Tissue-specificity of heparan sulfate biosynthetic machinery in cancer

Anastasia V Suhovskikh1,2, Natalya V Domanitskaya1, Alexandra Y Tsidulko1, Tatiana Y Prudnikova1, Vladimir I Kashuba3, and Elvira V Grigorieva1,2,3,*

1Institute of Molecular Biology and Biophysics SD RAMS; Novosibirsk, Russia; 2Novosibirsk State University; Novosibirsk, Russia; 3MTC; Karolinska Institute; Stockholm, Sweden

Keywords: biosynthesis, breast cancer, colon cancer, expression pattern, extracellular matrix, heparan sulfate, proteoglycan, prostate cancer, tissue-specificity

Heparan sulfate (HS) proteoglycans are key components of cell microenvironment and fine structure of their polysaccharide HS chains plays an important role in cell-cell interactions, adhesion, migration and signaling. It is formed on non-template basis, so, structure and functional activity of HS biosynthetic machinery is crucial for correct HS biosynthesis and post-synthetic modification. To reveal cancer-related changes in transcriptional pattern of HS biosynthetic system, the expression of HS metabolism-involved genes (EXT1/2, NDST1/2, GLCE, 3OST1/HS3ST1, SULF1/2, HPSE) in human normal (fibroblasts, PNT2) and cancer (MCF7, LNCaP, PC3, DU145, H157, H647, A549, U2020, U87, HT116, KRC/Y) cell lines and breast, prostate, colon tumors was studied. Real-time RT-PCR and Western-blot analyses revealed specific transcriptional patterns and expression levels of HS biosynthetic system both in different cell lines in vitro and cancers in vivo. Balance between transcriptional activities of elongation- and post-synthetic modification-involved genes was suggested as most informative parameter for HS biosynthetic machinery characterization. Normal human fibroblasts showed elongation-oriented HS biosynthesis, while PNT2 prostate epithelial cells had modification-oriented one. However, cancer epithelial cells demonstrated common tendency to acquire fibroblast-like elongation-oriented mode of HS biosynthetic system. Surprisingly, aggressive metastatic cancer cells (U2020, DU145, KRC/Y) retained modification-oriented HS biosynthesis similar to normal PNT2 cells, possibly enabling the cells to keep like-to-normal cell surface glycosylation pattern to escape antimetastatic control. The obtained results show the cell type-specific changes of HS-biosynthetic machinery in cancer cells in vitro and tissue-specific changes in different cancers in vivo, supporting a close involvement of HS biosynthetic system in carcinogenesis.

Introduction

Heparan sulfate proteoglycans (HSPGs) are an essential part of cell microenvironment and play important roles in cell-cell and cell-matrix communication, growth factor signaling, morphogenesis, and development.1-3 Cooperation between heparan sulfate (HS) and its core protein allows HSPGs to perform multiple functions, where in many cases HS has a critical role in cell interactions with extracellular matrix components and numerous protein ligands.4,5 Specificity of regulation of signaling by HS is provided by fine structure of HS, which exhibit a high heterogeneity in their composition, chain length, sulfation, acetylation, and epimerization patterns.6,7 The structure of HS is affected by the different developmental and pathophysiological conditions including cancer.8-10

Cancer-related changes are typically characterized by degradation of heparan sulfate (HS) chains/sulfation patterns via the endo-6-O-sulfatases (Sulf1 and Sulf2) or by heparanase, which results not just in structural but also functional consequences, which significantly impact tumor microenvironment and cancer progression.11-13

Understanding of molecular mechanisms involved in the pathological changes in HS composition and fine structure in cancer tissues is directly related to the nature of their biosynthesis and degradation. A principal difference between template-based biosynthesis (proteins, DNA, RNA) and non-template driven process of HS biosynthesis exists.14 HS biosynthesis occurs in a tissue-specific fashion and are generated by the action of a large family of enzymes involved in nucleotide sugar metabolism, polymer formation (glycosyltransferases), and chain processing (sulfotransferases and an epimerase).15 A physical complex of the enzymes, a “GAGosome,” committed to the assembly of HS, is an appealing concept, in which each enzyme provides the preferred substrate for the next reaction in the pathway. These reactions do not go to completion, and this results in a high degree of structural diversity of synthesized HS sugar sequences.6,15 So, highly coordinated and successful work of all the components of HS biosynthetic machinery is vital for HS biosynthesis and proper HS composition and structure in normal cells,16 and deranged HS biosynthesis could cause diseases.17 However, fine organization and regulation of the HS biosynthetic system in...
normal cells and tissues and their changes during malignant transformation remain unclear. An involvement of some individual HS metabolic enzymes in carcinogenesis are shown. Tumor-suppressor function is suggested for EXT family genes, \cite{18-20} NDST\cite{41} and D-glucuronyl C5-epimerase (GLCE); \cite{22-25} overexpression of heparan sulfate D-glucosaminyl 6-O-sulfotransferase-2 (HS6ST2) in colorectal cancer is associated with a poor survival, \cite{26} decreased expression of HS-3-O-sulfation (3-OST) genes is associated with chondrosarcoma progression, \cite{27} HS modifying enzymes O-sulfatases (SULF1/2) \cite{12,13} and heparanase-1 \cite{15,28,29} significantly impact cancer progression and metastasis. All the results suggest that the individual alterations in HS biosynthesis-involved genes are related to HS-associated pathology, and HS biosynthetic enzymes would be potential candidates for drug targets in various diseases. \cite{17} These individual changes seem disbalance HS metabolism and create cancer-specific patterns of HS metabolic machinery in different cancer tissues.

The results suggest that not only expression levels of individual enzymes but also their ratio and common organization of the biosynthetic machinery are important to create a functionally active “GAGosome.” In few experiments, expression levels of a number of GAG biosynthesis-involved genes were determined simultaneously. According Affimetrix microarray analysis, only 9 of 100 GAG biosynthesis-involved genes were determined simultaneously. According Affimetrix microarray analysis, only 9 of 100 GAG biosynthesis-involved genes (EXT2, CHSY3, CSGALNACT1, HS3ST2, HS2ST1, CHST11, CSGALNACT2, HPSE, SULF2) are significantly different between normal and malignant human plasma cells, while the rest genes remain unchanged; \cite{30} 6 of 15 GAG biosynthesis-involved genes are changed in neuroendocrine tumors; \cite{31} 7 of 21 key HS-synthesis-involved genes are significantly deregulated in breast cancer. \cite{32} Although the obtained results were not analyzed in terms of overall configuration of GAG-biosynthetic machinery in tumors, they support a common disbalance of the system in cancer. Complex analysis of transcriptional activity of HS biosynthetic system in normal prostate tissue and tumors showed significant down-regulation of total transcriptional activity of HS biosynthetic system and changes in overall expression patterns in prostate cancer, \cite{33} whereas in colon cancer only qualitative changes in expression patterns are detected. \cite{34} All the results suggest direct involvement of HS biosynthetic machinery in carcinogenesis and show complex changes of HS metabolic enzymes expression in tumors. However, the matter remains still poorly investigated.

The aim of this study was to investigate common expression patterns of HS biosynthesis-involved genes in different normal and cancer human cell lines and tissues using the same methodical approach, perform a comparative study and check a hypothesis about tissue-specific organization of the machinery in normal and cancer tissues.

**Results**

Total transcriptional activity of HS-biosynthetic system varies in different cancer cell lines in vitro

To analyze tissue-specificity of transcriptional activity of heparan sulfate (HS) biosynthetic system, expression of key HS biosynthesis-involved genes was examined in human normal fibroblasts and prostate epithelial cells and cancer cell lines of different morphological origin (breast, prostate, colon, kidney, lung, brain) (Fig. 1).

According to the multiplex RT–PCR data, the total transcriptional activities of HS biosynthetic system in the tested cell lines were different, with up to 3 time difference between the less and

![Figure 1. Transcriptional patterns of HS-biosynthetic system in different normal and cancer cell lines. RT–PCR analysis, intensity of the amplified DNA fragments for each gene normalized to that of GAPDH. Stacked column compares the contribution of each value to a total across categories. FB – human fibroblasts, PNT2 – normal prostate epithelial cell line.](image)
most active cell lines and overall tendency to be decreased in cancer cell lines (Fig. 1). Interestingly, normal human cells of different morphological origin (fibroblasts, FB and prostate epithelial cells, PNT2) showed different transcription patterns for HS biosynthetic system. Fibroblasts actively expressed mainly HS-elongating genes EXT1/2 with low transcriptional activities of HS-modifying genes (NDST1/2, GLCE, O-sulfotransferases) and SULF1 as main de-sulfating gene, while PNT2 cells had less active expression of EXT1/2 and 3-fold increased expression of HS-modifying genes. SULF2 was dominating sulfate in the cells. The results suggest that fibroblasts could be devoted to active biosynthesis of HS chains without their extensive post-synthetic modification. In contrast, epithelial cells seem to have less active biosynthesis of HS chains and more extensive post-synthetic modification.

For different cancer cell lines, differential expression of the individual HS biosynthesis-involved genes was observed both at the mRNA and protein levels (Fig. 1 and 2). It resulted in the cell type-specific transcriptional patterns for HS biosynthetic machinery, where quantitative changes in total transcriptional activity of the system and qualitative difference in its structure could be delineated.

From quantitative point of view, overall activity of the HS biosynthetic system had signs of tissue-specificity, although the difference was not significant. For example, average expression activity of the system in 3 different prostate cancer cell lines was 5.14 ± 1.61, while lung cancer cell lines expressed HS biosynthesis-involved genes at lower average rate (2.95 ± 0.62).

Along with the quantitative differences in overall transcriptional activity of the system, evident differences in the HS biosynthetic machinery transcriptional patterns were observed in the cancer cell lines. Main HS biosynthesis-involved genes, EXT1 and EXT2 (responsible for HS chain elongation) were most stably expressed genes, although their mRNA levels varied in a wide range, composing from 30% up to 75% of total transcriptional activity of the HS biosynthetic machinery. However, post-synthetic modification-involved genes (NDST1, NDST2, GLCE, 3OST1) were more differentially expressed in the cell lines, from active expression to almost complete absence.

The obtained results clearly show that overall transcriptional activity of HS biosynthetic system is different in cancer cell lines of different morphological origin. However, differential expression of individual HS biosynthesis-involved genes results also in cell type-specific transcriptional patterns of HS biosynthetic machinery in the cells.

**HS biosynthesis step dominates in cancer cells related to more aggressive phenotype**

To analyze the qualitative differences on the ability of cancer cells to express all key genes necessary for “GAGosome” formation further, we suggested to use a parameter of relative “contribution” of each individual component to overall “system” and depicted the expression level of each individual gene as a percentage of the total expression activity of all genes under the study (Fig. 3A). Comparative analysis of the obtained results revealed disbalance between GAG elongation step (EXT1/2) and post-synthetic modification step (NDST1/2, GLCE, 3OST1) as a most significantly changed and informative parameter, associated with functional properties of normal and cancer cells. For normal epithelial cells (PNT2), active expression of HS modification-involved genes was shown (elongation/modification ratio 1:2), although most of the studied cancer cell lines demonstrated near the equal total expression levels of elongation- (EXT1, EXT2) and modification-involved genes (NDST1/2, GLCE, 3OST1) (elongation/modification ratio 1:1) (Fig. 3B). The elongation/modification ratio disbalance in cancer cells seems to be due to both activation of expression of elongation-involved genes and downregulation of modification-involved ones (Fig. 1 and 3).

Interestingly, the specific transcriptional patterns of HS biosynthetic system and elongation/modification ratio were associated more with functional properties of the cells (proliferation rate and/or metastatic activities etc) rather than tissue of origin. For example, the aggressive metastatic cancer cells (DU145, U2020, KRC/Y) demonstrated expression patterns of HS biosynthesis-involved genes most similar to PNT2 normal epithelial cells (elongation/modification ratio 1:2), while less aggressive cancer cell lines for the same cancer types (LNCaP, PC3, H157, H647, A549) had significantly disbalanced expression patterns (activation of elongation step and suppression of modification step, ratio up to 2:1) (Fig. 3B). Comparative analysis of HS biosynthetic system in cell lines of the same origin (prostate and

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**Figure 2.** Western blot for NDST1 and GLCE proteins in different cancer cell lines. FB – human fibroblasts, PNT2 – normal prostate epithelial cell line.
lung) supported the hypothesis. Although overall transcriptional activities of the system in prostate cancer cell lines (LNCaP, PC3, DU145) did not show significant differences with those in normal PNT2 cells (Fig. 1), their expression patterns were specific for morphologically different prostate cancer cells (Fig. 3). Hormone-dependent non-metastatic LNCaP epithelial cancer cells had moderate disbalance of HS biosynthetic machinery compared with normal PNT2 cells, which was further disbalanced in hormone-independent metastatic PC3 cells and similar to that in completely different cell type such as fibroblasts. Unexpectedly, expression pattern of the system in aggressive DU145 prostate cancer cells was very similar to that in normal PNT2 cells (Fig. 3). For lung cancer cell lines, a clear difference in expression patterns between U2020 small-cell lung cancer cells and H157, H647, A549 non-small-cell lung cancer cells was shown. While all the non-small-cell lung cancer cell lines demonstrated the similar expression patterns for HS biosynthetic system, small-cell lung cancer cell line U2020 had a characteristic one similar to those in PNT2 cells (Fig. 3A).

In summary, the obtained results show that normal human fibroblasts and epithelial cells have specific total expression activities and transcriptional patterns for HS biosynthesis-involved genes. Cancer cells of different morphological origin are characterized by variable overall transcriptional activity of HS biosynthetic system (up to 3-fold difference between the cell lines) and qualitative changes in the expression patterns. Disbalance in the expression of the genes involved in different functional steps of HS biosynthesis (elongation of HS chain and its post-synthetic modification) is the most informative marker of malignant transformation, although its normal function in advanced cancers could be a predictive marker for high metastatic potential of the cells.

### Tissue specificity of HS biosynthesis system in different cancers in vivo

To check whether transcriptional activity of HS biosynthetic system is tissue-specific also in cancer tissues in vivo, breast, prostate and colon cancer clinical samples and appropriate control tissues were used for the subsequent experiment (16–24 samples for each tissue). Expression levels of the individual HS biosynthesis-involved genes and overall transcriptional patterns of HS biosynthetic machinery were determined by multiplex RT-PCR analysis with GAPDH gene as an internal control (Fig. 4).

Comparative analysis revealed relatively balanced expression levels and transcriptional patterns of HS-biosynthetic system in normal human breast, prostate and colon tissues. However, their changes in appropriate cancer tissues had tissue-specific character and lied in both significant overall suppression of HS biosynthetic machinery in prostate tumors and its formal increase (primarily because of activation of SULF1, SULF2 expression) in colon tumors. However, not taking into account the significant SULF1, SULF2 activation, overall transcriptional activity in colon tumors also showed suppression mainly due to down-regulation of NDST1, NDST2, GLCE expression. At the same time, no significant changes in HS biosynthetic system were detected in breast cancer compared with normal human breast tissue. Finally, different cancers (breast, prostate, colon) demonstrate totally different tissue-specific
transcriptional patterns and expression levels for HS biosynthetic machinery, suggesting different molecular mechanisms of deregulation of HS biosynthesis in different cancers.

**Discussion**

The main idea of the study was to take a look at the biosynthetic machinery as a whole and investigate a hypothesis about tissue-specific organization of the machinery as a basis for tissue-specific HS structure and composition in different tissues.\(^{35-37}\) Earlier, a similar idea was applied in few studies, where expression of a number of GAG biosynthesis-involved genes in different normal and cancer tissues were investigated simultaneously,\(^{30-32}\) however the obtained results were not analyzed in terms of a common expression pattern of the system.

To analyze patterns of relationship of different genes, we suggested non-conventional parameter such as a total transcriptional activity of the system consisting in the formal summation of the expression levels of the individual HS biosynthesis-involved genes. Also, an additional information was raised from the qualitative analysis of the expression pattern of the system in 100%-normalized scale. It let us estimate a complex balance between the individual HS biosynthesis-involved genes and its changes during malignant transformation.

For the first time, it was shown that all tested normal tissues (breast, prostate, colon) had similar expression levels and transcriptional patterns of HS biosynthetic system, with relatively balanced expression of the individual genes (Fig. 4).

Unfortunately, there are no direct data on this matter to compare with but the obtained result looks logical because all normal cells are intended to synthesize HS, which are essential part of any tissue. As it was noted by Bishop et al., with a few exceptions, all multicellular organisms produce HSPGs, from ancient cnidarians (*Hydra*) to modern Mammalia, and the overall structure of HS seems largely conserved, whereas the core proteins have undergone expansion in number and diversity.\(^{38}\)

An interesting aspect of the results is that the balance between key genes was maintained in all normal tissues, in spite of 1,5-fold difference in total transcriptional activities, supporting an existence of molecular mechanism tightly coordinating the expression of the HS biosynthesis-involved genes.\(^{33}\) The mechanism seems include feedback loop, where any gene is able to appropriately coordinate an expression of other gene(s). It was shown that the expression levels of exostosin proteins EXT1 and EXT2 oppositely affect the amount of glucosaminyl N-deacety-lase/N-sulfotransferase NDST1 in the cell, which, in turn, greatly influences HS structure;\(^{39}\) knock-out of sulfatase genes (Sulf) dynamically modulates the expression of the Hs2st and Hs6st heparan sulfate sulfotransferase genes resulting to the changes in HS sulfation and significant effects on fibroblast growth factor signaling;\(^{40}\) ectopic over-expression of D-glucuronyl C5-epimerase (GLCE) results in proportional up-regulation of expression of key genes and total transcriptional activation of HS-biosynthetic machinery in prostate cancer cells.\(^{33}\) The presented results support an existence of the self-regulating mechanism for the coordinated transcription of HS metabolism-involved genes.
As to cancer-related changes of the HS biosynthetic machinery, the obtained data show tissue-specific changes in total expression level and transcriptional pattern of the system in different tumors. They clearly recognize a disbalance of the system in cancer as a common trait, although the observed changes occur in a tissue-specific manner and mainly due to deregulation of post-synthetic modification-involved genes related to the HS sulfation (OST1/HS3ST1, SULF1, SULF2). It perfectly fits previous data about important contribution of SULFs to the generation of organ-specific sulfation patterns of heparan sulfate in experimental animal model; 41 SULFs expression is very variable in different cancers and is related to HS structure and growth factor signaling in the tissues. 42 The combined effects of heparanase and HSULFs could account for the lack of biologically active HS in tumor cells rather than deficiencies in the biosynthetic enzymes. 43 Possibly, do deterioration of molecular mechanisms regulating the coordination and tissue-specific pattern of HS biosynthetic system in normal cells results in heterogeneous changes in HS biosynthesis in cancer cells and microenvironment and appearance tumor-specific HS in the cells.

At this point, an unexpected moment is related to more profound changes in HS biosynthetic machinery in less aggressive cancer cell lines than in more aggressive ones (Fig. 3). From this observation, one could hypothesize that the similarity of HS biosynthetic machinery in highly aggressive metastatic cells (DU145, U2020, KRC/Y) to that in normal PNT2 cells let the cancer cells to save close-to-normal proteoglycan expression pattern at their cell surface and escape immune control therefore. The hypothesis is supported by the related data on complex and context-dependent changes in proteoglycan expression in different grade tumors. It was shown that syndecan 2 levels are very low or undetectable in normal neuroendocrine cells, increasing significantly in well-differentiated neuroendocrine tumors (NETs), and decreasing in poorly differentiated NETs. In contrast, normal neuroendocrine cells are positive for glypican 1, well-differentiated tumors have increased expression of glypican 1, but this is reduced as tumor grade increased. 31 Most of the studied genes for HS biosynthetic machinery show no changes or similar expression changes in non-metastatic or metastatic infiltrating ductal adenocarcinomas (IDCs) but sulfotransferase HS3ST5 is decreased in non-metastatic tumors and not changed in metastatic tumors, and SULF1 and SULF2 show overexpression in non-metastatic and no change in metastatic tumors. 32 All the data advocate for close association of HS biosynthetic system with tumor development and metastatic process although molecular mechanisms of the association remain unknown. Possibly, malignant cells with deteriorated HS composition at the cell surface may survive inside the tumor node only, where they do not contact with other cell types and could not be recognized and eliminated by immune system. Aggressive metastatic cancer cells (U2020, DU145, KRC/Y) maintain modification-oriented HS biosynthesis similar to normal PNT2 cells and seem have unchanged HS pattern enabling them to survive in tumor microenvironment during invasion and metastatic process.

Overall, the main results of the study support the initial hypothesis about specific HS biosynthesis organization in different cells and tissues, and show a complex mode of its changes in cancer. Possibly, there is a common molecular mechanism coordinating of the expression levels of the HS biosynthesis-involved genes in order to create functionally active HS biosynthetic machinery. Analysis of HS biosynthetic system could be a promising biomarker for personalised cancer type diagnostics and elaborating a new anticancer treatment strategy based on the correction of HS biosynthesis in cancer cells and tissues. In perspective, a special low density array/microarray could be developed or transcriptome data could be used for the analysis of deregulation of HS biosynthetic system in cells/tissues, with disbalance between elongation and modification steps as a main diagnostic parameter.

Materials and Methods

Patients and tissue samples
All tissue samples were obtained from primary tumors during radical surgery at the Central Municipal Hospital N1, Novosibirsk, Russia (breast and prostate tumors) and National Institute of Cancer Research, Kiev, Ukraine (colon tumors). Tissues were “snap-frozen” in liquid nitrogen and stored at −70°C. Regions were manually dissected from the frozen blocks to provide a consistent tumor cell content of more than 70% for analysis. The prevalent histological type of the breast tumors was duct infiltrating cancer, colon and prostate tumors - adenocarcinoma. The majority of cancer patients were at the II–III stage of malignancy progression according the TNM staging system. All patients provided written informed consent and the study protocol was approved by the Local Ethics Committees in accordance with the Helsinki Declaration of 1975.

Cell lines and cell culture
The PNT2 normal human prostate epithelial cell line was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The human TERT-immortalised fibroblasts, LNCaP, PC3, DU145, MCF7, T47D, U2020, A549, H157, H647, KRC/Y, U87, HCT116 human cancer cell lines were obtained from MTC (Karolinska Institute, Sweden). The fibroblasts, PNT2, LNCaP, PC3, DU145 cell lines were maintained in RPMI medium and all other cell lines were maintained in IMDM medium supplemented with L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37°C in a humidified 5% CO2 incubator.

Analysis of HS biosynthetic enzymes expression using multiplex RT-PCR
Multiplex RT-PCR analysis of HS biosynthesis-involved genes expression was performed as previously described. 35 Briefly, total RNA was extracted from the cells using the PureLink Total RNA Purification System (Invitrogen, USA) according to the manufacturer’s instructions. cDNA was synthesized from 1–2 μg of total RNA using a First Strand cDNA Synthesis kit (Fermentas, USA) and 1/10th of the product was subjected to PCR analysis. The following conditions were used for multiplex
RT-PCR: 95°C for 10 min, 95°C for 15 sec, 59°C for 15 sec and 72°C for 1 min, with a final elongation step at 72°C for 10 min using a Tercik PCR machine (DNA-technology, Russia). The total reaction volume was 10 μl. Target genes and GAPDH (housekeeping gene) were amplified for 33–34 and 22 cycles, respectively (Table 1). The amplified products were separated on 1.5% agarose gels. The gels were scanned using the "DNA Analyzer" system (Moscow, Russia) and expression levels of the genes were estimated from the intensity of the amplified DNA fragments normalized against the intensity of GAPDH (TotalLab program, Nonlinear Dynamics, UK). The PCR primers and conditions are listed in Table 1.

Western blotting
Cells were lysed with RIPA-buffer (1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris, pH 7.4) containing “Complete” Protease Inhibitor Cocktail (Roche), centrifuged for 10 min at 12 000 g. The protein concentration was quantified using QuantiT Protein Assay Kit (Invitrogen, USA). Total proteins (30 μg) were treated with NuPAGE LDS Sample Buffer (Invitrogen, USA) containing 10% β-mercaptoethanol for 5 min at 100°C, resolved in 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% milk for 1 h and incubated with rabbit anti-GLOCE polyclonal serum (GenScript Corporation), 1:2000 or mouse anti-NDST1 (Abnova), 1:500 overnight at 4°C, followed by secondary peroxidase-conjugated antibodies goat anti-Mouse IgG (Abcam, UK), 1:2000 or polyclonal goat anti-rabbit (Pierce), 1:10 000 for 1 h at RT. Proteins were detected with an Optiblot ECL Detect Kit (Abcam, UK) according to the manufacturer’s instructions.

Statistical analysis
Statistical analyses were performed using a computer program ORIGIN Pro 8.0; a value of p<0.05 was considered to indicate a statistically significant difference. Data are expressed as the means±SEM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Funding
The work was supported by the research grants from Russian Foundation for Basic Research (RFBR 12-04-01657a, RFBR 14-04-01283a) and Ministry of Education and Science of the Russian Federation.

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Table 1. The PCR primers and conditions

| Gene | Sequences | PCR conditions |
|------|-----------|----------------|
| EXT1 | F 5'-TTGGCTCTCAATGATCC-3' | 329 | 59 | 33 |
| EXT2 | F 5'-AAGCAACCGTTCTGTAAC-3' | 296 | 55 | 33 |
| NDS1 | F 5'-CAACAGAAGAATCTGCGC-3' | 513 | 64 | 33 |
| NDS2 | F 5'-GCTTCAGGCTACCC-3' | 575 | 64 | 33 |
| GLCE | F 5'-CTACAAAGGGACCTAAACGC-3' | 665 | 59 | 33 |
| 3-OST1 | F 5'-CGGCTTCCAGGATGGTCG-3' | 382 | 64 | 33 |
| SULF1 | F 5'-CTACAGCTGCGGAAAGAC-3' | 371 | 59 | 33 |
| SULF2 | F 5'-GGGAGAATTCGACGCTTCA-3' | 302 | 39 | 33 |
| HPSE | F 5'-TTGATCCCAAGAAGATGC-3' | 720 | 59 | 33 |
| GAPDH | F 5'-GGGCGTCCTGACGAC-3' | 350 | 59 | 22 |

Table 1. The PCR primers and conditions
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