

Properties of melanoma mitochondria to form tumor nodules in the body of experimental animals

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Abstract

The aim hereof is to develop a method for revealing the properties of mitochondria isolated from melanoma of the skin to form melanoma tumor nodules in the body of mice in parenteral transplantation of melanoma mitochondria.

Materials and methods. We used experimental animals according to the following design: intraperitoneal transplantation with B16/F10 melanoma mitochondria in the Balb/c Nude male mice, $n = 8$. All animals underwent a one-time transplantation of B16/F10 melanoma mitochondria based on 5.2 mg of protein per 1 animal in 0.4 ml of saline solution.

The Balb/c Nude male mice were used as the references, which were transplanted with 0.4 ml of saline solution once into the muscle and intraperitoneally. Morphological examination of sections of the internal organs and the B16/F10 melanoma foci was carried out after specimen paraffin embedding and hematoxylin-eosin staining with microscopy of specimens with Axiovert (Carl 44 Zeiss, Germany) and Axiovision 4 image visualization software (Carl Zeiss, Germany).

Results. Upon intraperitoneal transplantation of mitochondria isolated from melanoma to the Balb/c Nude mice, in the autopsies in 100% of the animals after 2 weeks of the experiment the total damage to the internal organs by the tumor nodules was revealed.

The morphological examination of the internal organs of the Nude mice 2 weeks after the intraperitoneal transplantation of B16 melanoma mitochondria demonstrated a tumor lesion of the visceral tissue and the abdominal organs.

Conclusion. Thus, the use of parenteral transplantation of mitochondria isolated from B16/F10 melanoma in the C57BL/6 and

Balb/c Nude male mice caused the growth and development of melanoma foci in the body. Our study revealed previously unknown properties of mitochondria isolated from B16/F10 skin melanoma, consisting in their ability to induce the development of melanoma tumor nodules in the body of animals upon the tumor cell parenteral transplantation.

Keywords

Mitochondria, Balb/c Nude mice, Experimental B16/F10 melanoma

Imprint

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Topicality

Melanoma, the deadliest form of skin cancer, develops as a result from an oncogenic transformation of cells belonging to the melanocytic lineage [1]. Although the study of genetic changes is essential to understanding the mechanisms involved in the onset of melanoma, the available evidence suggests that the mutational landscape of melanoma is insufficient to explain why metastatic spread occurs. Although oncogenic mutations may contribute to the metastatic process [2], they are unlikely to be the main driving forces thereof, since the time required for the emergence of new prometastatic mutations is longer than the period, during which metastases usually appear. A recent study conducted on 50 different types of tumors showed that certain mutations can often be found in tumors with specific metastatic patterns.

Tumor formation and spread of metastases depend on mitochondrial bioenergetics, mainly through respiration, signaling and dynamics [3]. Mitochondria are at the center of cell metabolism and signaling cascades [4]. These organelles must adapt to the environment in order to meet the demands of cellular metabolism. Similarly, the cell must “know” about mitochondrial activity and meet their needs through the activation of special gene expression programs. As a result, bidirectional communication systems between mitochondria and the nucleus evolved, and anterograde signals and

retrograde pathways from mitochondria to the nucleus began to act in concert to meet the specific metabolic needs of a cancer cell [5]. A number of authors also consider the role of the mitochondrial dynamics in the regulation of tumor formation and melanoma metastasis. For example, some markers of mitochondrial division and fusion, such as protein 1 (DRP1), mitochondrial division protein 1 (FIS1), and mitofusins, are found in higher quantities in patient tumor samples compared to their healthy tissues, and this fact is largely associated with metastasis [6]. Moreover, the levels of TMX1 and TMX3, the transmembrane proteins with oxidoreductase activity, which promote the ER-mitochondrial communication, are elevated in patients' melanoma cells and associated with a poor disease outcome [7].

Dysfunction of mitochondria contributes to the formation of tumors and metastases. However, the mechanisms linking mitochondrial dynamics with the development of metastasis remain poorly understood. In addition, for a successful fight against the malignant process, one should take into account the flexibility of mitochondria, which allows cancer cells to adapt to a changing micro-environment and stress events. Thus, a better understanding of the processes regulated by mitochondria and their complex interaction with mitochondrial biogenesis is the basis for new promising therapeutic strategies in cancer treatment [8].

Research in the last decade has shown the existence of horizontal mitochondrial transfer between eukaryotic cells. Several *in vitro* studies have shown that intercellular transfer of mitochondria occurs naturally. A study by Kitania T. et al. (2014) proves that exogenous human mitochondria are internalized into isogenic cells during *in vitro* experiments. To avoid false positive results, the authors thereof attached the probe to donor mitochondria and recipient cells. Transmission electron microscopy confirmed that isolated and enriched mitochondria retained a morphologically intact outer membrane. In addition, their surface was negatively charged, indicating that the potential of the mitochondrial membrane was maintained after isolation. Fluorescence microscopy showed that the DsRed-labeled mitochondria were internalized into GFP-expressing isogenic endometrioid carcinoma cells by simple co-incubation. In addition, GFP-expressing endometrioid carcinomas (EMCs) carrying DsRed-labeled mitochondria have been found to be single-nucleated that excludes the possibility of cell

fusion between the GFP-expressing EMCs and the EMCs containing DsRed-labeled mitochondria. Flow cytometry methods have confirmed the presence of a separate population of the double positive GFP and DsRed cells [9].

Another study also showed that mitochondria isolated from the mouse liver tissue by xenogenic transfer into human cells, deprived of functional mitochondria (p0 cells), restored the respiratory function [10]. These results prove the possibility of using mitochondrial transplantation in the treatment of mitochondrial diseases [11].

There is increasing evidence that mesenchymal stem cells (MSCs) are able to transfer mitochondria to various cells, including endothelial cells, cardiomyocytes, and epithelial cells, to repair tissue damage [12]. Mitochondrial transfer occurs via tunneling nanotubes (TNT), extracellular vesicles, and cell fusion [13, 14]. Mitochondrial transfer via TNT is used as the main mode of the mitochondrial transfer, and it is also an important, efficient, cell-to-cell communication pathway that mediates cell-to-cell interactions. Recent studies have shown that malignant cells with altered oxidative phosphorylation import healthy (functional) mitochondria from surrounding stromal cells to stimulate pyrimidine synthesis and cell proliferation. It has been shown that high-energy mitochondria are of fundamental importance in the processes of migration, invasion, and metastasis of tumor cells [15].

Under the physiological conditions, mitochondria can move between cells using various contact methods, including nanotube tunneling, extracellular vesicles, intercellular fusion, and GAP junctions [16,17]. Based on this knowledge, McCully and colleagues used mitochondrial transplantation as a therapeutic approach to treat ischemia, both experimentally and clinically (in children) [18]. A number of authors report on mitochondrial transplantation as a "magic" cure [19], since healthy organelles isolated from an intact tissue, after injection into the ischemic focus, move to damaged cells, restore the ATP energy production and improve the contractile function of cardiomyocytes within 10 minutes [20].

Thus, it is known that a change in the metabolism of mitochondria is necessary for melanoma to carry out the process of metastasizing, and it is also known that mitochondria can independently move between cells. However, all of the above movement is applicable to intact mitochondria only, which regulate

the disturbed metabolism of various cells. And such a property of melanoma-related mitochondria as the ability to form tumor structures in the body is not investigated.

Therefore, **the aim** hereof is to develop a method for detecting the properties of mitochondria, isolated from melanoma of the skin, to form melanoma tumor nodules in the body of mice in parenteral transplantation of this sort of mitochondria.

Materials and Methods

We used experimental animals according to the following design: intraperitoneal transplantation of B16/F10 melanoma mitochondria in the Balb/c Nude male mice, $n = 8$. All animals underwent a one-time transplantation with B16/F10 melanoma mitochondria based on 5.2 mg of protein per 1 animal in 0.4 ml of saline solution.

The Balb/c Nude male mice were used as the references, which were transplanted with 0.4 ml of saline solution once into the muscle and intraperitoneally, respectively.

Reproduction of melanoma B16/F10. The C57BL/6 male mice were transplanted into their skin of the back below the angle of the left shoulder blade with 0.5 ml of a suspension of the B16/F10 mouse melanoma tumor cells in saline at a 1:20 dilution. For this purpose, observing all the conditions of asepsis, an assistant fixed the mouse with its back up, having previously treated the skin with a 5% alcohol solution of iodine. The experimenter with a hand in a sterile glove grasped a skin fold, in the center of which he pierced the skin and injected the B16/F10 tumor cell suspension. After removing the needle, the injection site was tightly pressed with a cotton swab dipped in a 70% alcohol with a small addition of iodine, for 1 minute, to prevent leakage of tumor material.

Isolation of mitochondria from melanoma. The C57BL/6 mouse with transplanted B16/F10 skin melanoma was decapitated using a guillotine, and the melanoma tissue was removed. Mitochondria were isolated using differential centrifugation with a high-speed refrigerated centrifuge Avanti J-E, BECMAN COULTER, USA according to the method of Egorova M.V., Afanasiev S.A. (2011) and Gureev A.P. et al. (2015) [21,22]. To destroy intercellular junctions, the cell wall, and plasma membranes, mechanical processing of tissues was used with grinding with scissors and homogenization in a glass homogenizer with a Teflon

pestle (Potter-Elvehjem homogenizer). For each gram of tissue, 10 ml of sterile isolation medium (0.22 M mannitol, 0.3 M sucrose, 1 mM EDTA, 2 mM TRIS-HCL, 10 mM HEPES, pH 7.4) was added. The tissues were homogenized and centrifuged for the first time for 10 min at a speed of 1000 g, at a temperature 0-2 °C, the second and third centrifugation is carried out at 20000 g, for 20 min, at a temperature 0-2 °C. Between the centrifugations, the mitochondrial pellet was resuspended in the isolation medium. Mitochondria were further purified from lysosomes, peroxisomes, melanosomes, etc. by centrifugation in a 23% Percoll gradient. The suspension of subcellular structures was layered on the Percoll gradient, centrifuged for 15 min at 21000 g, after which separation into 3 phases was observed, the lower layer of mitochondria was left, and resuspended in the isolation medium. The next washing of mitochondria was carried out by centrifugation for 10 min at 15000 g, at a temperature 0-2 °C. Mitochondrial samples were diluted with a 0.9% NaCl solution to a protein concentration of 5.2 mg of protein in 0.4 ml of saline solution.

All animals were sacrificed by guillotine decapitation 2 weeks after the parenteral transplantation of mitochondria isolated from C57BL/6 melanoma.

Morphological examination of sections of the internal organs and the B16/F10 melanoma foci was carried out after specimen paraffin embedding and hematoxylin-eosin staining with microscopy of specimens using Axiovert (Carl 44 Zeiss, Germany) and Axiovision 4 image visualization software (Carl Zeiss, Germany).

Results

Features of the development of the melanoma foci in intraperitoneal transplantation of mitochondria, isolated from melanoma, into Balb/c Nude mice are shown in a photo given herein (see Figure 1 herein).

In 100% of the animals, in autopsy after 2 weeks of the experiment, a total lesion of the internal organs by tumor nodules was found.

The morphological examination of the internal organs of the Nude mice conducted 2 weeks after the intraperitoneal transplantation of B16 melanoma mitochondria indicated a tumor lesion of the visceral tissue and the abdominal organs.

First of all, a total lesion of the mesentery was revealed, where there were many small and large nodules of cells of a melanocytic nature, transformed from ele-

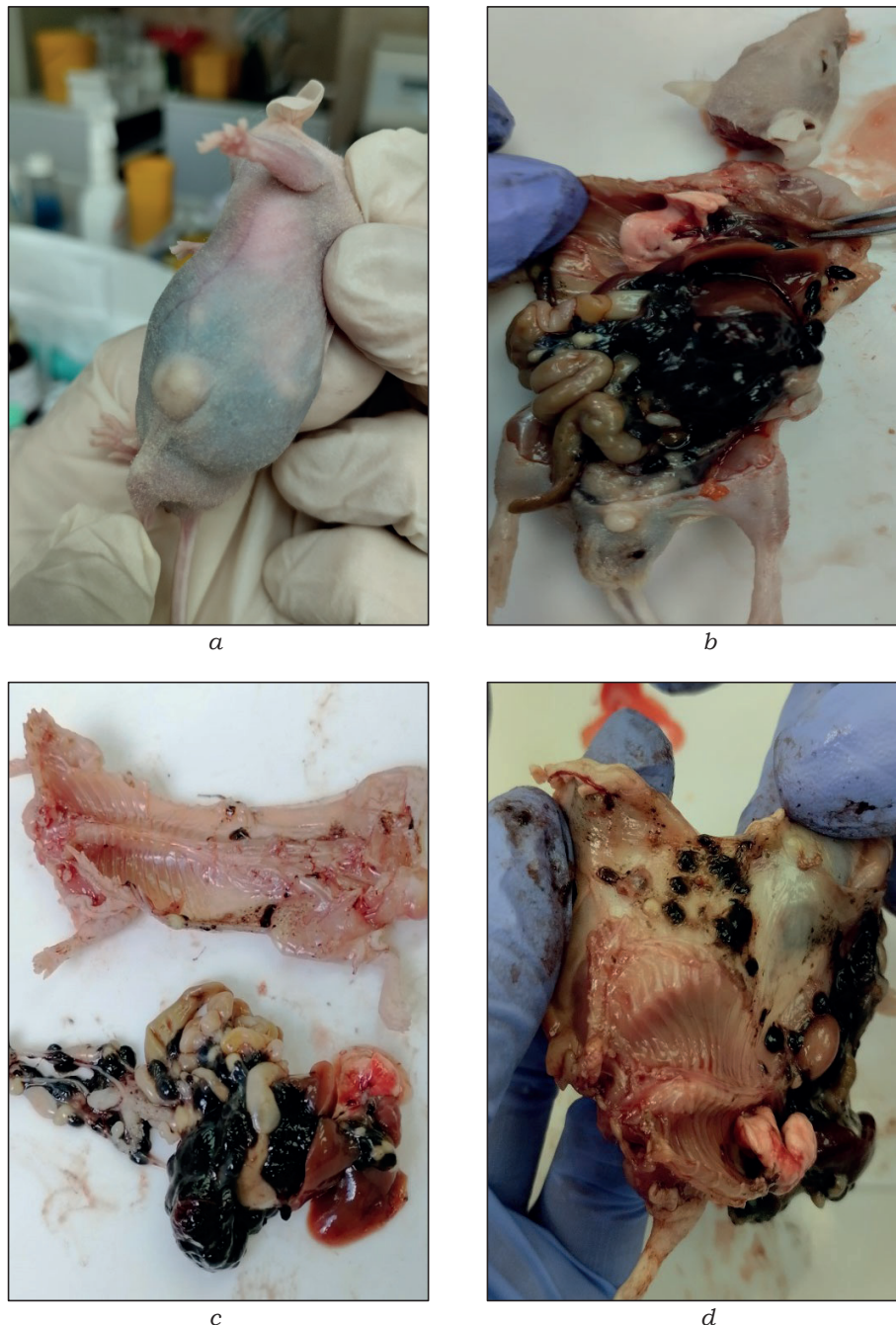


Figure 1. Intraperitoneal transplantation of mitochondria isolated from B16/F10 melanoma. Male mice of the Balb/c Nude line. View of an animal with intraperitoneal transplantation of mitochondria isolated from B16/F10 melanoma (a). Tumor lesions of the abdominal organs after intraperitoneal transplantation of mitochondria isolated from B16/F10 melanoma (b). Mouse organ complex after intraperitoneal transplantation of mitochondria isolated from B16/F10 melanoma (c). Tumor lesion of the skin after intraperitoneal transplantation of mitochondria isolated from B16/F10 melanoma (d).

ments of loose connective tissue with the participation of components of fat, blood, fibroblasts recruited by mitochondria, and lymphoid elements. The neoplastic tumor cells represented a cell structure typical of epithelioid melanoma with several large nucleoli and a significant content of melanin pigment granules (see Figure 2 herein).

In the intestinal loops in the Nude mice, as a result from the intraperitoneal transplantation of B16 melanoma mitochondria, the formation of tumor nod-

ules of various sizes was noted, tightly adhering to the walls of the small and large intestines, but however not growing into their cavity. An examination of the tumor nodules with a magnification x100 revealed the structural cell pattern of a dominant round shape typical for melanoma at different stages of pathological mitosis, as well as dispersed melanin pigment with a characteristic shade of brown (see Figure 3 herein).

Significant damage to the structure of the liver was noted, in areas of which large and small foci of

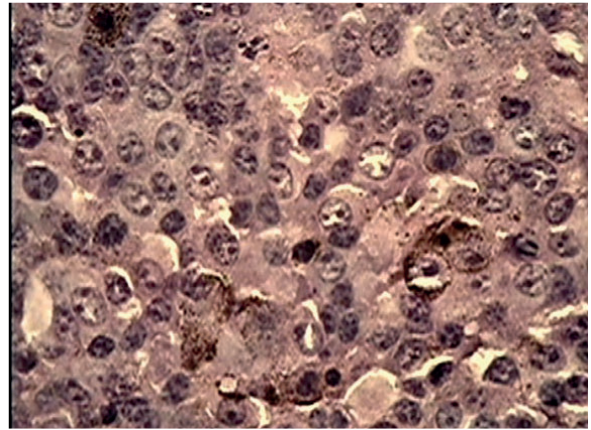
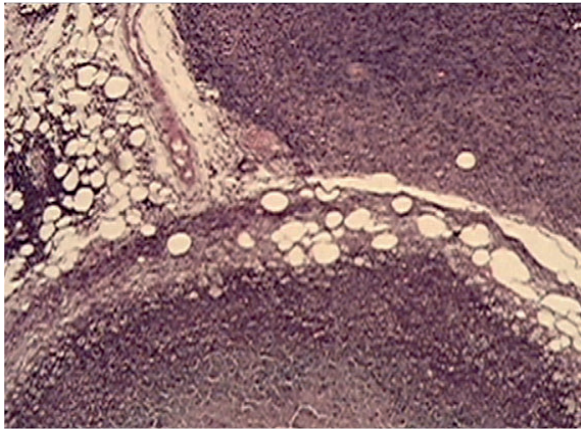
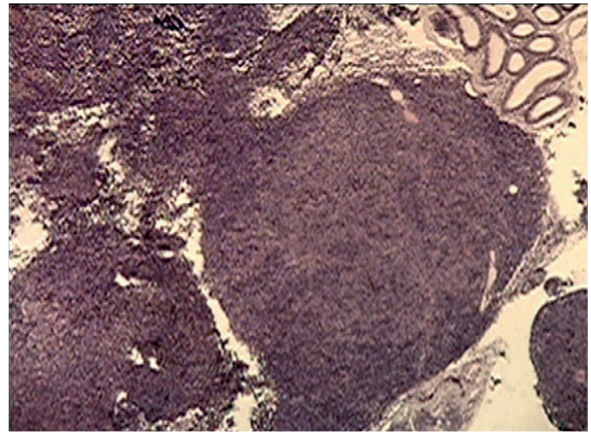
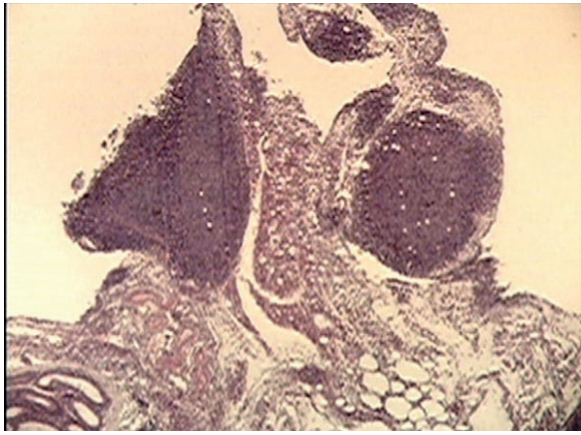


Figure 2. Microimage of numerous tumor nodules of various sizes formed in the mesentery of Nude mice in intraperitoneal transplantation of B16 melanoma mitochondria. Hematoxylin-eosin staining. Magnif. x10, x100

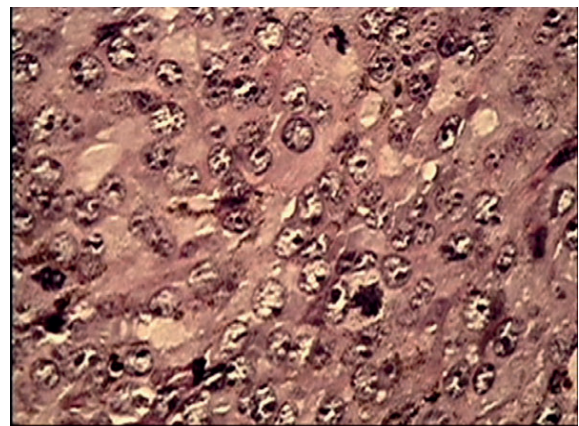
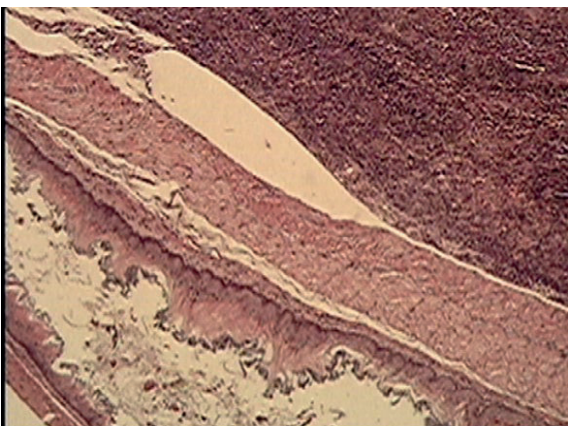
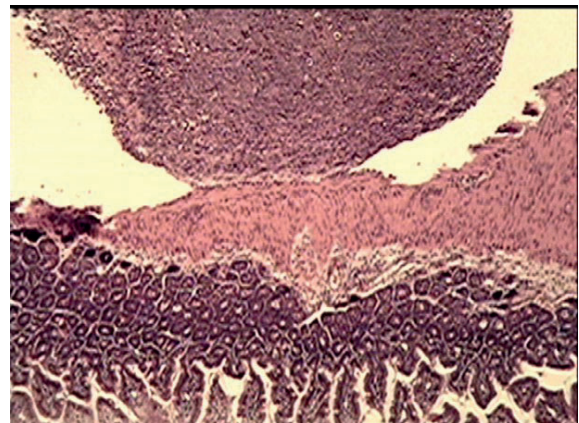
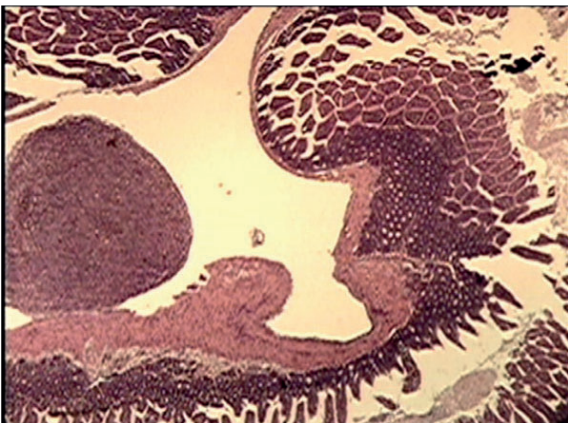


Figure 3. Microimage of tumor nodules formed in intestinal loops of Nude mice after intraperitoneal transplantation of B16 melanoma mitochondria. Hematoxylin-eosin staining. Magnif. x10, x100

hemorrhage were remarkable. In the presented images (see Figure 4 herein), considerable conglomerations of transplanted mitochondria of an elongated shape are clearly visible, adjacent to single melanocytes or embedded in tumor bulges or pseudosyncytia.

In the stomach area, as well as in the intestines of the Nude mice, many tumor nodules adjacent to the muscular wall of the stomach, but however not growing into the stomach cavity, were noted. A detailed examination of those nodules clearly showed the structural pattern made by densely located melanocytes of a round or ovoid shape with a characteristic distribution of nuclear material and the presence of dispersed or grouped melanin pigment granules (see Figure 5 herein).

The morphological examination of the spleen of the Nude mice in intraperitoneal transplantation of B16 melanoma mitochondria has confirmed the full disappearance of the organ architectonics as a result of the progressive tumor growth. The attacking dynamics of malignant cells included both the formation of spindle-shaped melanocyte strands and the introduction of large tumor bulges containing several neoplas-

tic round-shaped melanoma cells. When examining multiple fields of view, no areas with preserved white pulp follicles were noted, and a large number of megakaryocytes with signs of degradation and destruction were found in the red pulp. Obviously, the initial response by the spleen to the tumor invasion was directly related to the activation of blast cells such as megakaryocytes, which are delegated to replenish the cell population composition of the spleen (see Figure 6 herein).

The microimage material of the tumor lesion of the paranephric tissue in the Nude mice in intraperitoneal transplantation of mitochondria isolated from melanoma B16 was of particular interest, since we conducted a similar study in intramuscular transplantation of mitochondria isolated from the same melanoma from mice of another line. We have found a similar mechanism for the involvement of loose connective tissue resources (adipose tissue, blood, fibroblast recruitment) for the melanocyte neoplasm and the formation of a tumor nodule (see Figure 7 herein). As can be seen from the presented microphotos, the paranephric tissue serves as an adequate metabolic niche for the full

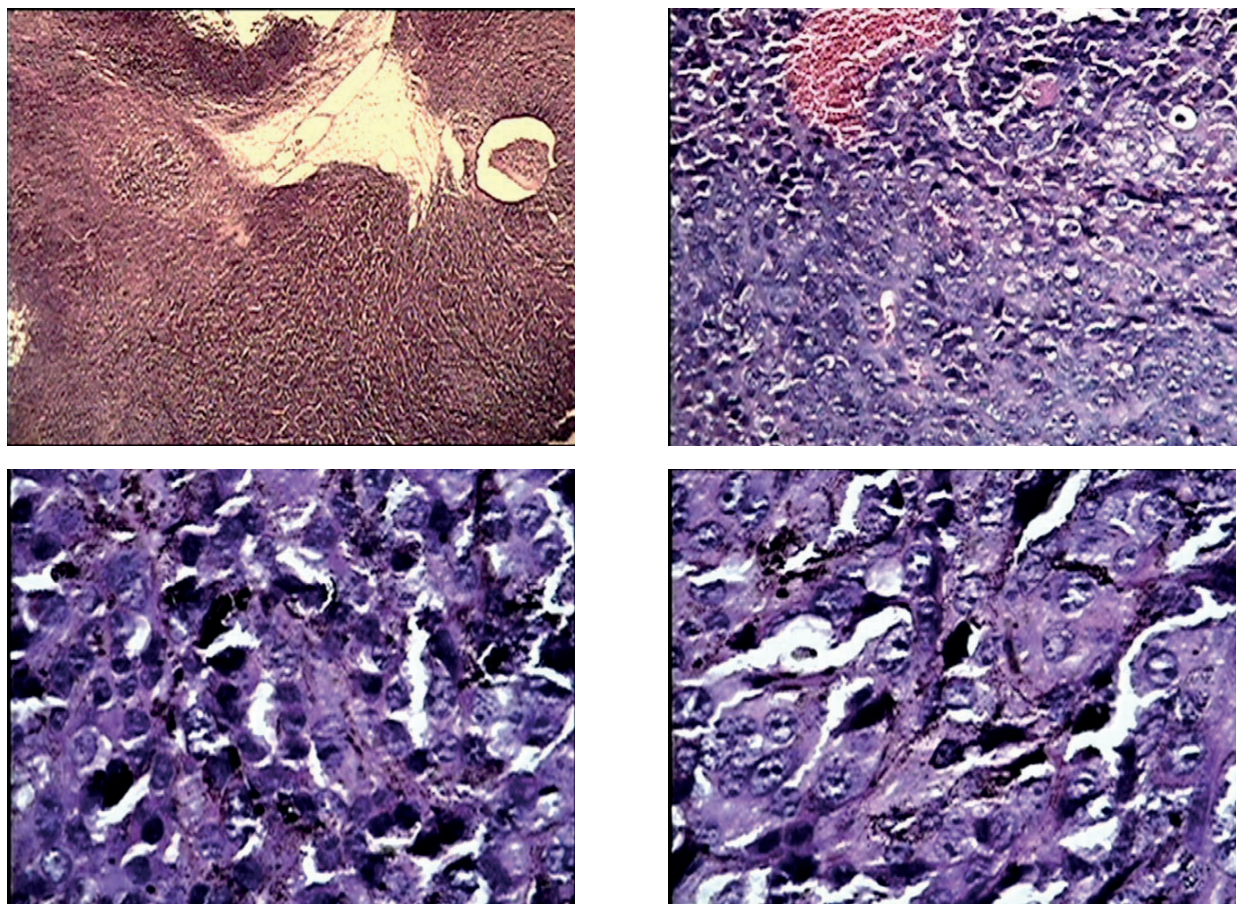


Figure 4. Microimage of liver tumor lesions in the Nude mice after intraperitoneal transplantation of B16 melanoma mitochondria. Hematoxylin-eosin staining. Magnif. x10, x40, x100.

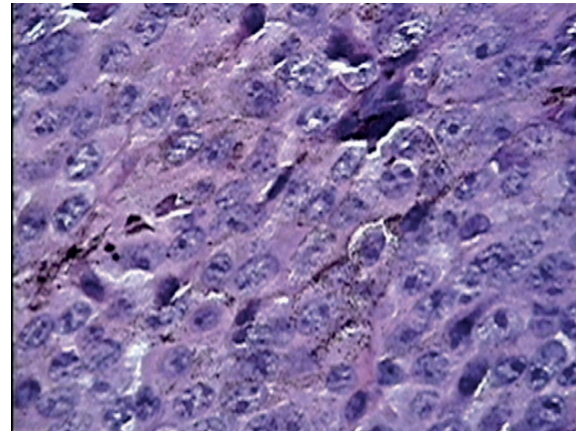
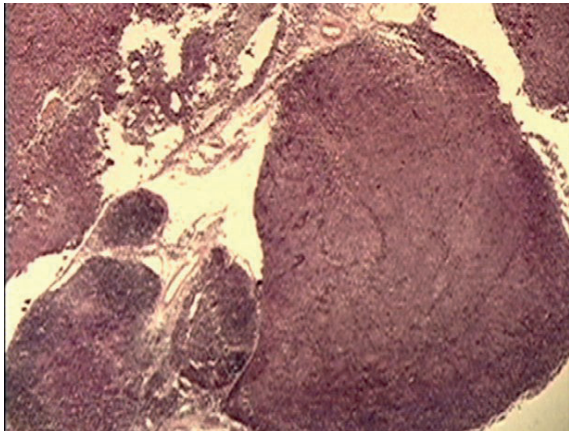
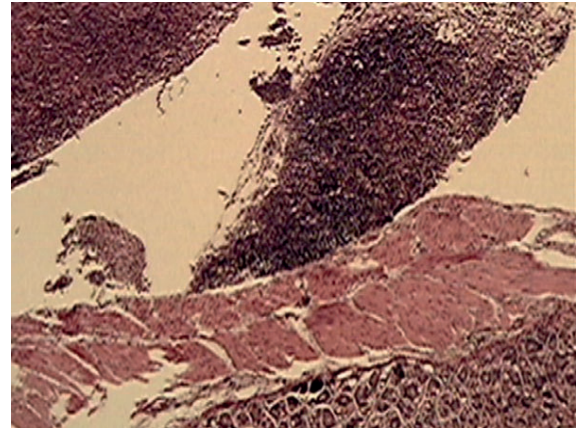
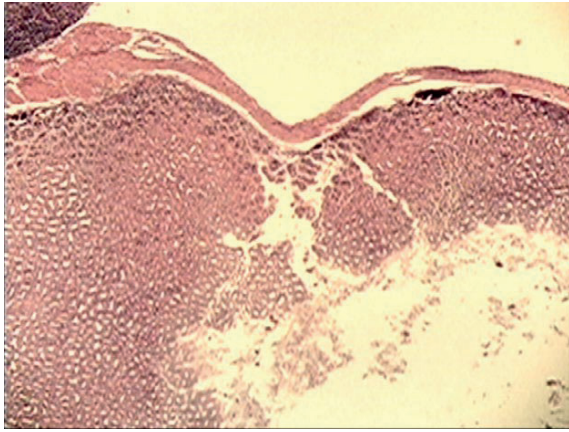


Figure 5. Microimage of multiple tumor nodules in the perigastric region, not affecting the walls and cavity of the stomach in the Nude mice in intraperitoneal transplantation of B16 melanoma mitochondria. Hematoxylin-eosin staining. Magnif. x10, x40, x100.

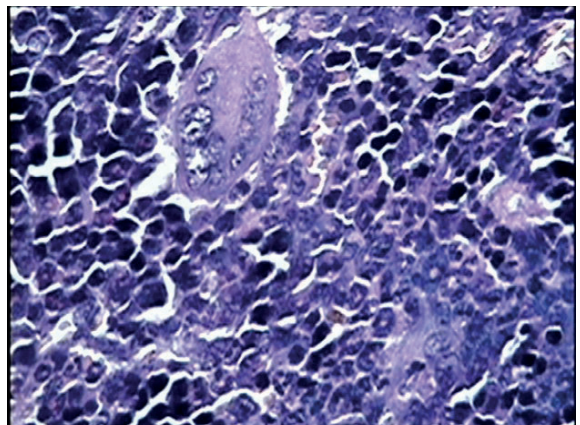
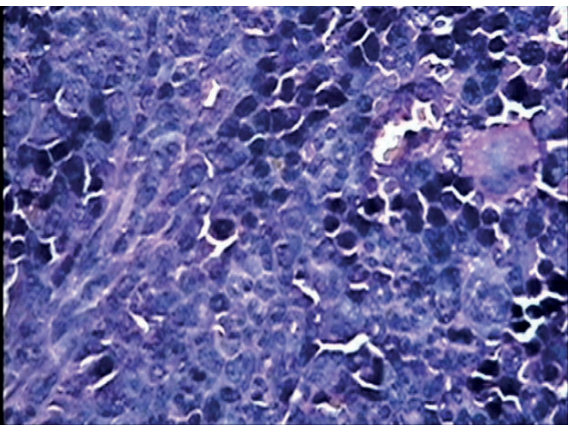
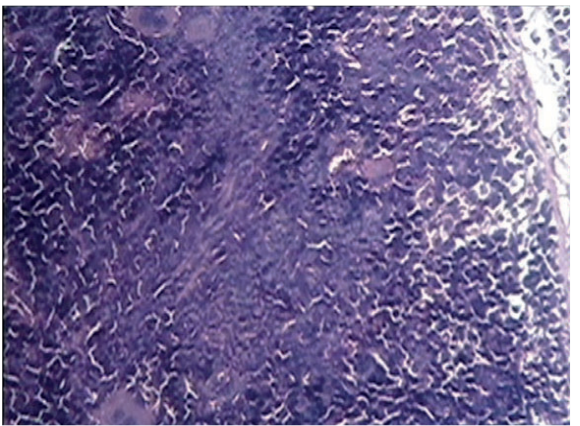
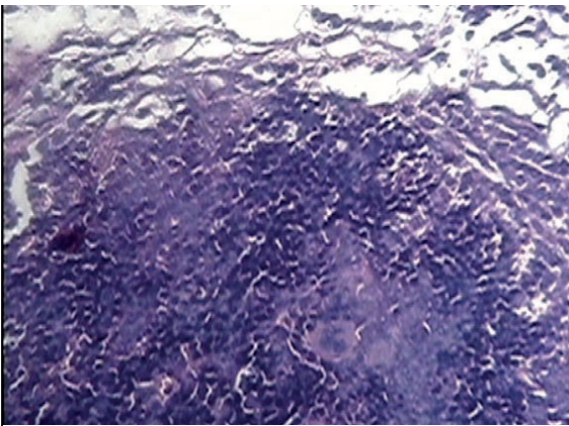


Figure 6. Microimage of the tumor lesion of the spleen in the Nude mice after intraperitoneal transplantation of B16 melanoma mitochondria. Full disappearance of the architectonics of the organ, many destroying megakaryocytes, rapid growth of tumor melanocytic cells in the form of strands and tumor bulges. Hematoxylin-eosin staining. Magnif. x10, x40, x100.

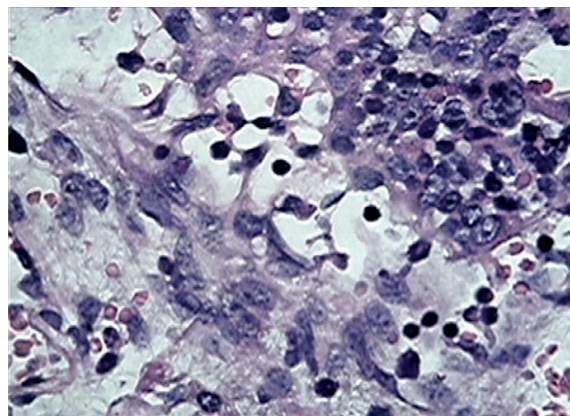
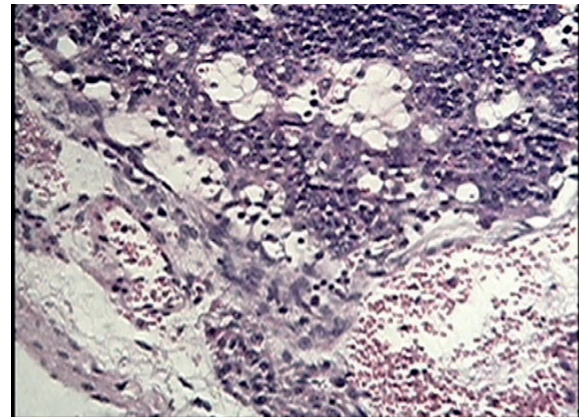
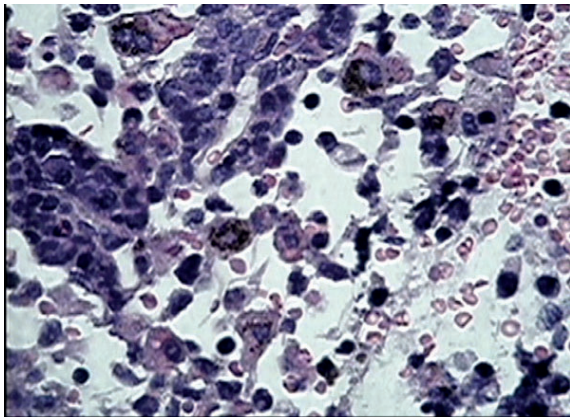
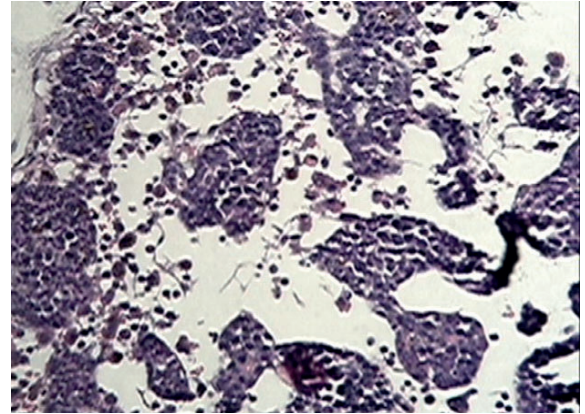
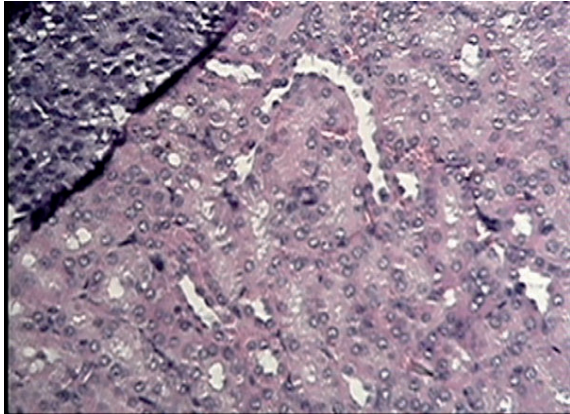
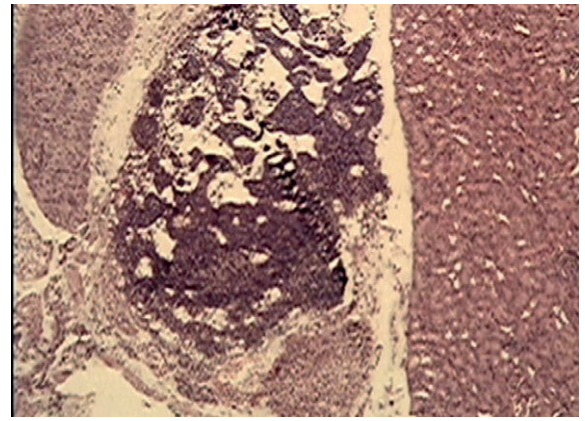
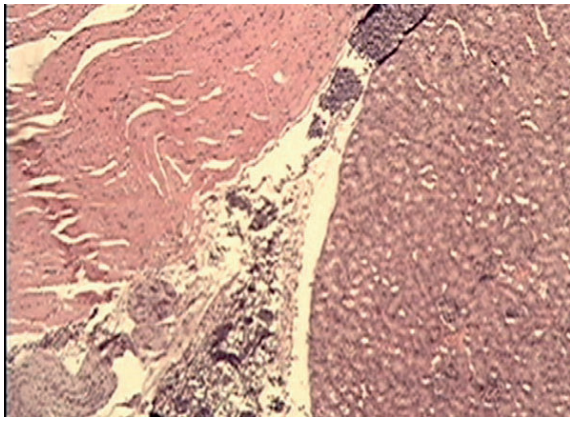


Figure 7. Microimage of a tumor lesion in the perirenal tissue in the Nude mice after intraperitoneal transplantation of B16 melanoma mitochondria. A similar mechanism involves loose connective tissue resources (adipose tissue, blood, fibroblast recruitment) for melanocyte neoplasm. Hematoxylin-eosin staining. Magnif. x10, x40, x100.

implementation of the mitochondrial program. The small foci of tumor cells that appear on its surface in the full sense have a fairly powerful constructing resource and metabolic components, including lipids, polysaccharides, proteins necessary for cellular energy, and a biochemically active free radical medium.

The intraperitoneal transplantation of mitochondria isolated from B16 melanoma was accompanied by the formation of several, not very large, nodules of the melanocytic tumor in the subcutaneous tissue and muscle layer (see Figure 8 herein). On the microphoto one can see how the invasion of microgranules of mitochondria of melanoma B16 takes place against the background of stratification of the muscle fibers and stretching elongated clusters of transformed malignant myocytes. As can be seen, the frequently found foci of hemorrhage make up the area of advancement of the tumor cell array, which repeat the pattern of the muscle fiber bundles. At the maximum magnification of the microscope (x100), the unique construction pattern of the neoplastic melanocytes in parallel bundles, similar to the muscle fiber patterns, was deter-

mined, that indicates a low differentiation and high adaptability of malignant cells.

The morphological analysis of the tissue of the heart and the lungs has born witness to the absence of a tumor lesion of these organs. At the same time, significant blood filling of the vessels, areas of hemorrhage, expansion of the bronchial tree and alveoli were noted in the lungs. Small foci of hemorrhage and necrosis could be seen in the cardiac muscle.

A study of the microstructure of the visceral organs in the Nude mice experienced the intraperitoneal transplantation of B16 melanoma mitochondria suggests the following. In our opinion, the process of mitochondrial carcinogenesis is the realization of some specific properties of mitochondria, primarily associated with the genetic program of mitochondrial DNA, which self-assembles the lost cellular structure of melanocytes from the available resources of the loose connective tissue of the recipient organism, including lipids, polysaccharides, proteins, as well as undertakes recruitment of fibroblasts, as the main scaffold for reprogramming healthy cell populations. This is con-

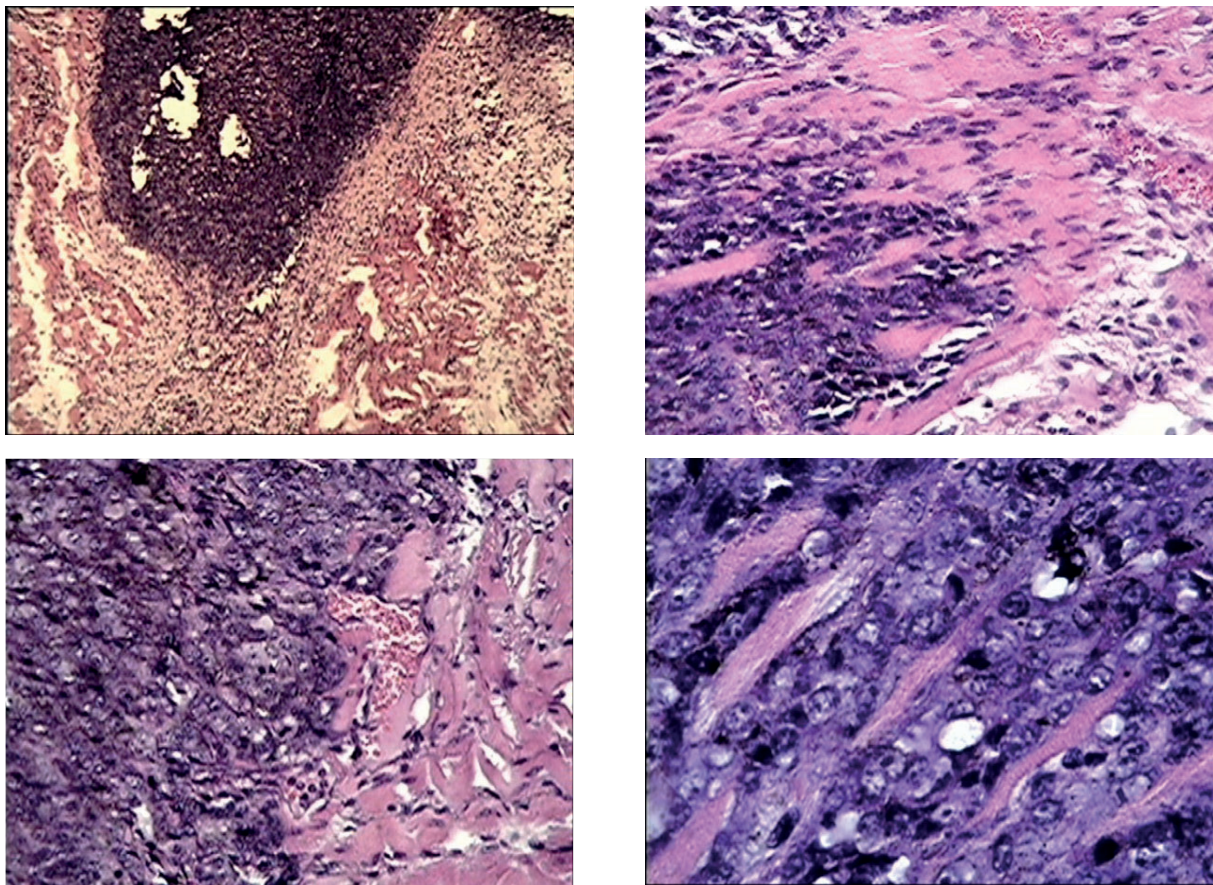


Figure 8. Microimage of the formation of tumor nodules in the skin in the Nude mice after intraperitoneal transplantation of B16 melanoma mitochondria. Tumor-forming conglomerations of mitochondria, which penetrate in the form of strands into the muscle layer of the skin. Hematoxylin-eosin staining. Magnif. x10, x40, x100.

firmed by the development of multiple large and small tumor nodules in the respective tissues forming the paranephric tissue, the mesentery, the interloop spaces of the small and large intestines, the spermatic cord tissue, the subcutaneous tissue and the muscle layer, and a total lesion of the liver and spleen parenchyma is also recorded. It has been noted that with this given 2-week period of mitochondrial carcinogenesis, there is no tumor invasion into the hollow organs: the stomach and the intestines, despite the close adherence of the tumor nodules to their surface. The lungs and the heart are outside the range of the direct tumor influence that does not exclude an indirect influence of the malignant process, confirmed by the morphological signs of functional stress of these vital organs.

The results of the morphological examination of the changes in the internal organs in the experimental animals have made it possible to form the basis for the primary evidence base, which represents a visual series of events recorded in the body of the recipient mice. The latter circumstance indicates the mechanisms of mitochondrial carcinogenesis and the ability of “free” forms of malignant cell mitochondria to accelerate the induction of the secondary process of the tumor progression.

The conducted studies revealed previously unknown properties of mitochondria isolated from B16/F10 skin melanoma, which, when transplanted parenterally, cause the development of the melanoma tumor nodules in the animals, that is confirmed by the morphological examination. This is a previously unknown fact in experimental oncology, which makes it possible to study new properties of melanoma cell mitochondria.

Conclusion

Thus, the use of parenteral transplantation of mitochondria isolated from B16/F10 melanoma in the C57BL/6 and Balb/c Nude male mice caused the growth and development of the melanoma foci in their body. Our study revealed previously unknown properties of mitochondria isolated from B16/F10 skin melanoma, consisting in their ability to induce the development of the melanoma tumor nodules in the body of the animals upon the parenteral transplantation. This makes it possible to study the pathogenesis of malignant growth under the influence of the transplantation of mitochondria isolated from melanoma, and the mechanism of mitochondrial action that induces the development of tumor foci (metastases?) of

melanoma that is important for the clinical practice, since it proves the presence of a new, previously unknown, mechanism of melanoma dissemination.

Statement on ethical issues

Research involving people and/or animals is in full compliance with current national and international ethical standards.

Conflict of interest

None declared.

Author contributions

The authors read the ICMJE criteria for authorship and approved the final manuscript.

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