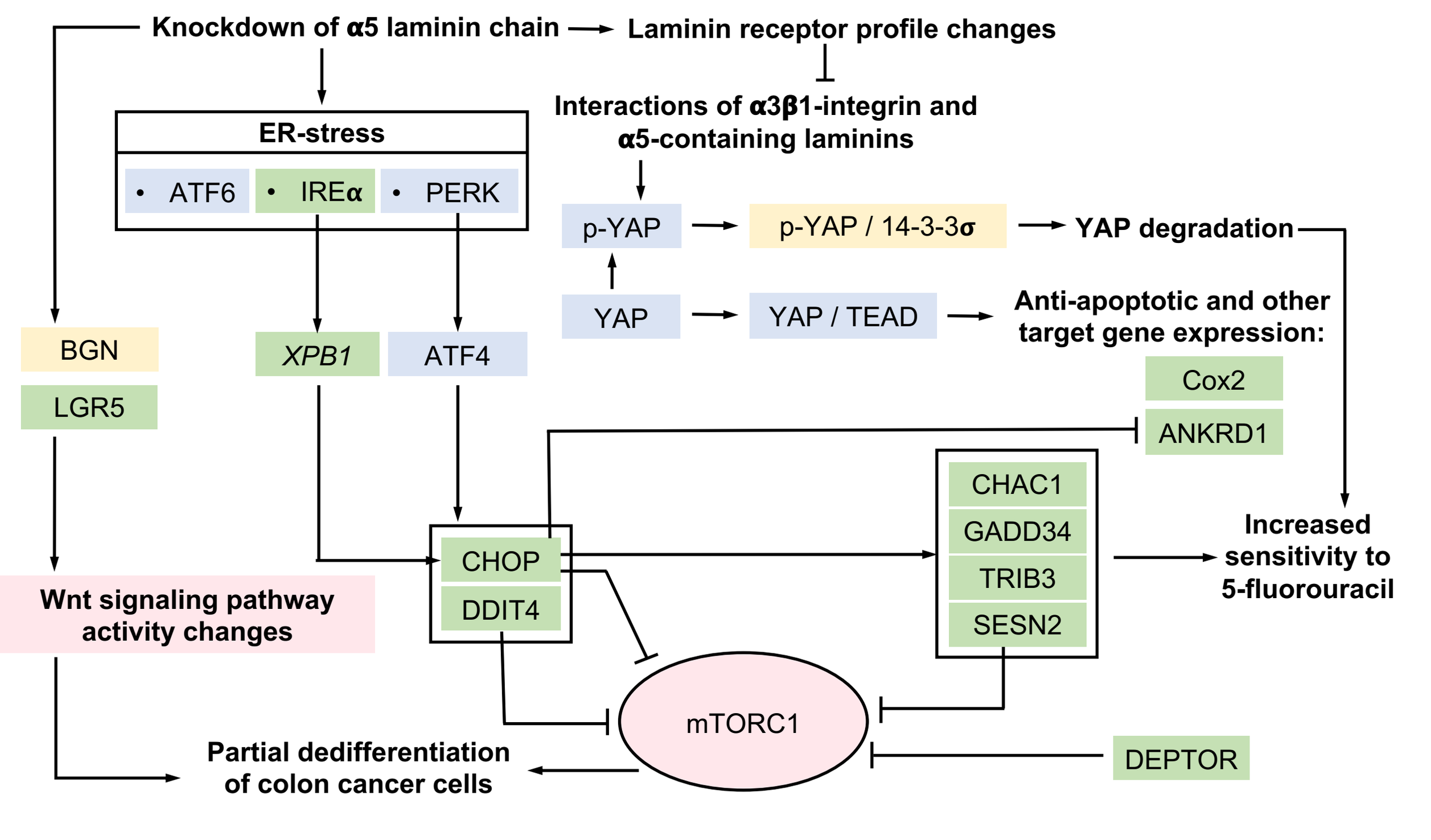
To appear in: *Biochimie*

Received Date: 26 January 2020

Revised Date: 13 April 2020

Accepted Date: 14 April 2020

Please cite this article as: D. Maltseva, M. Raygorodskaya, E. Knyazev, V. Zgoda, O. Tikhonova, S. Zaidi, S. Nikulin, A. Baranova, A. Turchinovich, S. Rodin, A. Tonevitsky, Knockdown of the α5 laminin chain affects differentiation of colorectal cancer cells and their sensitivity to chemotherapy, *Biochimie* (2020), doi: https://doi.org/10.1016/j.biochi.2020.04.016.


Knockdown of α5 laminin chain
Laminin receptor profile changes
Interactions of α3β1-integrin and α5-containing laminins
ER-stress
• ATF6
• IREα
• PERK
p-YAP
p-YAP / 14-3-3σ
YAP degradation
YAP
YAP / TEAD
Anti-apoptotic and other target gene expression:
BGN
LGR5
XPB1
ATF4
Cox2
ANKRD1
CHAC1
GADD34
TRIB3
SESN2
CHOP
DDIT4
Increased sensitivity to 5-fluorouracil
Wnt signaling pathway activity changes
mTORC1
DEPTOR
Partial dedifferentiation of colon cancer cells

Knockdown of the α5 laminin chain affects differentiation of colorectal cancer cells and their sensitivity to chemotherapy

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Abstract

The interaction of tumor cells with the extracellular matrix (ECM) may affect the rate of cancer progression and metastasis. One of the major components of ECM are laminins, the heterotrimeric glycoproteins consisting of α-, β-, and γ-chains (αβγ). Laminins interact with their cell surface receptors and, thus, regulate multiple cellular processes. In this work, we demonstrate that shRNA-mediated knockdown of the α5 laminin chain results in Wnt- and mTORC1-dependent partial dedifferentiation of colorectal cancer cells. Furthermore, we showed that this dedifferentiation involved activation of ER-stress signaling, pathway promoting the sensitivity of cells to 5-fluorouracil.

Keywords: colorectal cancer; laminin; dedifferentiation; intestinal epithelium stem cell marker; HT29; *LAMA5*

Abbreviations

ECM	Extracellular matrix
ER-stress	Endoplasmic reticulum stress
EMT	Epithelial to mesenchymal transition
FBS	Fetal bovine serum
hPSC	Human pluripotent stem cell
HRP	Horseradish peroxidase
LAMA5	Gene encoding α5 laminin chain
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
qPCR	Quantity Polymerase Chain Reaction
shCtrl	shRNA with scrambled sequence
shLAMA5	shRNA to *LAMA5* gene

1. Introduction

Laminins is a family of cell adhesion glycoproteins capable to assemble into αβγ trimers, which are major components of the basement membranes [1]. The interaction of laminins with cells is mediated mainly by binding of C-terminal globular domains of their α chains to various laminin-specific cell surface receptors, including integrins, Lutheran protein, 67 kDa laminin receptor, α-dystroglycan and some others, which leads to transmitting downstream signals relevant for the maintenance of both normal and tumor cells functioning. Besides, laminins play various roles in certain stages of the metastasis process [2]. Moreover, for many tumor types, the expression levels of individual laminin chains have been shown to carry prognostic significance [3]. For instance, in case of colorectal cancer, an increase in the ratio of expression levels of genes encoding α4 and α5 laminin chains (*LAMA4* / *LAMA5*) was associated with a poor prognosis [4]. Importantly, the changes in the laminin composition may both affect physical properties of basement membrane (for example, by forming denser and more rigid polymer "network" due to increased content of the α5 chain [2]), and tumor cell properties due to formation of more tight intercellular contacts [5]. Finally, changes in the expression profile of laminin chains have been also observed during epithelial-mesenchymal transition (EMT) [2,6,7]. Accordingly, α5 laminin chain has been previously shown to participate in the morphogenesis and differentiation of the epithelium in mouse small intestine [8].

Under standard culture conditions, human colorectal cancer cells HT29 are non-polarized, but carry a potential to differentiate. These cells form so-called "flat-foci" characterized predominantly by the epithelial phenotype, although some mesenchymal traits are also preserved [9]. Another feature of HT29 line is its heterogeneity, since it is comprised of cells producing a mucin-like matrix, as well as other the cells capable to differentiate into enterocytes of the small intestine [10]. Interestingly, the differentiation of HT29 cells is reversible, indicating their plasticity (the ability to change properties along the axis of "undifferentiated — fully differentiated cells") - the property which contributes to metastatic spread of tumor cells [11–13]. Extracellular matrix (ECM) plays a substantial role in this process [14–16].

In this work, we aimed to investigate the effects of α5 laminin chain knockdown in HT29 colon cancer cells and observed the changes in the transcriptome and proteome profiles pointing at their partial dedifferentiation after the α5 laminin chain knockdown which was accompanied by an increase in the sensitivity to 5-fluorouracil.

2. Experimental

2.1. Cell culture and generation of LAMA5 knockdown HT-29 cells

The routine culturing of the colorectal adenocarcinoma HT-29 cells as well as cells transduced by lentiviral particles containing either *LAMA5* shRNA or control scrambled shRNA was

performed as described previously [7]. The HT-29 cells were additionally tested for mycoplasma contamination using the MycoReport PCR kit (Evrogen, Russia). DNA oligonucleotides containing the *LAMA5* shRNA sequences (shLAMA5) flanked by BamHI and EcoRI sites (Table 1) were chemically synthesized by Evrogen (Russia). The shRNA having the scrambled sequence was used as negative control (shCtrl).

Table 1

shRNA sequences.

shRNA	Sequence
shLAMA5#1	5'-GATCCGCCTACGTCCTCATCAAGTTTCTCGAGAAACTTGATGAGGACGTAGGCTTTTTG
shLAMA5#2	5'-GATCCCCCTGGATAAATCCTATGACTCTCGAGAGTCATAGGATTTATCCAGGGTTTTTG
shLAMA5#3	5'-GATCCACTGGATCAGGCTGACTATTTCTCGAGAAATAGTCAGCCTGATCCAGTTTTTTG
shCtrl	5'-GATCCCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTTG

Next, double-stranded DNA fragments were cloned into the lentiviral pGPV vector containing CopGFP sequence (System Biosciences) using BamHI and EcoRI sites, and the obtained nucleotide composition was confirmed by Sanger sequencing. Each of the obtained lentiviral vectors was mixed with helper plasmids encoding viral structural proteins as well as glycoprotein of the envelope of vesicular stomatitis virus (VSV-G) and used for the lentiviral particle production in HEK293T cells. The HEK293T cells were cultivated in DMEM medium (PanEco, Russia) supplemented with 10% FBS (BioSera) and penicillin (50 IU/ml) and streptomycin (50 μg/ml). The transfection was performed using FuGene6 reagent (Promega) according to the manufacturer's protocol. The culture medium containing lentiviral particles was collected 48h after transfection, centrifuged at 300 g and filtered through 0.45 μm Millipore filter. The lentiviral preparations were titrated by serial dilutions of the concentrated vector stocks on HeLa cells (maintained DMEM with 10% FCS) in 24-well plates. The number of GFP-positive cells was analyzed 72 h post-transduction. To generate clones with stable expression of shRNAs, HT-29 cells (maintained McCoy's medium (Gibco) with 10% FCS) were seeded at 1 x 10^5 cells per well in 6-well plates and transduced after 24 hours with each type of GPV lentiviral particles in the volume corresponding to a multiplicity of infection of 10 particles per cell. The CopGFP expression levels were measured using Sony SH800S Cell Sorter (Sony) 3 days after transduction. The efficiency of the transduction was at least 90%.

2.2. Cell proliferation assay

Cell proliferation rates were analyzed using the MTT reagent (Sigma) during cultivation on plastic plates or on surfaces covered with laminins 332, 411, or 511 as described in [7] and [17]. Specifically, cells were seeded into 96-well-plates at 1×10^4 cells in 100 μl per well (8 replicates per cell line), and quantified 48 h later by adding 10 μl of MTT solution (5 mg/ml in DPBS) to each well

and incubating for 4 h at 37°C and 5% CO_2. Then, the medium was completely removed from the wells and the formazan salt was dissolved in 150 µl of DMSO. Absorption measurements were carried out on SpectroMax iD3 Multi-Mode Microplate Readers at a wavelength of 570 nm. The data presented are the results of three independent experiments. The statistical analysis was performed using publicly available on-line Mann-Whitney U Test Calculator (https://www.socscistatistics.com/tests/mannwhitney/default2.aspx).

2.3. Cell migration assay

The wells of the 96-well plates (Sarstedt, Germany) were coated with one of the laminins 332, 411, and 511 as described in [17]. The wells without coating were used as a control of cell motility on plastic. Next, the stoppers of the Oris Cell Migration Kit (PlatypusTechnologies, USA) were inserted into the wells and the cells (5×10^4 in 100 µl) were seeded into wells (8 replicates per cell line) coated with one of the laminins or without coating. After letting cells to attach for 16 h, the stoppers were removed from the wells and first images of the cells on the bottom of wells were taken. Then cells were allowed to migrate into the clear field after removal of the stoppers. The cells on the bottom of wells were photographed at 48 h time points. The pre-migration and post-migration images were analyzed using the ImageJ software (https://imagej.nih.gov/ij/). The statistical analysis was performed using publicly available on-line Mann-Whitney U Test Calculator https://www.socscistatistics.com/tests/mannwhitney/default2.aspx.

2.4. RNA isolation and qPCR analysis of gene expression

The cells were cultivated in 25 cm^2 flasks for adhesive cultures to achieve 80% confluency. Afterwards, the cells were treated by 0.25% trypsin-EDTA solution with Hanks salts (PanEco) and seeded into new flasks at the ratio of 1:4. The culture medium was removed after 72 hours, the cells were washed thoroughly with DPBS (Gibco) and lyzed in Qiazol Lysis Buffer (Qiagen) and the total RNA was isolated using miRNeasy mini kit (Qiagen) according to the manufacturer protocol including DNase I treatment step. The RNA quantity assessment and quality control were performed as described in [18,19]. The RNA integrity number (RIN) values were higher than 9.0. The isolated RNA samples were used for qPCR and microarray gene expression analysis. The reverse transcription and qPCR analysis were performed as described in [20]. Genes *ACTB*, *EEF1A1*, and *HUWE1* were used as the references for normalization as described in [7,9]. The sequences of primers used to evaluate of the expression of *LAMA5* gene was described in [7]. The sequences of primers used to evaluate the expression of other genes are shown in Table 2.

Table 2

The sequences of oligodeoxynucleotide primers used for qPCR and the values of qPCR efficiency

Gene	Primer Sequence	qPCR efficiency
ANXA13	f-5′-CAAGCAGTTACGAGCCACCTTTCA-3′ r-5′-CTCCTCATCGGTCCCCGCAC-3′	2.08±0.10
CDH1	f-5′-CGACACCCGATTCAAAGTGG-3′ r-5′-TCCCAGGCGTAGACCAAGAA-3′	1.97±0.10
KRT20	f-5′-CTCCTCATCGGTCCCCGCAC-3′ r-5′-ACACGACCTTGCCATCCACTACT-3′	1.91±0.09
MUC13	f-5′-AGACTGCGGATGACTGCCTCA-3′ r-5′-CATTGCTTGTGCTGTGCGTTGC-3′	1.88±0.07
SI	f-5′-AGCAGTTCTTATGGGGTCCAGCA-3′ r-5′-CGAGCATTGGGGACGTAGGC-3′	2.10±0.10
SOX4	f-5′-ATGACCCGAGAACCCCGTTGG-3′ r-5′-TGACCGTGAACCCCCTTCCA-3′	2.01±0.10
VEGFA	f-5′-TGGCAGAAGGAGGAGGGCAG-3′ r-5′-AGGGGCACACAGGATGGCTT-3′	2.02±0.07

2.5. In-Cell ELISA analysis of laminin α5 chain expression

HT-29 cells transduced by lentiviral particles with plasmids encoding shCtrl and shALAM5#3 were seeded at a density of 3×10^4 cells per well into both standard transparent 96-well plates and white plates for a luminescence assay (Corning). After 48 h in culture (at 37°C and 5% CO_2) the cells were fixed by incubation with a mix of 1% PFA and 0.1% of glutaraldehyde in 1x PBS (Gibco) for 30 min. After three-time washing in 1x PBS cells in the white plate were permeabilized with 1% Triton X-100 (Panreac) diluted in 1x PBS for 30 min. Afterwards, the solution in wells was changed to PBST-BSA buffer (1x PBS, 0.05% Tween20 (Panreac), 0.1% BSA (Fermentas)), incubated for 1 h. The primary antibodies to laminin α5 chain (Atlas Antibodies, AMAb91124) were diluted with PBST-BSA in a 1:300 ratio and incubated with cells overnight at 4°C. After thorough three times washing of wells with PBST buffer (1x PBS, 0,05% Tween20) the conjugate of goat anti-mouse secondary antibodies with HRP (Sigma) were immediately added to the cells at 1:1000 dilution and incubated for 2 h at 37°C. Finally, the wells were washed five times with PBST before the luminol solution (Bio-Rad) was added. The level of the laminin α5 chain expression was estimated using SpectraMax i3x (Molecular Devices). The cells in the standard transparent plate were used for the number of cell estimation using a 0.1% Crystal Violet solution (Sigma).

2.6. Microarray gene expression analysis

The microarray experiments were performed according to the manufacturer's instructions (TermoFisher Scientific UserGuide P/N 703174) and as described in [14,21]. Procedures for cDNA

synthesis and labeling were carried out according to the GeneChip WT PLUS Reagent Kit (Applied Biosystems) protocol using 500 ng of total RNA as the starting material. Target DNA fragmentation, labeling, hybridization on Affymetrix Gene Chip Human Transcriptome Array 2.0 microarrays, array washing, staining, and scanning were performed as described in [22,23]. The raw microarray data were processed using the Transcriptome Analysis Console 3.0.

The raw microarray data (CEL-files) are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7754. The processed microarray data are presented in the supplementary table (Table S1).

GSEA analyses were performed in the Pathway Studio environment (www.pathwaystudio.com, accessed in Jan 2020).

2.7. Proteome analysis

The cells were growing in 25 cm^2 flasks were allowed to reach 80% confluency. Afterwards, the cells were treated by 0.25% trypsin-EDTA solution with Hanks salts (PanEco) and seed in new flasks in the ratio of 1:4. After 72 h the culture medium was taken away, cells (about 1×10^7) were washed thoroughly in DPBS (Gibco) and lysed in 1 ml of 4% SDS and 0.1 M DTT in 0.1 M Tris- HCl (pH 7.6) at room temperature and briefly sonicated to reduce the viscosity of the lysate. Total protein content in samples was measured according to the BCA method [24]. A total protein amount of 100 μg for each sample was used for tryptic digestion according to the common FASP protocol [25]. Briefly, detergents in the samples were exchanged with 100 mM Tris-HCl (pH 8.5) using Microcon filters (10 kDa cut off, Millipore, Bedford, MA, USA). Protein disulfide bridges were reduced with 100 mM 1,4-dithiothreitol in 100 mM Tris-HCl (pH 8.5), alkylation of thiols was performed with 55 mM iodoacetamide in 8 M urea/100 mM Tris-HCl (pH 8.5). Tryptic digestion with trypsin (Sequencing Grade Modified, Promega, Madison, WI, USA) to protein ratio of 1:100 was carried out overnight at 37 °C in a 50 mM tetraethylammonium bicarbonate (pH 8.5). To obtain the peptide solution the filter samples were centrifuged at 11000 g for 15 min in a thermostated centrifuge at 20 °C. The filters were then washed with 50 μL of 30% formic acid solution by centrifugation at 11000 g for 15 min in a thermostat centrifuge at 20 °C. The filtrates were dried in a vacuum concentrator and dissolved in 20 μl of 5% formic acid for subsequent mass-spectrometry.

One microgram of peptides in a volume of 1 μl was loaded directly onto the 15-cm long C18 column (Acclaim® PepMap™ RSLC inner diameter of 75 μm, Thermo Fisher Scientific, Rockwell, IL, USA) at a flow rate of 0.3 μL/min for 12 min in an isocratic mode of Mobile Phase C (2% acetonitrile, 0.1% formic acid). Then the peptides were separated with high-performance liquid chromatography (HPLC, Ultimate 3000 Nano LC System, Thermo Scientific, Rockwell, IL, USA) in a 15-cm long C18 column (Acclaim® PepMap™ RSLC inner diameter of 75 μm, Thermo Fisher Scientific, Rockwell, IL, USA). The peptides were eluted with a gradient of buffer B (80% acetonitrile, 0.1% formic acid) at a flow rate of 0.3 μL/min. Total run time was 130 minutes, which

included initial 12 min of column equilibration to buffer A (0.1% formic acid), then a gradient from 5 to 35% of buffer B over 95 min, then 6 min to reach 99% of buffer B, flushing 10 min with 99% of buffer B and 7 min re-equilibration to buffer A.

MS analysis was performed at least in triplicate with a Q Exactive HF-X mass spectrometer (Q Exactive HF-X Hybrid Quadrupole-OrbitrapTM Mass spectrometer, Thermo Fisher Scientific, Rockwell, IL, USA). The temperature of the capillary was 240°C and the voltage at the emitter was 2.1 kV. Mass spectra were acquired at a resolution of 120,000 (MS) in a range of 300−1500 *m/z*. Tandem mass spectra of fragments were acquired at a resolution of 15,000 (MS/MS) in the range from 140 *m/z* to m/z value determined by a charge state of the precursor, but no more than 2000 *m/z*. The maximum integration time was 50 ms and 110 ms for precursor and fragment ions, correspondently. AGC target for precursor and fragment ions were set to 1×10^6 and 2×10^5, correspondently. An isolation intensity threshold of 50,000 counts was determined for precursor's selection and up to the top 20 precursors were chosen for fragmentation with high-energy collisional dissociation (HCD) at 29 NCE. Precursors with a charge state of +1 and more than +5 were rejected and all measured precursors were dynamically excluded from triggering of a subsequent MS/MS for 20 s.

The raw data were analyzed with MaxQuant 1.6 [26]. Peptides were identified using the UniProt human reference proteome (Proteome ID UP000005640) [27]. iBAQ algorithm was used to quantify the protein content [28]. Subsequent data processing was performed using Perseus 1.6 [29]. The data were filtered, log2-transformed, median normalized and missing values were imputed from a normal distribution. Statistical data processing was performed using the programming language R 3.5 with the integrated development environment RStudio 1.1. To determine the statistical significance of the observed differences, t-test with FDR correction for multiple comparisons was used. The processed proteome data are presented in the supplementary table (Table S2). The number of detected proteins was 1400.

2.8. Cell survival under treatment with 5-fluouracil

The measurements of the cytotoxic effect of 5-fluorouracil on control cell line HT29-shCtral and the cell line with *LAMA5* knockdown HT29-shLAMA5#3 during cultivation on plastic and on laminin 521 were carried out as described in [17] with some modifications. Cells were seeded into 96-well plates coated with laminin 521 and without coating at 5000 cells per well (in 100 µl of culture medium) and incubated at + 37°C in a CO_2 incubator for 24 h. Then 10 µl of culture medium containing 5-fluorouracil was added in each well so that the final concentration of the chemotherapeutic agent was 0, 0.1, 1, 4, 10, 40, 100, 250, 500 and 1000 µM. Cells were incubated at +37°C in a CO_2-incubator for 120 h. After 72 h the medium was taken from the wells, replaced with a portion of fresh one and 10 µl of 5-fluorouracil solution at the same concentration was immediately added. Cell viability was assessed using MTT reagent (Sigma). To this end, 10 µl of

MTT solution (5 mg/ml) in DPBS was added to each well and incubated for 4 h. Then, the medium was completely taken from the wells and the formazan salt was dissolved in 150 µl of DMSO. Absorption measurements were carried out on SpectroMax iD3 Multi-Mode Microplate Readers at a wavelength of 570 nm. The data presented are the results of two independent experiments. Each concentration of 5-fluorouracil was set in five replicates on each plate. The statistical analysis of survival curves and the calculation of the doses of half-maximum inhibition of cell viability (IC_{50}) were performed as described in [30] using the statistical processing software RStudio and the *drc* software package extension [31].

3. Results

3.1. Knockdown of LAMA5 gene decrease the proliferation of HT29 cells

The HT29 cell line is a standard model in colorectal cancer research. We have transduced the HT29 cells by lentiviral constructs encoding *LAMA5* gene shRNAs. Out of three different shRNAs used (Table 1), only one (shLAMA5#3) mediated significant downregulation of *LAMA5* mRNA expression (1.7-fold decrease, Fig. 1A). To evaluate the efficiency of the *LAMA5* knockdown on a protein level, both the intracellular and basement membrane fractions of laminin α5 chain protein were quantified. In HT-29 cells treated with shLAMA5#3, a 3.8-fold decrease in total levels of laminin α5 chain protein was detected ($p < 0.001$) (Fig. 1B), without concomitant changes in cell morphology.

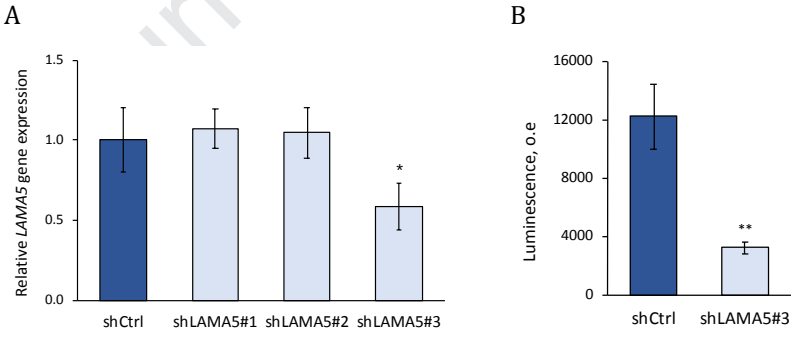

Fig. 1. A comparison of shRNA efficiency in knockdown of the *LAMA5* gene by qPCR (A) and by In-Cell ELISA (B). (A) Relative levels of *LAMA5* mRNA in shLAMA5-treated HT-29 cells as compared to shCtrl. (B) The levels of laminin α5 chain expression in HT-29 cells treated by shLAMA5#3 and by shCtrl. The mean values obtained in three independent experiments are shown. * $p = 0.012$, ** $p < 0.001$.

Furthermore, the knockdown of *LAMA5* gene led to a significant decrease in proliferation of HT29 cells ($p < 0.001$) as compared to scramble-transfected controls. Similar magnitudes (1.6-fold) of the difference in proliferation were observed when cells were grown on plastic and on various laminin substrates for a period of 48 h (Fig. 2).

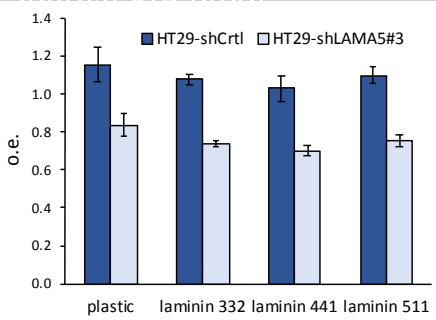

Fig. 2. The knockdown of *LAMA5* affects proliferation of HT-29 cells grown on plastic and on various laminin substrates. Experiments were repeated in three biological replicates.

3.2. The LAMA5 knockdown alters cell migration on laminin substrates

The migration activity of HT29-shCtrl and HT29-shLAMA5#3 cells were accessed by Platypus Technologies assays as described in MMs section. When growing on plastic supports, the both cell lines demonstrated similarly low motility - less than 20% of the initial clear field after removal of the stoppers, for a period of 48 h (Fig. 3). On the wells coated with laminin 332 the HT29-shCtrl cells showed up to a 1.8-fold increase in migration ($p < 0.001$) as compared to plastic without coating. While for HT29-shLAMA5#3 cells such effect was not observed (Fig. 3). Next, laminin 411 promoted only a relatively small increase in migration of the HT29-shCtrl cells (×1.5 times, $p < 0.01$) compared to plastic, while mediating no effect on cells with *LAMA5* knockdown. Interestingly, in the case of laminin 511, the impact was the opposite. Thus, HT29-shCtrl cells did not change their migration on this substrate, while HT29-shLAMA5#3 cells showed 2.8 times slower migration than on plastic without coating ($p < 0.05$). When comparing HT29-shCtrl and HT29-shLAMA5#3 lines to each other, the lower motility was observed for shLAMA5-transfected cells on laminin 332 (1.7-fold, $p < 0.001$) (Fig. 3). The cells growing on laminins 411 and 511 showed similar trends, but the results had not reached a statistical significance.

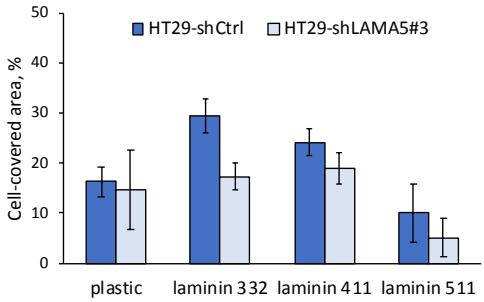

Fig. 3. The *LAMA5* knockdown decreases the cell migration on laminin substrates. The migration activity of HT29-shCtrl cells and HT29-shLAMA5#3 cells.

3.3. Gene expression profile by microarrays reveal a partial dedifferentiation of HT29 cells and an activation of the ER-stress signaling pathway

A comparative analysis of gene expression profiles in HT29-shLAMA5#3 and HT29-shCtrl cells was performed using microarrays. The obtained data indicate that the knockdown of the *LAMA5* had a relatively mild effect on HT29 cells transcriptome. Thus, a total of 56 gene probesets showed differential expression 2-fold or more (Table S1). After the removal of duplicates and non-protein coding genes, a total of 41 distinct protein-coding genes (downregulated: N=17, upregulated: N=24) were subjected to further enrichment analysis. The latter, in turn, showed that alterations of various membrane transport function were the most prominent among the affected Molecular Functions, along with a predominance of "Stress Response" and "Steroid Hormone/Glucocorticoid Response" and "Transport" centered gene lists in the most altered Biological Processes (Table S3). When the analyses of upregulated (Table S4) and downregulated (Table S5) genes were performed separately, biological processes and molecular functions performed by upregulated components were minimally perturbed when compared to that of the entire list of genes with altered expression (Table S3). Surprisingly, the central theme of Cellular Components perturbed by downregulation was "vesicle/exosome," while the Top of the lists of downregulated Biological Processes and Molecular Functions was dominated by gene lists reflecting various aspects of cellular response to external stimuli and, especially, miRNA signals (Table S5).

For the genes which altered their expression twofold or more, and also for some other interesting candidates showing smaller changes, the microarray results were verified by qPCR analysis (Table 3).

3.3.1. The impact of LAMA5 knockdown on certain cell differentiation markers

Interestingly, the *LAMA5* knockdown mediated 1.7-3.0 fold downregulation of expression levels of several markers of intestinal epithelial cell differentiation including *KRT20*, *MUC13*, *ANXA13* [32–34] (Table 3), as was confirmed by RT-qPCR. Besides, as the intensity level of probeset for enterocyte differentiation marker *SI* was below the threshold of microarray sensitivity, the expression of this gene was quantified by RT-qPCR. Marked downregulation of *SI* was detected in cells after the *LAMA5* knockdown (Table 3).

In addition, the genes important for stem cell differentiation such as *NDRG1* and *BGN* have been also found downregulated (Table 3) in 2.1 and 2.2 times, respectively. Importantly, the *NDRC1* (N-myc downstream-regulated gene 1) is known as an important regulator of cancer progression and metastasis, as well as negative regulator of the epithelial-mesenchymal transformation in colorectal cancer cells [35,36]. In addition, upregulation of NDRC1 expression in response to differentiation signals have been reported for various tumor cells [37]. Finally, the *BGN* gene encodes biglycan, a component of the extracellular matrix that can also act as a signaling molecule

[38]. By interacting with LRP6, a co-receptor of Wnt receptors biglycan activates the canonical Wnt signaling pathway [39]. Note that the Wnt signaling pathway is the most important regulator of self-renewal and maintenance of stem cell properties, the process of differentiation, the polarization of cells, and embryogenesis [40].

Table 3

Effects of the *LAMA5* knockdown in HT29 cells on gene expression.

Gene symbol	Gene expression changes, times [a]	
	Microarrays	qPCR
Markers of intestinal epithelial cell differentiation		
ANXA13	-3.0	-2.0
KRT20	-1.8	-1.4
MUC13	-1.7	-1.8
SI	nd	-2.0
Important for stem cell differentiation		
BGN	-2.2	-
NDRG1	-2.1	-
Markers of intestinal stem cells		
LRG5	1.6	-
Associated with EMT		
CDH1	-1.3	-1.4
SOX4	1.9	1.5 [b]
VEGFA	1.8	1.4 [b]

[a] A negative value means that the expression of the corresponding gene was downregulated in response to the *LAMA5* knockdown. «nd» means that gene expression is not detected by this method. "-" indicates that there is no data for this gene.

[b] $p < 0.1$

Interestingly, many genes involved in the endoplasmic reticulum stress (ER-stress) were found upregulated (Table S6) after *LAMA5* knockdown. Those included *ERN1,* which encodes one of the ER-stress sensors IRE1α, as well as *CHAC1*, *DDIT3*, *DDIT4*, *PPP1R15A*, *SESN2*, *TRIB3*, *VEGFA*, and *XBP1,* which are activated by PERK and IRE1α branches of ER-stress [41–43]. Notably, *DDIT3*, *DDIT4*, and *SESN2* genes encode proteins that inhibit the activity of the mTORC1 complex [44,45]. We also found an increase expression of one more mTORC1 inhibitors *DEPTOR*. The loss of mTORC1 activity leads to the appearance of pluripotency properties, and, conversely, the activity of the mTORC1 complex is required for cell differentiation [46]. In addition, in HT29-shLAMA5#3 cells, an increase in expression of the *LGR5* gene (Table 3), encoding the R-spondin receptor, a well-known marker of intestinal epithelial stem cells, was detected [47].

We also have drawn our attention to genes associated with EMT. However, expression changes of only a few of them were revealed (Table 3).

Thus, comparative transcriptome analysis of HT29-shLAMA5#3 and HT29-shCtrl cells points that the knockdown of the *LAMA5* may be associated with partial dedifferentiation of cells or a shift of their characteristics on the axis "stem cells - differentiated cells" to the side of stem cells. The detected activation of the ER-stress signaling pathways apparently serves as an adaptation mechanism of HT29-shLAMA5#3 cells to the absence of α5-containing laminins and helps to maintain cell viability. It is worth noting that the ER-stress plays an important role in stemness, pluripotency and development [48].

3.4. Proteome analysis supported the partial dedifferentiation of HT29 cells and revealed changes in the laminin receptor composition on the cells

Comparative proteome analysis of HT29-shLAMA5 and HT29-shCtrl cell lines revealed 87 proteins which showed at least 2-fold differential expression ($p < 0.05$). Thus, inhibition of α5 laminin chain expression had an only mild effect at the cell proteome. The intersection of the list of genes encoding differentially expressed proteins and the list of differentially expressed genes revealed six common ones. The small intersection of the lists may due to the mass spectrometric analysis allows detecting mainly the most abundant proteins in cells, whereas the most proteins encoded by differentially expressed genes are not. We cannot exclude also the importance of post-transcriptional and post-translational regulation of gene expression and protein degradation [49–51]. Nevertheless, it is important that among the differentially expressed proteins there were markers of differentiation of the intestinal epithelium annexin 13 and cytokeratin 20 encoded by the *ANXA13* and *KRT20* genes, respectively, which was downregulated by transcriptome analysis (Table 3). At the protein level the decrease was even more pronounced (11.8 times for annexin 13 and 3.4 times for cytokeratin 20).

Interestingly, we detected a 2.6-fold increase in the Lutheran protein, a specific receptor for laminins containing the α5 chain with a high affinity (K_d 7.9 nM) [52,53], in HT29-shLAMA5#3 cells. Besides, we observed a 1.8-fold increase in the α6 integrin subunit and a 1.8-fold decrease in the β4 integrin subunit which are comprised the integrins α6β4 and α6β1, two other laminin receptors [52]. Since the expression of the β1 integrin subunit was not altered after *LAMA5* knockdown, the redistribution of laminin receptors may occur yielding more α6β1 integrin on the cell surface in response to the knockdown. Note that α6β1 integrin has the highest affinity to laminin 511 containing α5 laminin chain (K_d 0.73 nM) [54].

Interestingly, it was previously shown that binding of the β1 and α6 integrin subunits is necessary to maintain a self-renewal of human pluripotent stem cell (hPSC) [55]. The formation of α6β1 integrin inhibits the signaling activity of the β1 subunit and interferes with the differentiation of hPSC.

Thus, our proteome data supports the hypothesis of dedifferentiation of HT29 cells assuming that the *LAMA5* knockdown may have caused changes in the laminin receptor composition on the cell surface as well as in the regulated intracellular signals.

3.5. Sensitivity of cells to chemotherapy treatment on plastic and laminin 521

As a part of the basement membrane, laminins provide a survival signal against death by anoikis and apoptosis [2], which may reflect in differential sensitivity to chemotherapy. Indeed, *LAMA5* knockdown resulted in a 2.2-fold increase ($p < 0.05$) in the sensitivity of HT29 cells to 5-fluorouracil (IC_{50}: 102 μM vs 224 μM, $p < 0.05$) used for colorectal cancer treatment (Fig. 4A). It is possible that this increase is due to the activation of ER-stress signaling pathways we detected in the course of the transcriptome analysis.

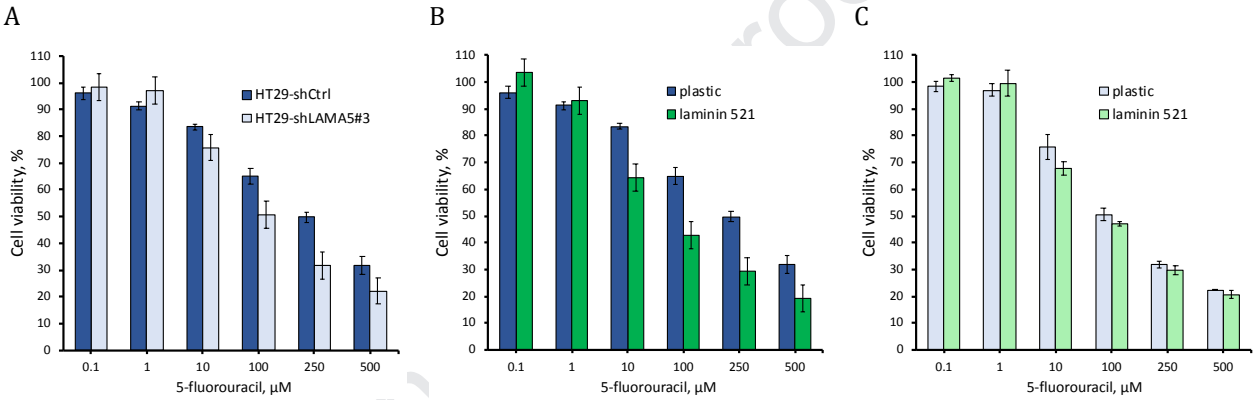

Fig. 4. The *LAMA5* knockdown increased the sensitivity of HT29 cells to 5-fluorouracil on plastic and rejected the effect of laminin 521. (A) The viability of HT29-shCtrl and HT29-shLAMA5#3 cells under 5-fluorouracil treatment on plastic. The effect of laminin 521 on the survival of HT29-shCtrl cells (B) and HT29-shLAMA5#3 cells (C).

Previously, we have shown that colorectal cell lines RKO and SW480 treated with chemotherapy agent 5-fluorouracil and regorafenib, respectively, showed significantly better survival (resistance) on laminin 521, as compared to the same cells cultured on other laminins or plastic without coating [17]. Therefore, in this work we also tested the impact of laminin 521 support on the survival of the HT29-shCtrl and HT29-shLAMA5#3 cells (Fig. 4B and C). Surprisingly, we found that control cells growing on laminin 521 had approximately 2.4-fold higher sensitivity to the 5-fluorouracil drug (IC_{50} = 92 μM), as compared to cell growing on plastic support. At the same time, shLAMA5-transformed HT29 cells did not show any substrate-dependent differences in 5-fluorouracil sensitivity.

4. Discussion

Malignant transformation of human tissues is initiated by genetic aberrations, whereas the rate of disease progression and the metastatic process highly dependent on the interaction of tumor cells with their cellular and non-cellular microenvironment [15]. In particular, tumors actively remodel ECM, which then promotes pathogenesis and compromises treatment efficacy. Further, ECM interaction with integrins elicits the signaling essential for the maintenance and differentiation of adult stem cells [15]. In many tissues, stem cell niches are dependent on specific ECM proteins, with the loss of integrin expression or ECM adhesion reducing the stem cell population [15,56]. Similarly, cancer stem cells also depend on integrin signaling and certain ECM proteins for their maintenance and self-renewal [56].

In this work, we investigated the role of laminins, the major components of the basement membrane, in the carcinogenic process. In particular, we focused on the role of laminins containing α5 chain (e.g. laminins 511, 521) in colorectal cancer. The laminin α5 chain is widely distributed in body tissues [57]. Furthermore, in the adult small intestine, there is a gradient of the α5 laminin chain expression along a crypt-villus axis with the predominant location of α5 chain in villus and absence in crypts, which are intestinal stem cell niches [58,59].

Knockdown of the *LAMA5* gene in HT29 cells led to a decrease in expression of mRNA of intestinal epithelial differentiation markers *KRT20*, *ANXA13, MUC13*, and *SI* as well as the increase in the marker of intestinal epithelial stem cells *LGR5* (Table 3). Of these, the *KRT20* and *ANXA13* were also found downregulated at the protein level by proteome analysis. Surprisingly, the transcriptome data revealed the upregulation of the set of ER-stress genes, thus, indicating the activation of ER-stress signaling pathways. ER-stress plays a dual role in the cellular response to stress conditions [60]. On the one hand, ER-stress may restore cellular homeostasis and support survival, as a part of adaptive response. Indeed, in HT29-shLAMA5#3 cells, we detected the increase in expression of *SESN2* and *ANKRD1* (Table S6), which inhibit ER stress-induced apoptosis [61,62]. On the other hand, when cellular homeostasis is significantly perturbed, the ER-stress may induce apoptosis. Along these lines, the observed increase in ER-stress contributes to an increase in sensitivity to 5-fluouracil of cells after the *LAMA5* knockdown (Fig. 4A).

Notably, the molecular cascades orchestrated by ER-stress also play a crucial role in cell stemness, pluripotency and differentiation [48]. Thus, the transcriptome analysis showed the upregulation of certain genes associated with ER-stress including *DDIT3* (CHOP protein), *DDIT4*, *SESN2* as well as *DEPTOR* (Table S6). Each of these genes encodes proteins which inhibits the activity of mTROC1 complex [44,45]. The activity of the mTORC1 is, in turn, important for stem cell differentiation [46,63]. In particular, in the intestinal epithelium, mTORC1 controls differentiation of Paneth and goblet cells [64], and its suppression promotes pluripotency [46]. In addition, mTORC1 was shown to modulate signaling cascades crosstalk between Wnt and Notch pathways [64,65], which play a critical role in differentiation of intestinal epithelial stem and progenitor cells [66]. While undifferentiated state of the intestinal cells are maintained by robust levels of Wnt

signaling, and are characterized by expression of LGR5 [47], the intestinal stem cell marker upregulated after the *LAMA5* knockdown in HT29 cells (Table 3). The binding of the LGR5 to its receptor inhibits the activity of transmembrane ubiquitin ligases ZNRF3 and RNF43, thus, contributing to the accumulation of Frizzled receptors on the cell surface [67]. We have also found an increase in expression of the *BGN* gene (Table 3), which encodes another activator of the canonical Wnt pathway [37].

The above results suggest that the knockdown of the *LAMA5* gene leads to partial dedifferentiation of HT29 by shifting their characteristics towards higher stemness. Possible molecular mechanisms underlining the changes observed in HT29 cells after the *LAMA5* knockdown are charted on Figure 5. Notably, these cell line-derived data are consistent with previous observation that knockout of the *Lama5* gene in mice impairs differentiation of the intestinal epithelium through the impairment of Wnt signaling [68].

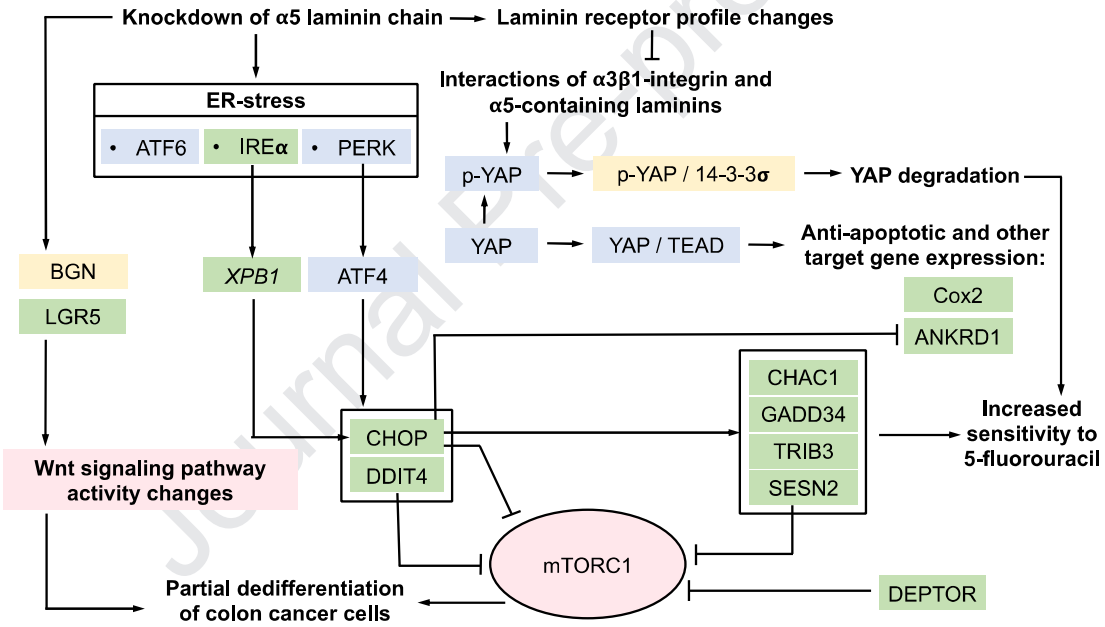

Fig. 5. Scheme of possible molecular mechanisms of partial dedifferentiation of HT29 cells after the knockdown of *LAMA5* gene. Proteins for which there is a decrease or increase in the expression of their genes are highlighted in yellow and green, respectively.

Integrins are considered the main laminin receptors on the cell surface [2]. According to the transcriptome and proteome data, both HT29-shCtrl and HT29-shLAMA5#3 cells express the following set of integrin subunits: α3, α6, β1 and β4, which is consistent with that observed in the parental HT29 cell line [9]. These integrin subunits may form laminin-specific integrin heterodimers α3β1, α6β1, and α6β4. It is important, however, that all α6 subunits expressed in HT29 cells are represented by α6β4 complexes [69]. According to our proteomics data, the *LAMA5* knockdown mediated the elevation of α6 integrin subunits levels and decrease in β4 integrin

subunit content. Since the expression of the partner β1 integrin subunit did not change, the redistribution of integrin heterodimers might likely to occur, resulting in α6β1 integrin dimers to appear on the cell surface. Interestingly, the β1 integrin subunit is evenly distributed along the intestinal crypt-villus axis [39], while both α6 and β4 subunits are enriched in crypt and villus regions, respectively. Because of that, in the small intestine, the levels of α6β1 integrin should inversely correlate with the expression of the α5 laminin chain (see above), which is consistent with the changes in integrin expression observed in response to the *LAMA5* knockdown.

Interestingly, shLAMA*5*-transformed HT29 cells had lower motility as compared to control cells, which could possibly be explained by the expression changes in laminin specific receptors. As α6β1 integrin has the highest affinity to laminin 511, an increase in its cell surface levels could promote the adhesion of HT29-shLAMA5#3 cells on laminin 511 coated surface and impede migration. Another adhesion enhancer upregulated in HT29-shLAMA5#3 cells is a product of the *CLEC3A* gene (Table S1), a C-type lectin domain family 3 member augmenting adhesion to laminin 332 [70].

The interaction of integrin α3β1 with laminins may lead to the activation of FAK, PI3K, and Akt kinases, thus, promoting inactivation of the apoptosis-associated proteins Bad and YAP [71,72] via their phosphorylation and subsequent binding to the complexes 14-3-3 ζ / p-Bad and 14-3-3 σ / p-YAP. The formation of such complexes leads to the retention of Bad and YAP proteins in the cytoplasm followed by their subsequent degradation. The genes encoding 14-3-3 ζ and 14-3-3 σ regulatory proteins were expressed both in control and shLAMA5-transformed HT29 cells (Table S1 and S2). At the protein levels, Bad and YAP could not be detected, however, the levels of their mRNA transcripts significantly differed (more than 30-fold) with the prevalence of *YAP1* in both cell lines (Table S1). It should be noted that YAP can mediate both antiapoptotic and proapoptotic activity depending on a binding partner (YAP / TEAD and YAP / p73 complexes being predominantly formed in respective regulatory pathways) [73]. Notably, in both the control and the *LAMA5* knockdown cell lines, the expression of the *TP73* gene was very subtle (if any) while the expression levels of *TEAD1* and *TEAD2* genes encoding the corresponding proteins of TEAD family were relatively high (Table S1). This observation is consistent with the anti-apoptotic role of YAP in the intestine [73]. We surmise that the increase in the sensitivity of HT29-shCtrl cells to 5-fluorouracil in the presence of laminin 521 (Fig. 4B) may rely on the following regulatory sequence: the binding of α3β1 integrins to laminin 521 leading to activation of FAK / PI3K / Akt signaling pathway, which, in turn, leads to the formation of 14-3-3 σ / p-YAP complex that inhibits YAP transport to the nucleus and blocks its anti-apoptotic effect (Fig. 5). It should be noted that other protein kinases could be also involved in the inhibitory phosphorylation of YAP [74], however their exact repertoire is to be characterized.

The *LAMA5* knockdown which leads to downregulation of *SNF* gene encoding 14-3-3 σ subunit (Table S6) and, possibly, to redistribution of β1 subunit between the integrin heterodimers,

could, in turn, facilitate nuclear YAP transport and subsequent induction of its target genes (Fig. 5) precluding the response of HT29-shLAMA5#3 to the presence of laminin 521 under 5-fluorouracil treatment (Fig. 4C). Thus, in HT29-shLAMA5#3 cells, elevation of YAP activity was indirectly evidenced by an increase in expression of YAP-target genes *ANKRD1* and *PTGS2* (Cox2 protein) (Table S6). Despite the fact that the ER-stress pathway suppresses the *ANKRD1* gene expression [75], the latter remains upregulated in HT29-shLAMA5#3 cells indicating that the redistribution of laminin receptors on the cell surface could be a factor with a relatively larger biological role.

To summarize, we showed that knockdown of the α5 laminin chain results in Wnt- and mTORC1-dependent partial dedifferentiation of colorectal cancer HT29 cells. Furthermore, this dedifferentiation involved activation of ER-stress signaling, and, possibly, an increase in susceptibility to apoptosis, which confers sensitivity of cells to 5-fluorouracil, the first-line drug of colon cancer chemotherapy. A syngeneic mouse model with subcutaneous HT29-shCtrl and HT29-shLAMA5#3 tumors could provide further insights on a possible clinical relevance of the findings.

Funding

The study was supported by the Russian Science Foundation (project 17-14-01338).

Availability of data and materials

The microarray datasets generated and analyzed during the current study are available in the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7754.

Author contributions

Conception and design of the experiments: A. Tonevitsky, S. Rodin, D. Maltseva; collection of the data: D. Maltseva, M. Raygorodskaya, E. Knyazev, V. Zgoda, O. Tikhonova; proteome data processing: S. Nikulin; analysis and interpretation of the data: D. Maltseva, S. Zaidi, A. Baranova, A. Tonevitsky; writing—original draft preparation: D. Maltseva; critically revising the article: A. Baranova, S. Rodin, A. Turchinovich, A. Tonevitsky. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

The authors gratefully acknowledge the assistance of the “Human Proteome” Core Facility (Institute of Biomedical Chemistry, Moscow, Russia) in proteomic data generation.

Appendix A. Supplementary data

Table S1: The processed microarray data.

Table S2: The processed proteome data.

Table S3: The results of enrichment analysis of entire list of differentially expressed genes.

Table S4: The results of enrichment analysis of upregulated genes.

Table S5: The results of enrichment analysis of downregulated genes.

Table S6: Differentially expressed genes associated with ER-stress, mTORC1 activity and YAP-signaling.

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- Knockdown of the α5 laminin chain induced partial dedifferentiation of HT29 cells.
- Dedifferentiation of HT29 cells was due to Wnt and mTORC1 signaling changes.
- Knockdown of the α5 laminin chain was associated with ER-stress activation.
- ER-stress facilitated sensitivity of HT29 cells to 5-fluorouracil.