IN VIVO BIOLUMINESCENCE IMAGING OF TUMOR CELLS USING OPTIMIZED FIREFLY LUCIFERASE LUC2

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The present study was aimed to establish a tumor cell line stably expressing luciferase luc2, and to develop the technique to observe primary tumor nodes and metastases using *in vivo* bioluminescence imaging.

Materials and Methods. In this research we used pLuc2-N plasmid, lentiviral vector pLVT-1, Colo 26 cell line and BALB/c mice to generate new bioluminescent tumor model. Bioluminescence imaging *in vitro* и *in vivo* was carried out on IVIS-Spectrum system (Caliper Life Sciences, USA). Primary tumor model was created by subcutaneous injection of 500 000 Colo 26-luc2 cells. Model of metastases was generated by i.v. injection of 75 000 Colo 26-luc2 cells. Histological analysis was performed to verify the results of the imaging.

Results. We created the lentiviral vector containing luc2 gene using molecular cloning. Then Colo 26-luc2 tumor cell line was generated. We assessed the sensitivity of luc2-based bioluminescence imaging. The intensity of bioluminescent signal *in vitro* averaged about 5000 photon/s per cell, *in vivo* — 250 photon/sec per cell. *In vivo* monitoring of Colo 26-luc2 primary tumor and metastases was demonstrated. The results of bioluminescence imaging correlated with histological analysis data.

Conclusion. The present work shows the possibility of bioluminescent system based on optimized luciferase luc2 for *in vivo* noninvasive high-sensitive whole-body imaging of tumors.

Key words: bioluminescence imaging; optimized firefly luciferase luc2; primary tumor; metastases model

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Recently, whole-body optical imaging has become a powerful tool in the experimental oncology. This technique is based on the use of low-power long-wavelength visible and near infrared light, which is noninvasive for living cells due to a small quantum energy. The optical imaging is known to be a quick, accurate and relatively low-cost technique [1].

Optical imaging includes fluorescence and bioluminescence imaging modalities. Different fluorophores are used as contrast agents in fluorescence imaging; they can be administered exogeneously in an animal body (e.g., indocyanine green), or produced by tumor cells that contain a gene encoding fluorophore in the genome (e.g., GFP-like proteins). The limitations of fluorescence imaging first of all result from the tissue autofluorescence, which produces a high background signal reducing the method sensitivity [2].

Bioluminescence imaging is based on the oxidation reaction of an appropriate substrate by luciferase enzyme, which is accompanied by emission of light. DNA encoding luciferase is integrated in a genome of target cells, while the substrate is injected systemically. Since autoluminescence of tissues is extremely low, this approach provides high-sensitive signal detection.

Luciferases occur in various living organisms — bacteria, fungi, dinoagellates, radiolarians, about 700 species in total. The most extensively studied and widely used system is firefly luciferase from *Photinus pyralis* [3]. In the presence of MgATP and molecular oxygen, the enzyme oxidizes its substrate, D-luciferin, into oxyluciferin, emitting yellow-green light. Firefly luciferase is a 62 kDa molecular weight oxygenase consisting of 550 amino-acid residues [4]. The molecule folds into two distinct domains: a large N-terminal (433 a.a.) and a small C-terminal domains (115 a.a.), connected by a flexible loop. To preserve enzymatic activity and bioluminescence, both parts should be very close to each other and form a single structure [5].

In 2005 Promega Corporation introduced a new product — optimized firefly luciferase (luc2) with enhanced properties. Luc2 is the brightest firefly luciferase construct for deep tissue *in vivo* bioluminescence imaging. This enhanced luciferase is codon optimized for mammalian cell cytoplasmic expression and demonstrates more efficiency compared to its predecessor [6].

Currently, bioluminescence imaging based on luc2 is a rapid, highly sensitive and noninvasive technique for cancer research both *in vitro* and *in vivo*. Many applications for luciferases are developed as a reporter gene to investigate cellular functions, in particular, the pattern of gene expression, cellular receptors activity, signal transduction pathway, RNA processing and protein-protein interactions [7, 8]. Stem cells [9] and apoptosis studies [10] are considered to be prospective. Luc2-based bioluminescence imaging is applied to observe the growth of primary tumor nodes and to detect metastases [11, 12].

It should be noted that the assessment of tumor metastatic activity has become an indispensable step to develop new antitumor treatment modalities. Metastatic lesions are frequently too small to be detected by macroscopic observations and instead require careful, time consuming histological evaluation of selected and processed tissues. In addition, lesions distributed to multiple tissues within an animal are challenging to locate, and unexpected sites can be overlooked by a traditional histological approach that samples only targeted organs. Very often macro- and microscopic analysis can not provide a complete picture of neoplastic processes in animal body [13]. Bioluminescence imaging gives the opportunities for detection of primary and metastatic tumors at early stages in live animals and in real-time. Therefore, the creation of metastasis models and the development of approaches for their observation using bioluminescence imaging proved to be an actual issue.

The aim of the present study was to establish a tumor cell line stably expressing luciferase luc2, and develop the technique to observe primary tumor nodes and metastases using *in vivo* bioluminescence imaging.

Methods

Lentiviral vector generation. pLuc2-N plasmid and lentiviral vector pLVT-1 were kindly provided by the Laboratory of molecular technologies (Institute of Bioorganic Chemistry of RAS, Russia). The fragment corresponding to luc2 open reading frame was amplified from pLuc2-N matrix and treated by restriction endonuclease Not I with DNA-polymerase I *E. coli* (Klenow fragment). Then luc2 fragment was processed by restriction endonuclease Nhe I. pLVT-1 was also initially processed by restriction endonuclease Sal I with DNA-polymerase I *E. coli* (Klenow fragment), and after that — by restrictase Nhe I. pLVT-1/Nhe I-Sal I* and fragment luc2/Nhe I-Not I* were ligated and *E. coli* XL1-Blue cells were transfected by the ligation product according to a standard protocol. Recombinant clones were screened by polymerase chain reaction (PCR) from bacterial colonies using luc2 gene-specific PCR-primers. The results were verified by sequencing.

Cell culture. Murine colon carcinoma Colo 26 was obtained from Gertsen Scientific Research Institute of Oncology (Moscow, Russia). The cells were grown in DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin and 4 мМ L-glutamine. Cells were seeded onto flasks (25 cm^2) at 37°C and 5% CO_2. At every time point the cells were trypsinized and counted using Gorjaev's chamber. Subculturing was performed every 2–3 days, at 80% confluency of culture.

Transfection and stable cell line generation. Lentiviral transduction protocol was performed. HEK 293T cell line was co-transfected by the mixture of lentiviral plasmids including pLVT-luc2 with calcium-phosphate technique. Target Colo 26 cells were transfected by lentiviral particles.

Animal and tumor models. BALB/c female mice, 18–20 g weight were used. The animals were maintained under standard vivarium conditions with a 12-hour light day. 25, 50, 100 and 200 Colo 26-luc2 tumor cells in 50 μl PBS were injected subcutaneously in the dorsal side of animals to assess the sensitivity of *in vivo* bioluminescence imaging. 500 000 Colo 26-luc2 cells in 100 μl PBS were injected subcutaneously in thigh area to create a primary tumor model. The size of a tumor node was measured with calipers on the 4^{th} and 7^{th} days after inoculation. 75 000 Colo 26-luc2 cells in 100 μl PBS were injected systematically via the tail vein to generate a metastasis model. The animals were sacrificed by cervical vertebrae dislocation on the

7th day after subcutaneous injection and on the 9th after intravenous (i.v.) injection of the tumor cells.

All the animal studies were approved be the ethical committee of the Nizhny Novgorod State Medical Academy.

Bioluminescence imaging. Bioluminescence images *in vivo* and *in vitro* were carried out using IVIS-Spectrum system (Caliper Life Sciences, USA). For *in vitro* luciferase assay Colo 26-luc2 cells were plated on 96-well plates at initial concentration of 25,000 cells/well with dilution series 1:2, three times. 150 μg/ml D-luciferin dissolved in deionized water was added to the wells. The measurements were taken in dynamics till a signal reached plateau. For *in vivo* imaging animals were given D-luciferin by intraperitoneal (i.p) injection at 150 mg/kg in PBS and anesthetized with 2% isflurane. The images were captured in series, 3 min after injection, every 2 min, during 20 min. Animals with primary tumor model were imaged on the 4th and 7th days after the injection of tumor cells, animals with metastasis model — on the 9th day, respectively. *Ex vivo* bioluminescence imaging was done immediately after sacrifice of animals with metastasis model. Bioluminescence signal was quantified using Living Image 4.2 software. The changes of bioluminescent signal intensity of a primary tumor Colo 26-luc2 were assessed as percentage ratio.

Histological analysis. Subcutaneous tumors and the lungs with metastases were fixed in 10% neutral formalin and embedded in paraffin according to standard technique. H&E staining was performed. Slides were examined pathomorphologically using the light microscope Leica DFC290 (Germany) at 100- and 400-fold magnification.

Results

Lentiviral construction creation and stable Colo 26-luc2 cell line generation

Molecular cloning resulted in lentiviral vector containing optimized firefly luciferase luc2 gene. As a result of lentiviral transfection, Colo 26-luc2 cancer cells stably expressing bioluminescent marker were created. The cells were cultured in several successive passages (over 20). When D-luciferin was added to the cells, the culture has preserved its bioluminescent properties for a long period of time. The cells were also found not to lose luciferase activity after freezing/thawing procedures.

Sensitivity estimation of bioluminescence imaging based on Colo 26-luc2

For *in vitro* assessment of bioluminescent signal intensity a series of diluted Colo 26-luc2 cells was prepared in culture plate. Bioluminescence was recorded after D-luciferin adding to the cells. Quantitative assessment showed that bioluminescent signal grew linearly with cell number. Luminescence intensity averaged 5000 photon/s per a cell (Fig. 1).

Then *in vivo* assessment of sensitivity of bioluminescence imaging based on Colo 26-luc2 was performed. Cell suspensions consisting of 25, 50, 100 and 200 Colo 26-luc2 in 50 μl PBS were injected subcutaneously to BALB/c mice. Immediately after that D-luciferin was i.p. injected. *In vivo* bioluminescence imaging showed that minimal number of cells that we could detect was about 50 (Fig. 2). Total bioluminescence intensity (TotalFlux) of 50 cells averaged 9600 photon/s, 100 cells — 15 100 photon/s, 200 cells — 80000 photon/s, that was on the average 250 photon/s

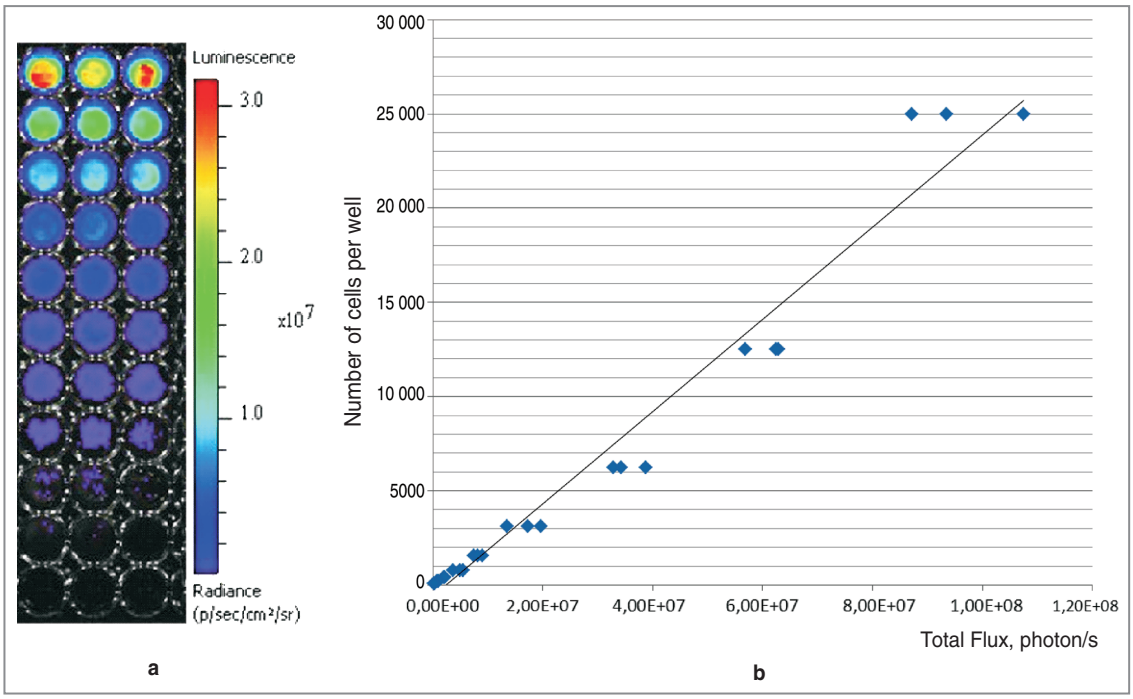


Fig. 1. *In vitro* sensitivity estimation of bioluminescence imaging based on Colo 26-luc2*: a* — bioluminescent image of 96-well plate with tumor cells; *b* — bioluminescence intensity increasing with Colo 26-luc2 cell number. D-luciferin, 150 μg/ml

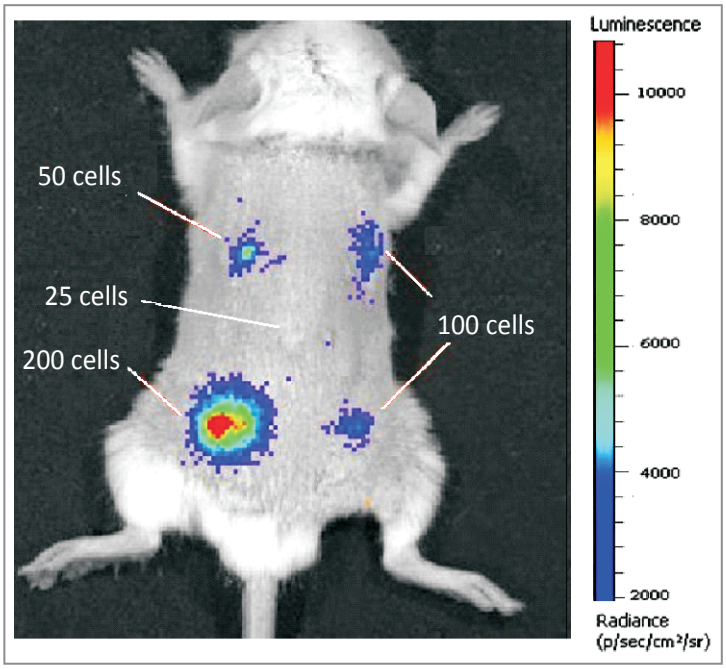
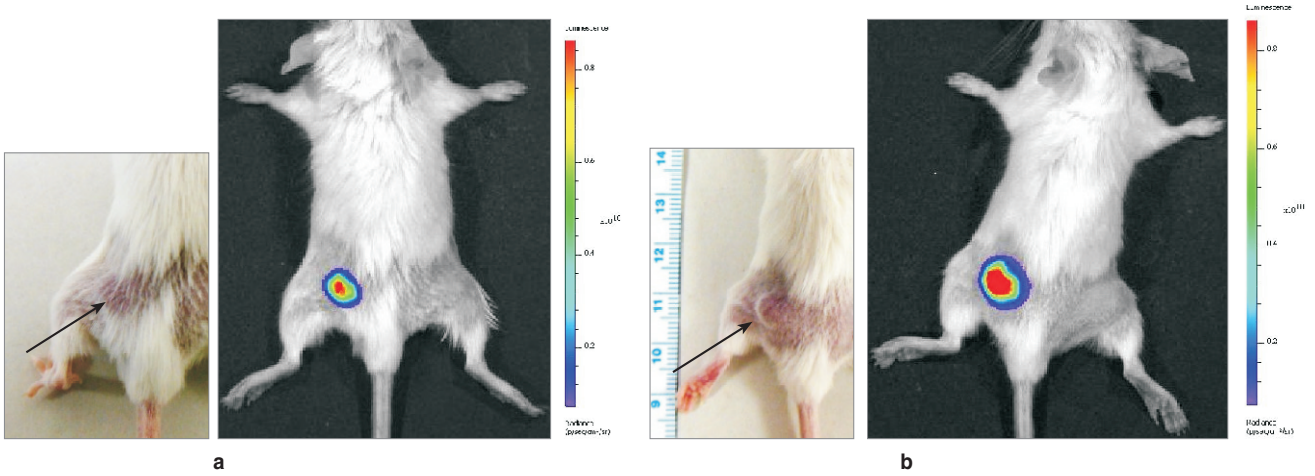


Fig. 2. *In vivo* sensitivity estimation of bioluminescence imaging based on Colo 26-luc2. Subcutaneous injections of Colo 26-luc2 cells in amounts of 25, 50, 100 and 200. D-luciferin, 150 mg/kg, i.p.



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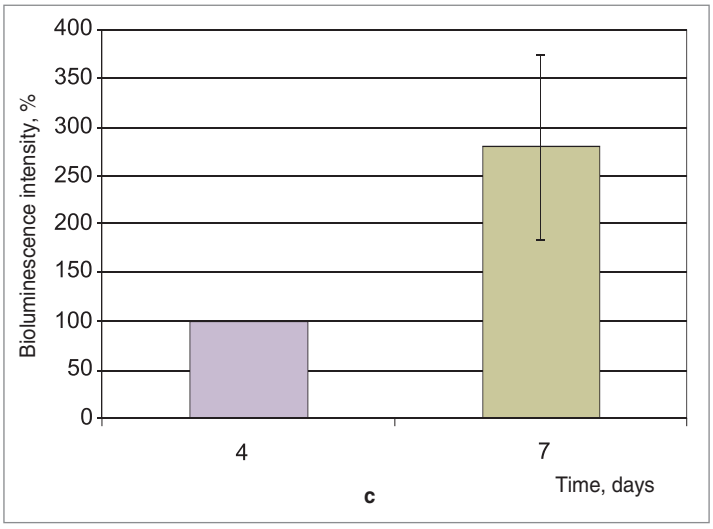

c

Fig. 3. *In vivo* bioluminescence imaging of Colo 26-luc2 primary tumor: *a* — the 4th day after inoculation; *b* — the 7th day after inoculation. The arrows show the tumor node formation in mice; *c* —bioluminescent signal growth in time. D-luciferin, 150 mg/kg, i.p

per cell. Bioluminescence was not recorded in case of 25 Colo 26-luc2 cells injected subcutaneously. Therefore, despite signal attenuation by skin was approximately 20 times, the sensitivity of bioluminescence imaging of cancer cells based on optimized luc2 luciferase appeared to be sufficiently high.

Bioluminescence imaging of subcutaneous tumor Colo 26-luc2

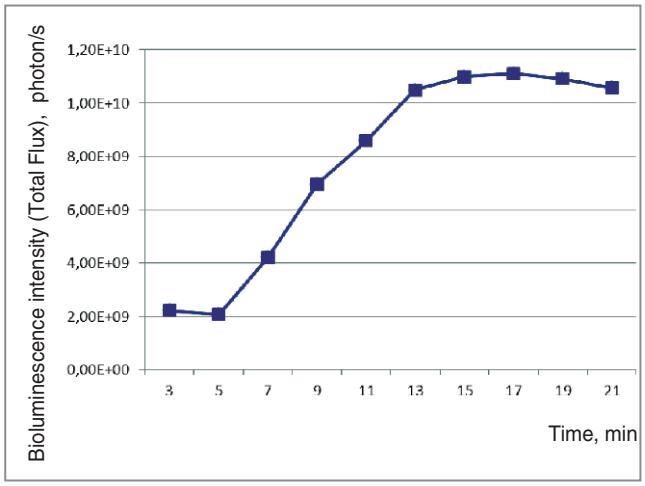

Fig. 4. Kinetics of *in vivo* bioluminescence reaction. D-luciferin, 50 mg/kg, i.p.

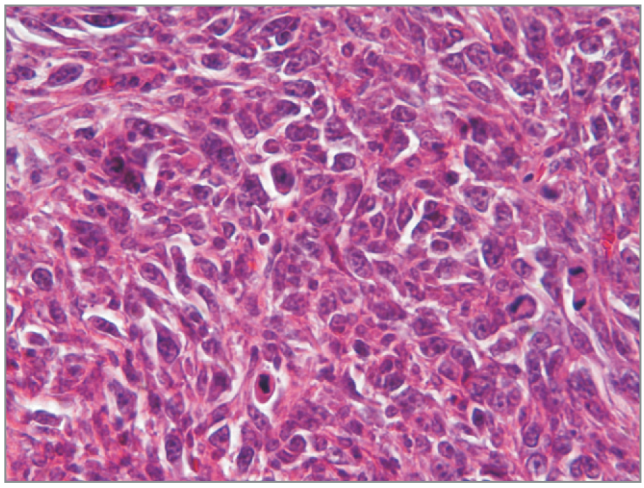

Fig. 5. Histological analysis of Colo 26-luc2 subcutaneous tumor. H&E staining, 400x

Monitoring of primary subcutaneous Colo 26-luc2 tumors showed that they had no difference with unmodified Colo 26 tumors on the growth rate.

In vivo bioluminescence imaging demonstrated the possibility of tumor node detection at early stage. An intensive bioluminescent signal was seen already on the 4th day after subcutaneous injection of Colo 26-luc2 cells in thigh area. It indicated the presence of living tumor cells, while the tumor has been was non-palpable yet. A palpable node of 9–10 mm^3 size was found on the 7th day after inoculation, and bioluminescent signal significantly increased. Figure 3 shows the increase of bioluminescent signal intensity of primary Colo 26-luc2 tumor during the growth.

A typical bioluminescent signal kinetic of labeled tumor after D-luciferin i.p. injection within 20 min is shown in Figure 4. Bioluminescent signal reached the maximum in 13 min after the substrate injection and then remained on the plateau level that corresponded to the literature data [14].

Histological analysis showed that Colo 26-luc2 tumors had a typical dense tissue structure with polymorphic cells of various sizes, with large nuclei consisting of diffusely distributed chromatin and 1–2 nucleoli (Fig. 5). The cytoplasm formed a thin ring around the nuclei and had a weak basophilic reaction. Tumor cells were organized in solid complexes and cords. The tumor had a developed vasculature and was poor in connective tissue. Numerous mitoses, the areas of hemorrhages and spontaneous necroses were found in the tumors.

Bioluminescence imaging of Colo 26-luc2 metastases

Metastasis model was generated by i.v. injection of Colo 26-luc2 cells to BALB/c mice. *In vivo* bioluminescence imaging was carried out on the 9th day the injection. Mice were imaged in 4 positions (dorsal side, ventral side, the left and the right side). An intensive bioluminescent signal was detected *in vivo* in thoracic area in all cases (Fig. 6).

Ex vivo bioluminescence imaging revealed metastases only in the lungs. The metastatic lesions were not found macroscopically due to their small size (Fig. 7).

Single metastases were verified by histological analysis of the lung tissue, and their localization was corresponded to bioluminescent areas. The metastases were represented by small populations of cancer cells. Small size and not well-

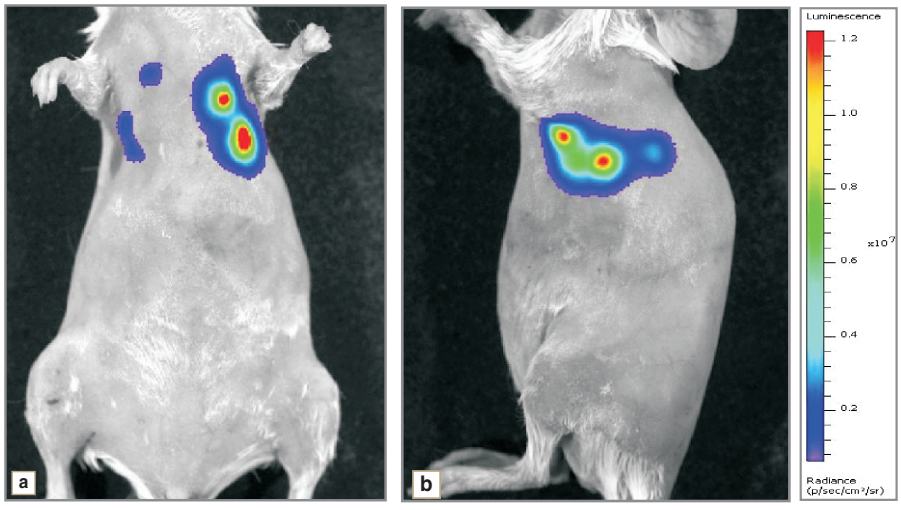

Fig. 6. *In vivo* bioluminescence imaging of Colo 26-luc2 metastases in the lungs on the 9th day after i.v. injection of tumor cells: *a* — the ventral side of mouse; *b* — lateral view. D-luciferin, 150 mg/kg, i.p.

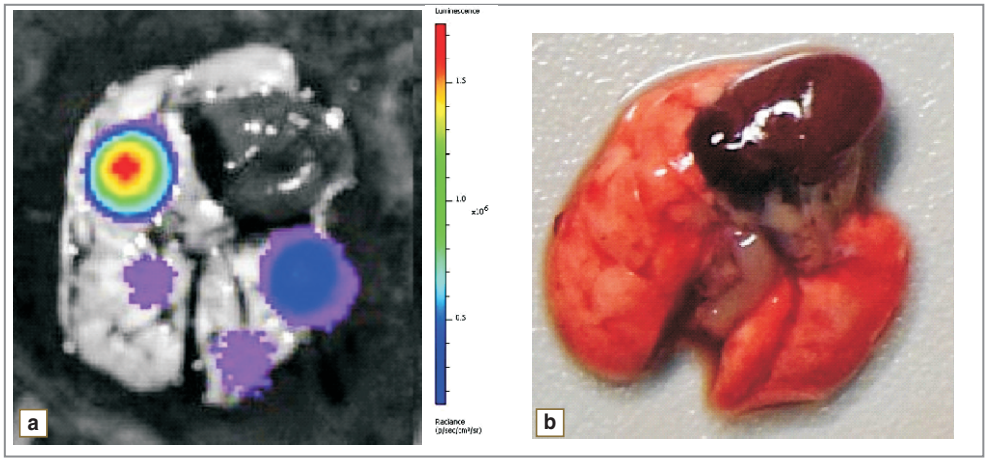

Fig. 7. Isolated lungs of BALB/c mouse on the 9th day after i.v. injection of Colo 26-luc2 cells: *a* — bioluminescence imaging *ex vivo*; *b* — photo

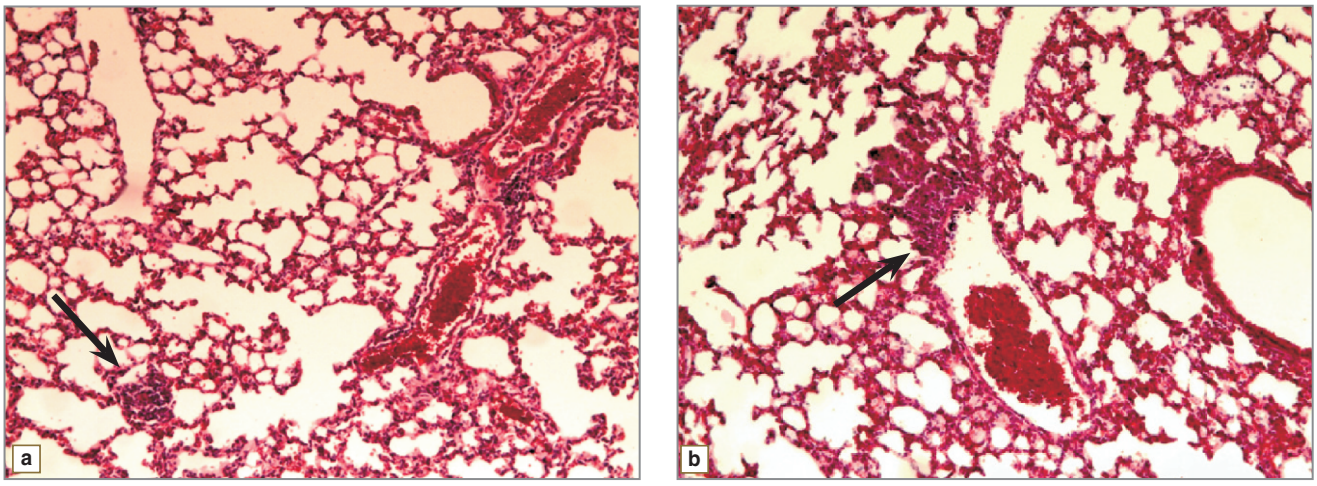

Fig. 8. Histological analysis of lung tissue: *a* — Colo 26-luc2 metastasis in the right lung; *b* — Colo 26-luc2 metastasis in the left lung. H&E staining, 100x. Arrows show the metastases

defined structure of the revealed cell complexes indicated a very early stage of tumor node formation (Fig. 8).

Discussion

Since last decade bioluminescence imaging has proved to be an irreplaceable instrument for tumors and metastases investigation using small animal models. It became possible due to the development of highly sensitive imaging and bright luciferin-luciferase systems [11, 12]. Today, there are commercially available human and animal tumor cell lines stably expressing bioluminescent marker luc2, such as human prostatic carcinoma (metastasizing) PC-3M-luc2, murine breast carcinoma 4T1-luc2, etc. Nevertheless, the extension of the range of labeled cancer cell lines seems to be a crucial issue for experimental oncology [15, 16].

In the present work Colo 26-luc2 cell line was developed. The choice of Colo 26 was determined by its extensive use in preclinical studies of antitumor drugs and testing of new diagnostic and treatment modalities [17, 18]. Colo 26 is characterized with a high metastatic potential and forms predominantly lungs metastases after i.v. injection [19].

Colo 26-luc2 cells were created by means of lentiviral transfection using a designed lentiviral vector containing luc2 gene. Lentiviral transfection technology is used as an effective technique to prepare stable cell lines. The application of lentiviral vectors enables to insert the genes of interest into genome of target cells owing to their ability to manage the efficient delivery, integration, and long-term expression of transgenes in cells both *in vitro* and *in vivo* [20]. It provides stable expression and production of proteins for a long period of time.

In vitro assessment of luciferase activity of Colo 26-luc2 culture showed that the cells responded adequately to substrate addition and generated intensive and stable signal. *In vivo* studies demonstrated that luc2-based bioluminescence imaging being a highly sensitive tool provided detection of a small number of cells (about 50) injected subcutaneously. For comparison, minimal number of cells labeled with red fluorescent protein TurboFP635 and recorded by epifluorescence imaging was about 90 000 [21].

We showed the ability to image a tumor node at early stages using the model of subcutaneous primary Colo 26-

luc2 tumor. Moreover, histological analysis confirmed that Colo 26-luc2 cells injected subcutaneously formed a node with tumor structure corresponding to unmodified Colo 26 tumor [22].

Currently, tumor growth monitoring based on optical (fluorescence or bioluminescence) images is considered to be more accurate compared to traditional measurements with calipers. Bioluminescence imaging allows to assess tumor size by total bioluminescent signal (TotalFlux, photon/s) that is proportional to the number of living cells expressing luciferase [23]. The possibility of living cells detection provides a lot of advantages to this technique over both fluorescence imaging and traditional caliper measurements. In the first case dead cells containing fluorescent protein maintain the capacity of light emission; in the second one necrotic tissue and stroma also contribute to tumor volume. Successful application of *in vivo* bioluminescence imaging to estimate tumor growth and response to the treatment has been demonstrated by a number of researchers [24–26]. In this work we demonstrated the increase of bioluminescent signal intensity with the growth of a tumor node.

Developed Colo 26-luc2 tumor cell line was used for in vivo observation of early metastases in mice. In Ref. 27 lung metastases is suggested to be counted on the 21st day after i.v. Colo 26 cell injection [27]. Using bioluminescence imaging single small-sized metastatic lesions in the lungs were detected on the 9th day. The previous studies we carried out using Colo 26 cells expressing red fluorescent protein KillerRed showed that the detection of lung metastases by their fluorescent signal seemed to be difficult even at late stages of the growth (unpublished data).

Conclusion

This work demonstrates the possibilities of bioluminescent system based on optimized luciferase luc2 for *in vivo* non-invasive whole-body imaging of tumors. Bioluminescence imaging using Colo 26-luc2 cell line provides not only qualitative, but highly sensitive quantitative *in vivo* assessment of tumor cells, and enables detection of early metastases. Stable Colo 26-luc2 tumor cell line and developed techniques of bioluminescence imaging of primary tumors and metastases in BALB/c mice are of interest for future studies in experimental oncology.

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