# Genetic Aberrations in Tissue Inhibitor of Metalloproteinases-3 Lead to Manifestations of the Myofibroblast Phenotype in Mouse Fibroblasts and Fibroblasts of Patients with Sorsby Fundus Dystrophy

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Mutations in the C-terminal domain of tissue inhibitor of metalloproteinases-3 (TIMP-3) lead to autosomal dominant hereditary disease of the retina (Sorsby fundus dystrophy). Mouse knock-out and heterozygous knock-in fibroblasts and fibroblasts from patients with Sorsby fundus dystrophy exhibit some characteristics of myofibroblasts. Genetic changes in the *timp-3* gene (*TIMP-3*) lead to a shift towards the myofibroblast phenotype in the fibroblast culture. It is hypothesized that Timp-3 (TIMP-3) plays a role of anti-transformation agent preventing myofibroblast transformation *in vivo* and that the pathogenesis of Sorsby fundus dystrophy is associated with the loss of the antitransformation function by the mutant protein.

**Key Words:** *Sorsby fundus dystrophy; tissue inhibitor of metalloproteinases-3*



Tissue inhibitor of metalloproteinases-3 (TIMP-3), a representative of the TIMP family, is located in the extracellular matrix (ECM). In addition to inhibition of metalloproteinase activity, this polyfunctional protein participates in many basic processes of cell physiology such as angiogenesis, apoptosis, and cell transformation [2-4]. Hyperproduction of this protein is observed in fibrosis of different location [7,10], while decreased expression is associated with malignant growth [5,8]. Mutations in TIMP-3 C-terminal domain are pathogenetically related to Sorsby fundus dystrophy, a rare autosomal dominant hereditary disease of the retina [14,15].

Transgenic knock-out and knock-in mice, carriers of Ser156Cys (Timp-$3^{S156C}$) mutation, occurring in one of genetic variants of Sorsby fundus dystrophy, were generated for studies of the pathogenesis of this disease [13]. This mutation results in the development of changes characteristic of the initial manifestations of Sorsby fundus dystrophy in the retina [13]. Biochemical characteristics of mouse mutant Timp-$3^{S156C}$, namely, its capacity to form high-molecular-weight complexes, correspond to those of human mutant TIMP-3 [13]. The morphological and biochemical phenotype of mouse knock-out fibroblasts and fibroblasts carrying the mutant protein considerably differs from normal [11]. The expression of mutant Timp-$3^{S156C}$ is constitutive and not regulated by mitogens phorbol-myristate-12-acetate, in contrast to normal protein [1]. Cells producing mutant protein acquire capacity to grow and multiply in serum-free medium without growth factors [1].

Specific morphological features of mutant cells and their behavior in the absence of growth factors suggest that genetic aberrations in Timp-3 associated with Sorsby fundus dystrophy cause transformation of fibroblast phenotype and these cells

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acquire signs of myofibroblasts (cells of mesenchymal origin, intermediate between fibroblasts and smooth-muscle cells) [9,12]. These flat ameba-like cells with large nuclei participate in normal and pathological tissue reparation, for example, in wound healing and fibrosis in foci of chronic inflammation. Myofibroblasts actively secrete cytokines and growth factors (inflammatory response mediators: IL-1, -6, and -10, TNF-α, transforming growth factor-β1, vascular endothelial growth factor, *etc.*) and ECM proteins (types I-VI and XVIII collagens, laminines, matrix metalloproteinases and TIMP) [9]. In addition to the typical morphological and physiological signs, myofibroblasts are characterized by expression of proteins belonging to the filament system of eukaryotic cells: α-smooth-muscle actin (α-SMA), desmin, and vimentin [9,12]. Expression of α-SMA is considered as one of the most demonstrative signs of myofibroblasts.

We evaluated the expression of α-SMA in fibroblasts with genetic aberrations in Timp-3 and studied the signs of myofibroblast transformation (expression of desmin, type I collagen, and growth factor release into conditioned medium) in knock-out mouse fibroblasts.

## MATERIALS AND METHODS

Mouse fibroblast cultures were obtained as described previously [11]. Human fibroblasts from patients with genetic variants of Sorsby fundus dystrophy heterozygous by the corresponding mutations were obtained from gingival mucosa biopsy specimens. Primary fibroblasts of passage 5 were used in the study. All cells were cultured in DMEM containing 10% FCS.

For protein electrophoresis and Western blot analysis the cells were cultured until ~80% confluence, lyzed in electrophoretic buffer for samples, calibrated for total protein using Bradford reagent (Bio-Rad), and electrophoretically separated under reducing conditions. The proteins were then transferred onto Immobilon polyvinylidene fluoride membrane (Millipore); nonspecific binding was supblocked by 18-h incubation in PBS with 0.1% Twin-200 and 0.05% BSA.

Immunodetection was carried out using monoclonal antibodies to α-SMA, β-actin, and desmin (all reagents from Sigma-Aldrich) and type I collagen (Calbiochem). Incubation with the first antibodies diluted 1:1000 in buffer was carried out for 2 h at ambient temperature. The membranes were then washed 3×10 min at ambient temperature in a buffer with 0.1% Twin-200. Second detection was carried out using peroxidase-labeled rabbit antibodies to mouse immunoglobulins (Calbiochem) diluted 1:1500 in a buffer with 0.1% Twin-200 and 0.05% BSA. Incubation with second antibodies was carried out for 1 h at ambient temperature. The membranes were then washed 3×10 min in a buffer with 0.1% Twin-200. The signal was detected using ECL reagent (Amersham).

For immunocytochemical study, the cells were cultured in DMEM with 10% FCS until 30% confluence in Nunc chambers [1]. The preparations were stained with fluorescein isothiocyanate-labeled monoclonal antibodies to α-SMA (Sigma-Aldrich) diluted 1:200 with a buffer with 0.1% Twin-200 and 0.05% BSA. The samples were incubated for 1 h at ambient temperature and washed 3×10 min in a buffer with 0.1% Twin-200. The cells were then examined under a microscope with Opton system (Zeiss).

In order to prepare conditioned medium, knock-out and normal fibroblasts were cultured until 80% confluence in DMEM with 10% FCS. The medium was then removed, the cells were washed 3 times in sterile PBS, and transferred into DMEM without FCS. The cells were left under these conditions for 48 h, after which conditioned medium was collected under sterile conditions and stored at -20°C for further experiments.

For evaluation of the properties of conditioned medium, normal mouse fibroblasts were plated in 9 dishes, cultured until 80% confluence in DMEM with 10% FCS, after which the medium was removed and the cells were washed 3 times with sterile buffer. The cells from 3 control dishes were harvested with trypsin, counted, the mean number of cells per dish was evaluated, and this value was used for calculating the survival/growth ratio for the remaining cells as the initial value (100%). The remaining cells (6 dishes) were cultured in conditioned serum-free medium from knock-out or normal fibroblasts (3 dishes for each variant) for 48 h, after which the cells were harvested with trypsin, their mean number per dish was counted, and survival was evaluated.

## RESULTS

The contents of α-SMA, desmin (myofibroblast markers), and type I collagen (indicator of accelerated ECM synthesis) were elevated in protein extracts of knock-out mouse fibroblasts in comparison with normal fibroblasts (Fig. 1, *a*).

α-SMA was chosen for demonstration of myofibroblast transformation of mouse fibroblasts carrying Timp-$3^{S156C}$ mutation associated with Sorsby fundus dystrophy and fibroblasts obtained from patients with genetic variants of this condition. He-

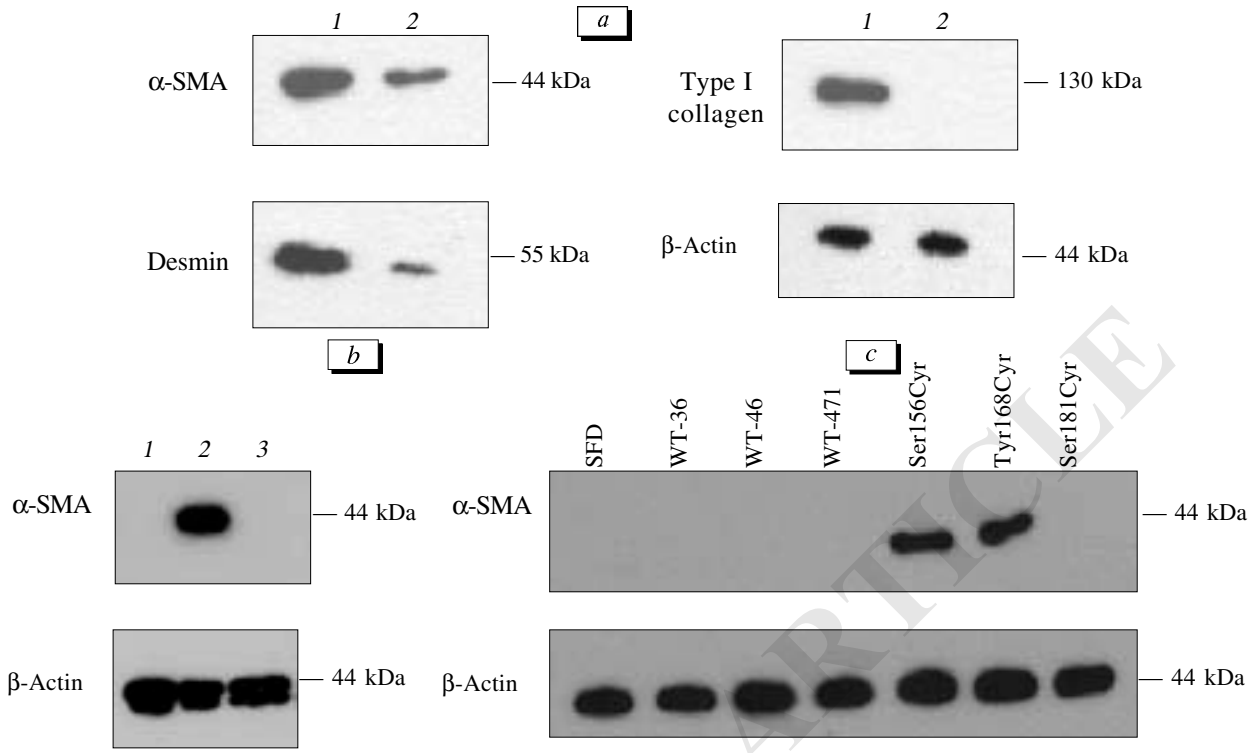


**Fig. 1.** Expression of proteins associated with myofibroblast phenotype by mouse and human fibroblasts. Western blot analysis with cell extracts. The samples are standardized for total protein. The level of constitutive expression of protein (β-actin) is shown for comparison. *a*) α-SMA, desmin, and type I collagen in fibroblast extracts from knock-out (*1*) and normal (*2*) mice; *b*) α-SMA in fibroblast extracts from normal mice (*1*), hetero- (*2*) and homozygous knock-in mice carrying S156C mutation (*3*); *c*) α-SMA in fibroblast extracts from patients with retinal dystrophy not associated with TIMP-3 mutation (SFD), from donors (WT-36, WT-46, WT-471), and from patients with genetic variants of Sorsby fundus dystrophy (Ser156Cys, Tyr168Cys, Ser181Cys).

terozygous knock-in mouse cells intensely expressed this protein, while normal and (unexpectedly) homozygous knock-in mouse fibroblasts did not (Fig. 1, *b*). Similarly, 2 of 3 heterozygous cultures (Ser156Cys and Tyr168Cys) from patients with Sorsby fundus dystrophy expressed α-SMA (Fig. 1, *b*). No α-SMA expression was detected in control fibroblasts from normal subjects (WT-36, WT-46, and WT-471), fibroblasts from a patient with Sorsby fundus dystrophy with Ser181Cys mutation, and fibroblasts from a patient with retinal dystrophy not associated with mutant TIMP-3 variants.

Staining of mouse fibroblasts with specific antibodies showed intensively fluorescing α-SMA fibers in knock-out and heterozygous knock-in Timp-$3^{S156C}$ fibroblasts (Fig. 2). No α-SMA was detected in normal and homozygous knock-in fibroblasts, which was in complete agreement with the data of Western blot analysis (Fig. 1, *b*).

Conditioned medium from knock-out fibroblasts maintained not only survival, but even proliferation (145% survival) of normal fibroblasts without FCS and other growth factors, while conditioned medium from normal cell did not provide sufficient conditions for cell growth (90% survival).

Signs of myofibroblast transformation in knock-out mouse fibroblasts indicate that normal Timp-3 prevents the shift towards the myofibroblast phenotype; hence, it exhibits characteristics of antitransformation agent, is a steroid hormone agonist, and mitogen and proinflammatory cytokine antagonist. This property of Timp-3 suggests a new interpretation of high expression of this protein in foci of chronic inflammation. For instance, hyperproduction of Timp-3 limits the pathological process, preventing myofibroblastic transformation, invasion, and disseminated fibrosis. Hence, high expression of TIMP-3 in fibrosis foci is an antifibrotic compensatory reaction, a defense mechanisms protecting from uncontrolled cell transformation. If Timp-3 production is partially or completely suppressed because of genetic or epigenetic factors, pronounced transformation and invasion are observed, up to the formation of malignant phenotype, like, for example, in glioblastoma [8], breast cancer [6], or prostatic cancer [5].

The expression of α-SMA by not only heterozygous knock-in mouse fibroblasts, but also by human heterozygous fibroblasts from patients with genetic variants of Sorsby fundus dystrophy indi-

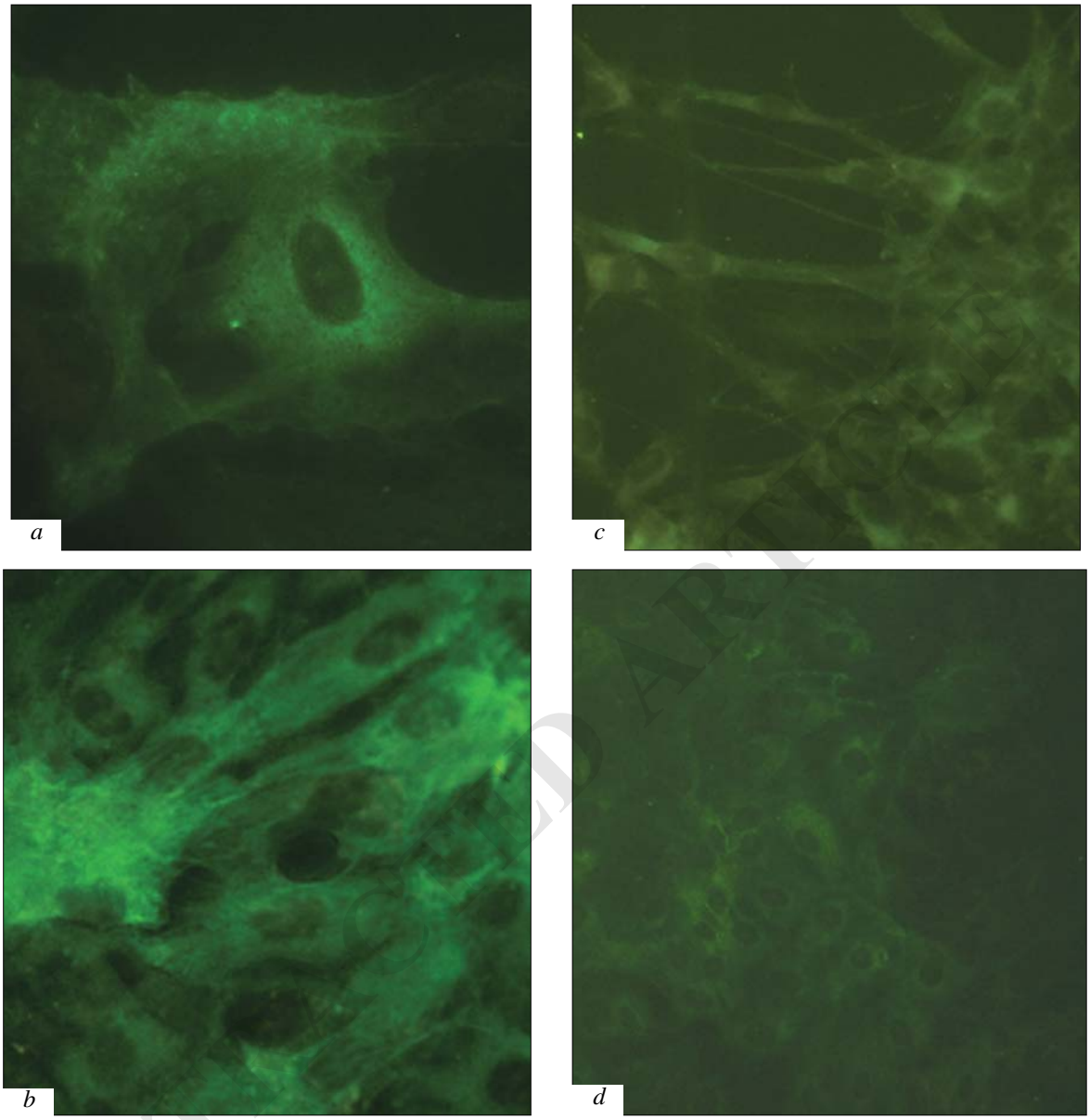


**Fig. 2.** α-SMA in mouse fibroblasts. Immunocytochemical analysis, ×400 (*a*, *b*), ×100 (*c*, *d*). *a*) fibroblasts from knock-out mice; *b*) heterozygous cells carrying Ser156Cys mutation; *c*) normal mouse fibroblasts; *d*) homozygous cells carrying Ser156Cys mutation. *a*, *b*: α-SMA fibers are well seen; *c*, *d*: the signal is not detected.

cates, on the one hand, similarity of the molecular mechanisms of pathological conditions in laboratory animals and humans, and hence, the adequacy of mouse model of Sorsby fundus dystrophy, and, on the other, association of the disease with mutations in TIMP-3 associated with the loss of function.

Homozygous knock-in fibroblasts do not express α-SMA, but exhibit morphological, biochemical, and physiological signs of transformed phenotype [1,11]. This means that Timp-$3^{S156C}$ mutation is presumably associated not only with loss of function of normal protein, but also with acquisition of new properties by the mutant protein. Presumably, mutant Timp-$3^{S156C}$ possesses characteristics of a mitogen stimulating fibroblast transformation in the direction other than the myofibroblast phenotype. We intend to investigate the characteristics of normal and mutant Timp-3 precisely.

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