SCOLIOSIS: A REPORT TO THE TEACHER

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It is with gratitude that I dedicate my work to the teacher, Ya.L. Tsivyan, who not only provided a subject for research, but also, on his own example of a person devoted to his work, brought up a generation of scholars for whom life and science are inseparable.
The paper presents the results of many years of research on idiopathic scoliosis in the form of a report to the teacher. Several fundamental topics were considered:
1) for the first time in world practice, it was established, on the basis of a study of 50 patients with idiopathic scoliosis, that the etiological factor of scoliosis is ectopic localization of neural crest derivatives, which are not genetically determined to chondrogenic differentiation and the growth process, in the vertebral body growth plate;
2) a local disturbance of chondrogenesis in the vertebral body growth plate is the cause of the growth asymmetry and formation of spinal deformity in idiopathic scoliosis;
3) the degree of structural changes in the spine and the prognosis of the deformity progression depend on the level of disturbance of the morphogenetic processes in the vertebral body growth plate embedded in embryogenesis;
4) it is supposed to confirm the proposed hypotheses by inhibition of the *PAX3* gene in the chick embryo model of idiopathic scoliosis and to get answers to many more unclear questions concerning scoliotic disease.
Key Words: idiopathic scoliosis, vertebral body growth plates, proteoglycans, scoliosis model.
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Science is not and will never be a closed book.
Each important success arouses new questions, and any development
reveals new, even deeper, challenges as time passes.
A. Einstein

It all started with a dialogue. Professor Ya.L. Tsivyan: "There is a disease, scoliosis, that is very difficult to study. Very little is known about it, and all the histologists have refused to deal with it. Would you like to accept this challenge? I know you can do it". Junior researcher A.M. Zaydman: "Scoliosis? Of course I will accept it! I was going to ask you to let me focus on this issue". Professor Ya.L. Tsivyan: "I am operating tomorrow. Please be prepared. I want you to be my assistant and examine everything we resect. You need to find what causes scoliosis. This is your way".

And that is how the new stage in treating idiopathic scoliosis, which had been introduced to orthopedic practice by professor Ya.L. Tsivyan, has started. For me, it has become a life-long study.

We were studying everything that was resected during the surgery: osseous tissue, intervertebral discs, growth plates, muscle tissue, etc. We operated patients with congenital, idiopathic, and paralytic scoliosis. From time to time the teacher would ask: "Have you found anything?" And one day I finally answered: "Yes, I have". It was the growth plate that has shed some light on the direction for future research, for the first time over these long years (ten experimental models, genetic studies of idiopathic scoliosis and Scheuermann's disease, major gene dependency and, finally, neural crest cells observed in the growth plates). "I think I have found what we are looking for..."

Many hypotheses related to idiopathic scoliosis have been proposed between the ancient period and today. Each research stage corresponded to social development levels and the existing methodology. None of the theories, including the genetic one, had revealed the essence of idiopathic scoliosis by that time. It would be incorrect to think that prior research has made no contribution to studying idiopathic scoliosis. Each hypothesis has laid the basis for reinterpreting and continuing the search at a new level of knowledge. Although significant progress in science has been made, each article focused on idiopathic scoliosis is currently still opened with the following sentence: "The etiology and pathogenesis of idiopathic scoliosis remain unknown". Why is it so? A plausible reason for that is the approach (the method) used to search for the etiological factor of idiopathic scoliosis. Let me dwell into the details of the concepts that are based on the imbalance of growth of the skeletal and nervous systems in addition to other factors (it is just impossible to study their sequence) [1, 2]. Indeed, the embryogenetic and postnatal development of the spine and spinal cord is characterized by certain periods of growth, like all other structures do. In humans, until the age of three months, the spinal cord is completely located within the spinal canal and both structures (the spinal canal and the spinal cord) have the same longitudinal dimensions (the periods of growth in animals are different, but the processes are similar). This corresponds to the level and needs of embryonic development. After the age of three months, the

lumbosacral spine starts to grow: it is the stage of cauda equina formation in humans and all mammals. As a result, the spinal cord and its roots rise. During growth, they form the cauda equina providing innervation to the formed pelvis and extremities. The embryo now can make its first movements. The lumbar curve acquires its lordosis. The thoracic spine is not involved in growth imbalance, but mild kyphosis develops according to the laws of biomechanics. This process of human and animal skeleton formation is rather slow. Scoliotic changes in the spine caused by growth imbalance never occur in animals. Why is torsion formed in the thoracic spine? The so-called imbalance of spine and spinal cord growth is an evolutionary genetically and conservatively fixed mechanism that results in formation of a stable structure of the animal and human bodies. This structure ensures a certain range of motion used both when performing physical work and creating various art masterpieces. The extensive experience of the author of this publication, who is simultaneously a clinician and a researcher, has made it possible to discuss the problem of idiopathic scoliosis from the perspective of general biology.

Idiopathic scoliosis is a disease (the "scoliotic disease"). The term "scoliotic disease" was coined by my teacher, Ya.L. Tsivyan. Each disease is characterized by the following parameters: 1) localization of the process; 2) the pathological substrate; 3) the prime cause (the etiological factor of the disease). The growth plate is a local factor in idiopathic scoliosis. Long-term (> 50 years) studies focused on the structural components of the spine (> 1,000 specimens) have shown that poorly differentiated chondroblasts are located in the vertebral body growth plate on the concave side of the deformity. These chondroblasts do not properly go through the differentiation stages, resulting in growth impairment. On the convex side of the deformity, histogenesis corresponds to that for the control samples (is unchanged). Therefore, the process takes place within the growth plate. The substrate of the disease is poorly differentiated cells (chondroblasts). The impaired cell differentiation and proliferation are the reasons for growth asymmetry and development of spinal deformity. The subsequent sections of this publication focus on the cause of growth asymmetry. This approach was a new stage in searching for the etiology of idiopathic scoliosis.

An analysis of gene expression in chondroblasts within vertebral body growth plates in patients with idiopathic scoliosis

Despite the numerous studies and a large body of available data, the genetic mechanisms of the disease remain unclear. However, if the genes whose hyper- or hypoexpression causes idiopathic scoliosis or is associated with it were identified, this would open the way for early diagnosis, prognosis, prevention, and probably correction of this pathology using modern molecular biology techniques such as genome editing (CRISPR) [3].

A joint study conducted by researchers of the Novosibirsk Research Institute of Traumatology and Orthopedics and the Institute of Cytology and Genetics, SB RAS, revealed that idiopathic scoliosis is a genetically determined spinal deformity; there is a gene mutation associated with it. It was shown the deformity does not develop in individuals not carrying this mutation [4]. Therefore, patients with idiopathic scoliosis carry the mutated gene whose dysfunction alter normal spine growth. Morphological and biochemical studies [7] in patients with idiopathic scoliosis show that growth asymmetry is the pathogenetic mechanism of spinal deformity development. It is fair to assume that growth asymmetry is based on genetic dysregulation of spine growth on the concave side of the deformity. Let me dwell on the key studies without mentioning the long way that has been travelled in attempts to find the truth.

During the postnatal period, the entire vertebral body undergoes osteogenesis, except for the narrow cartilaginous plate (the growth plate) that is responsible for vertebral growth. This growth takes place due to proliferation, differentiation of poorly differentiated chondroblasts into hypertrophic chondrocytes, and subsequent osteogenesis [8]. Since the growth plate is directly involved in vertebral growth, it is reasonable to study gene expression in the growth plate (i.e., in the region where the pathology is located), whereas blood samples from patients are used for analysis in most genetic studies [9, 10]. Therefore, we studied gene expression in the chondroblasts in vertebral body growth plates in patients with idiopathic scoliosis having the most prominent signs of the disease.

The genes regulating the function of the vertebral body growth plate in patients with idiopathic scoliosis were selected according to the biochemical and morphological data on the disturbed structural organization of cells and the growth plate matrix on the concave side of the spinal deformity. The concave side of the growth plate deformity has no zonal structure and is represented by randomly arranged poorly differentiated chondroblasts accompanied by the dystrophized matrix. The growth plate structure on the convex side of the deformity is preserved; the columnar layer is represented by well-differentiated cells (Fig. 1).

Expression of 17 genes was studied by real-time PCR. These genes included the *TGFR1*, *EGFR*, *IGF1R*, and *GHR* genes involved in regulation of spine growth; the *SOX9, PAX1, PAX9*, and *IHH* genes regulating chondrogen differentiation; the *ACAN*, *LUM, VCAN, COL1A1, COL2A1*, and *HAPLN1* genes determining the structural and functional features of the cartilage matrix, and the *SLC26A2*, *CHST1*, and *CHST3* genes involved in proteoglycan sulfation (i.e., the process that is fundamentally important for the extracellular matrix formation).

The study focused on expression of the genes involved in spine growth regulation has revealed a high level of mRNA of the transforming growth factor receptor 1 *(TGFR1)* gene in chondroblasts in patients with scoliosis compared to the control ($P < 0.05$; Fig. 2). *TGFR1* is a proteoglycan found in the membrane functioning as a TGF co-receptor. The intracellular substrates of activated receptors are involved in transcriptional regulation of the genes recruited in cartilage and bone forma-

tion [11, 12]. The key role of *TGFR* in cartilage tissue consists in negative regulation of chondrocyte differentiation.

The *EGFR* mRNA level in the samples derived from patients with scoliosis was higher compared to that in control samples (Fig. 2). EGFR is a cell surface protein binding the epidermal growth factor. The epidermal growth factor receptor gene positively regulates cell proliferation. *EGFR* signaling plays an important role in remodeling of the extracellular matrix of proteoglycan cartilage into bone tissue during intercartilaginous bone formation. The reduced *EGFR* expression level decreases cellular response to EGF, disrupts the differentiation of proteoglycan cells, and therefore slows down the growth [13].

Expression of the growth hormone receptor *(GHR)* gene (whose function is to positively regulate cell differentiation [14]) was lower in scoliotic cells compared to control chondroblasts (Fig. 2). Since growth hormone is one of the key hormones regulating the metabolism of cartilage tissue cells, it may indicate that the metabolic rate in cells is reduced in patients with idiopathic scoliosis.

Therefore, chondroblast differentiation in the vertebral body growth plate in patients with scoliosis may be disrupted because of the high expression level of TGF receptor gene and low expression level of *GHR* mRNA. In other words, the differentiation, metabolism, and growth in growth plate cells are slowed down, which agrees with the morphological data. The increased expression level of the *EGFR* gene potentially contributes to chondrocyte proliferation, while simultaneous changes in expression of the *TGFR1* and *GHR* genes lead to accumulation of poorly differentiated cells and/or disturbed differentiation of growth plate cells. The low level of *EGFR* expression in control chondroblasts compared to those derived from patients with scoliosis can be related to the embryonic development period (when endochondral ossification has not begun yet and no active proliferation of growth plate cells is required). The expression levels of the gene encoding insulin-dependent growth factor receptor *(IGF1R)* were identical in cells of scoliosis patients and in the control samples (Fig. 2). *IGF1R* exhibits a direct effect on insulin-like growth factor 1, which is produced by proliferating and hypertrophic chondrocytes and induces their proliferation [15]. Since chondroblasts of patients with scoliosis were not characterized by any statistically significant difference in *IGFR* expression level, it is fair to assume that *IGFR* is not involved in the disturbance of proliferation of growth plate cells.

An analysis of expression of the genes regulating chondroblast differentiation showed that the expression levels of the *PAX1* and *IHH* genes in growth plate cells in patients with scoliosis are statistically significantly higher, while the expression levels of the *SOX9* and *PAX9* genes are lower compared to those in the control samples (Fig. 3). Transcription factor *SOX9* is expressed both during the embryonic period (cartilage thickening) and during the later stages in growth plate chondrocytes. *SOX9* is involved in chondrogenesis by inducing chondroblast differentiation and endochondral bone formation [16, 17]. *COL2A1*, the gene encoding the major collagen in the cartilage, is one of its regulatory targets [18]. Therefore, improper chondrocyte differentiation in the growth plate in patients with scoliosis can be associated with low expression level of the *SOX9* gene.

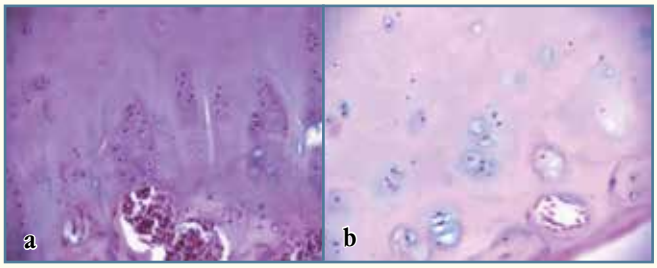

Fig. 1
The vertebral body growth plate in a patient with grade III–IV idiopathic scoliosis. **a** – the convex side of the deformity: the structure of the growth plate is preserved, the columnar layer consists of well-differentiated cells; the positive staining with Hale's colloidal iron stain is indicative of high degree of polymerization of proteoglycans in the cytoplasm and the matrix; 20 × 10; **b** – the concave side of the deformity: disturbed zonality, disorderly arrangement of poorly differentiated cells, staining with Hale's colloidal iron stain is insufficiently prominent; 20 × 10

PAX is the family of the genes encoding DNA-binding proteins, which are intranuclear transcription factors. The *PAX1* and *PAX9* genes play a critical role in axial skeleton development during embryogenesis and control cell differentiation and proliferation during early sclerotome formation [19]. The gene expression level plays a critical role in chondrogenic differentiation of sclerotome cells [19]. The expression levels of the *PAX1* and *PAX9* genes significantly contribute to chondrogenic differentiation of sclerotome cells [20]. Since the genes under study exhibit an interdependent effect, and the chondroblasts of patients with scoliosis show a reduced expression level of the *PAX9* gene, while expression of the *PAX1* gene is significantly increased ($P < 0.05$), this may indicate that the regulation of the genes responsible for chondrogenic differentiation is disturbed.

The *IHH* gene is normally expressed in prehypertrophic chondrocytes and plays a key role in differentiation of growth plate chondrocytes [21]. Through the feedback mechanism, it regulates the parathyroid hormone-related peptide (PTHrP), which in turn inhibits the differentiation of proliferating chondrocytes. *IHH* induces the synthesis of PTHrP, thus indirectly slowing down chondrocyte hypertrophy and maintaining cells in a nonhypertrophic proliferative state [22]. In the *IHH* pathway, parathyroid hormone related peptide is one of the two main signaling pathways controlling the chondrocyte proliferation and differentiation. Therefore, the disturbance of chondrocyte differentiation in the vertebral body growth plate in patients with scoliosis can be caused by the features of expression the genes regulating chondrocyte differentiation: an increased expression level of the *IHH* gene, a low expression level of the *SOX9* gene, and the disturbed interaction between the *PAX1* and *PAX9* genes.

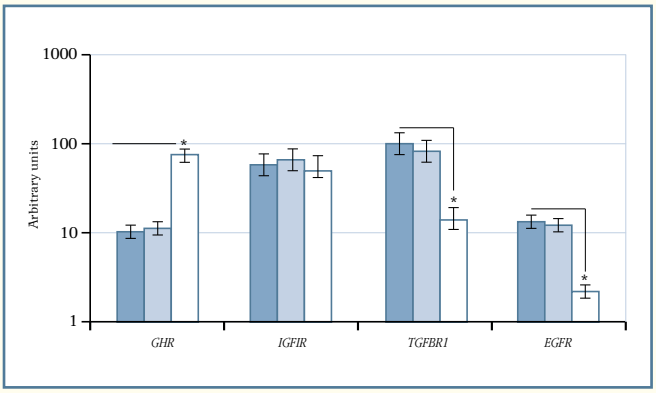

Fig. 2
The relative expression level of the genes involved in regulation of spine growth in chondroblasts of vertebral body growth plates in patients with idiopathic scoliosis and chondroblasts of the fetal spine: deep blue bars denote the convex side of the deformity; light blue bars denote the concave side of the deformity; white bars are the control (fetal spine). The data are presented as the mean value SD; *statistically significant differences between the samples collected from the patients with scoliosis and the control samples ($P < 0.05$). The relative expression level was calculated with respect to the GAPDH gene

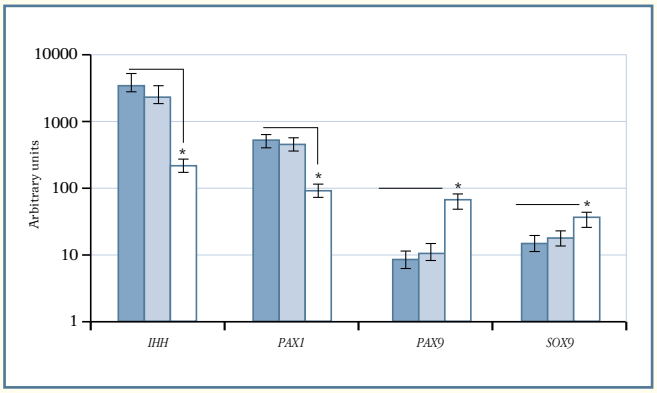

Fig. 3
The relative expression level of the genes regulating the chondrogenic differentiation in chondroblasts of vertebral body growth plates in patients with idiopathic scoliosis and chondroblasts of the fetal spine: deep blue bars denote the convex side of the deformity; light blue bars denote the concave side of the deformity; white bars are the control (fetal spine). The data are presented as the mean value SD; *statistically significant differences between the samples collected from the patients with scoliosis and the control samples ($P < 0.05$). The relative expression level was calculated with respect to the GAPDH gene

A study focused on expression of the genes responsible for the structural and functional features of the cartilage matrix revealed that the expression levels of mRNA genes encoding the synthesis of the major proteoglycans in cartilage tissue (*ACAN*, *LUM*, and *VCAN*) and the genes encoding type I and II collagen (*COL1A1* and *COL2A1*) in samples taken from patients with scoliosis and control ones did not differ significantly (Fig. 4). The *ACAN* gene having alternative transcript variants encodes aggrecan, the most representative chondroitin sulfate proteoglycan forming the main part of the extracellular matrix in cartilage tissue. Aggrecan is involved in binding of hyaluronic acid and proteins; together with type II collagen, it forms the cartilage matrix [16]. Versican (encoded by the *VCAN* gene), a large chondroitin sulfate proteoglycan, is also present in the extracellular matrix; it is involved in binding of glycosaminoglycans and hyaluronic acid and partakes in adhesion, proliferation, and cell migration. The *LUM* gene encodes lumican, the major keratan sulfate proteoglycan belonging to the family of leucine-rich proteoglycans. Lumican is a component of the structural extracellular matrix and is involved in collagen binding, as well as organization and regulation of collagen fibrils. Fibril-forming type I collagen is typical of the poorly differentiated cartilage tissue and is mainly found in bone and tendons. Type I collagen is a component of the structural extracellular matrix involved in endochondral ossification. The *COL2A1* gene encodes the alpha-1 chain of type II collagen specific to mature cartilage tissue. Being an essential component of the extracellular matrix, type II collagen is important for normal functioning of the cartilage tissue as it ensures tissue strength and elasticity [23, 16]. Since no differences in the expression levels of the genes coding for collagen and those encoding cartilage matrix proteoglycans were found in the samples derived from patients with scoliosis and control samples, it is fair to assume that no dysfunction of the genes under study takes place in patients with scoliosis.

The level of *HAPLN1* mRNA in the cells collected from patients with scoliosis was lower compared to that in the control samples. The low expression level of the *HAPLN1* gene indicates that the degree of polymerization of proteoglycans in the growth plate in patients with idiopathic scoliosis is reduced (the product of the *HAPLN1* gene binds hyaluronic acid residues [24], which agrees with the morphological data (the low degree of polymerization of proteoglycans)) [7].

The biochemical data [7] regarding the disturbed synthesis of glycosaminoglycans in the vertebral body growth plate on the concave side of the deformity in children with scoliosis inspired the researchers to study the genes encoding the proteoglycan sulfation process (i.e., the sulfotransferase 1 gene *(CHST1)*, the sulfotransferase 3 gene *(CHST3)*, and the sulfate transmembrane transporter gene *(SLC26A2)*). It has been shown that the lack of proteoglycan sulfation can occur due to inactivation of the genes encoding sulfate transporters, resulting in abnormal matrix structure and growth process [25]. The disturbance of proteoglycan sulfation may cause various pathologies [26]. An analysis of expression of the genes involved in proteoglycan sulfation showed that the mRNA levels for the *CHST3* and *SLC26A2* genes in the samples collected from patients with scoliosis were

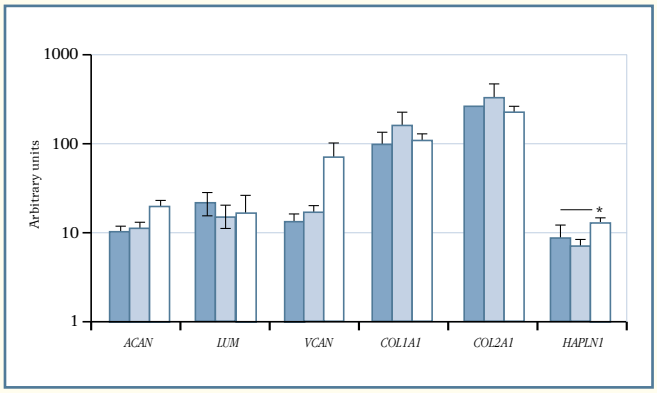

Fig. 4
The relative expression level of the genes responsible for the structural and functional features of the cartilage matrix in chondroblasts of vertebral body growth plates in patients with idiopathic scoliosis and chondroblasts of the fetal spine: deep blue bars denote the convex side of the deformity; light blue bars denote the concave side of the deformity; white bars are the control (fetal spine). The data are presented as the mean value SD; *statistically significant differences between the samples collected from the patients with scoliosis and the control samples ($P < 0.05$). The relative expression level was calculated with respect to the GAPDH gene

higher, while the expression level of the *CHST1* gene did not differ from that in the control samples (Fig. 5). The sulfate transporter *(SLC26A2)* is a transmembrane glycoprotein involved in the pathogenesis of several types of chondrodysplasia. The *SLC26A2* gene plays a critical role in proteoglycan sulfation in cartilage tissue and structural organization of the cartilage matrix, ensuring sulfate transfer to chondrocytes and adequate sulfation. The previously noted disturbance of proteoglycan sulfation [7] observed on the concave side of the deformity in patients with idiopathic scoliosis contradicts the high expression level of the *SLC26A2* gene. It is quite possible that disturbed sulfation occurs at a stage other than the sulfate transfer stage. The *CHST3* gene encodes an enzyme catalyzing sulfation of chondroitin proteoglycans, which undergo differentiation in the extracellular matrix and most cells. The biochemical data on reduced amount of chondroitin sulfate and simultaneously increased amount of keratan sulfate on the concave side of the deformity in patients with scoliosis [7] are not consistent with the data on the expression level of the *CHST1* and *CHST3* genes. It is quite possible that regulation of the function of these genes is performed at the transcription level and/or the genes involved in alternative reactions are also activated in these cells.

The resulting data on expression of all the genes under study were statistically analyzed. Factor analysis revealed fundamental differences in the gene expression profiles for the samples collected from patients with idiopathic scoliosis and the control ones (Fig. 6).

The morphogenesis of any tissue depends on expression of the respective genes. Disturbance of the gene interaction or any disruption in the "gene–the final product" chain yields abnormal structures [8]. The structure of cartilaginous tissue, as well as endochondral ossification, is determined by the coordinated function of cells and the matrix consisting of collagen fibers and proteoglycans, which depend on expression of the genes regulating the synthesis, transport, and attachment of the components of proteoglycan molecules. An analysis of expression of the genes determining the structural and functional features of the cartilage matrix showed no differences in mRNA levels in the genes in control and scoliosis samples. The genes encoding the major components of the cartilage matrix (aggrecan, versican, lumican, and type II collagen) are expressed in the cells of vertebral bodies and embryonic spine cells. These data agree with the fact that the growth plate is a section of cartilage tissue represented by the intercellular matter and chondroblasts having varying differentiation degrees; the proportion of poorly differentiated cells is quite high as evidenced by the high level of type I collagen. These findings confirm that the growth plate is a derivate of embryonic cartilage. All the cell differentiation processes taking place in the growth plate are actually the postnatal continuation of embryonic morphogenesis, which allows one to use the embryonic spine cells as a control [27].

An analysis of expression of the genes regulating chondroblast differentiation, as well as functioning and the components of the extracellular matrix in vertebral body growth plates in patients with grade III–IV idiopathic scoliosis, revealed genes whose expression levels were identical to those in control samples. Therefore, it is fair to assume that the detected disturbance in scoliosis patients is not related to the function of the *IGF1R*, *ACAN, LUM, VCAN, COL1A1, COL2A1,* and *CHST1* genes. On the other hand, genes having elevated or reduced expression levels in patients with scoliosis were identified. Morphological data (Fig. 1) show abrupt changes in structural organization of cells and the growth plate on the concave side of spinal deformity, zonality disturbance, disorderly arrangement of rare poorly differentiated chondroblasts, no proliferative activity or synthetic potential, and dystrophic changes in the matrix. These changes could be caused by the reduced expression level of the *GHR, PAX*, and *SOX9* genes, which play a key role in chondroblast differentiation. The reduced degree of proteoglycan sulfation [7] is probably caused by the low expression level of the *HAPLN1* gene in scoliosis patients. The high expression level of the *TGFR1* and *EGFR* genes compared to those in the control samples, as well as *SOX9* and *GHR* hypoexpression, demonstrate that the normal function of receptors to growth factors and transcription factors is disturbed in cells of scoliosis patients. The observed imbalance may indicate that there is a lack of growth factors or mediator molecules and can also be related to the formation of mixed-phenotype cells not responding to normal differentiation signals.

The hyperexpressed *CHST3, PAX1*, and *IHH* genes hold a special position among all the studied genes in patients with scoliosis. These genes seem to make the most significant con-

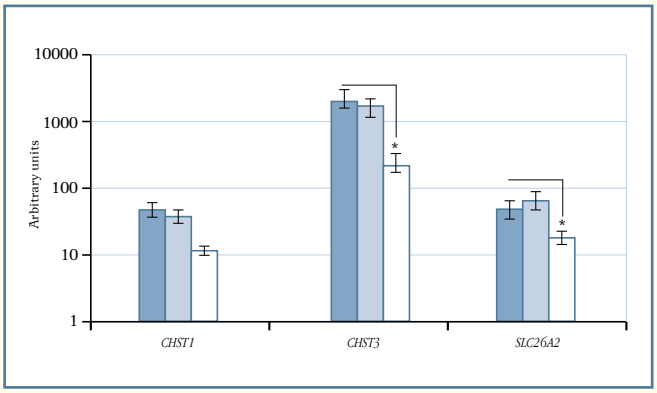

Fig. 5
The relative expression level of the genes encoding proteoglycan sulfation in chondroblasts of vertebral body growth plates in patients with idiopathic scoliosis and chondroblasts of the fetal spine: deep blue bars denote the convex side of the deformity; light blue bars denote the concave side of the deformity; white bars are the control (fetal spine). The data are presented as the mean value SD; *statistically significant differences between the samples collected from the patients with scoliosis and the control samples ($P < 0.05$). The relative expression level was calculated with respect to the *GAPDH* gene

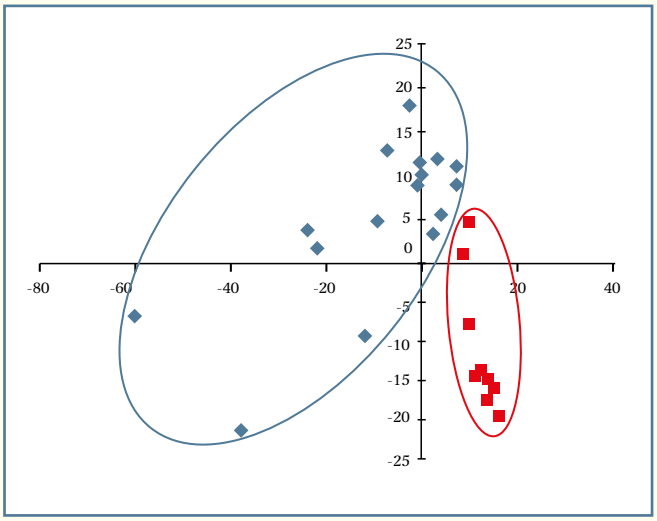

Fig. 6
The results of factor analysis: the expression profiles of the genes involved in regulation of spinal growth (*TGFR1, EGFR, IGF1R*, and *GHR*), the genes regulating chondrogenic differentiation (*SOX9, PAX1, PAX9,* and *IHH*), the genes responsible for the structural and functional features of the cartilage matrix (*ACAN, LUM, VCAN, COL1A1, COL2A1,* and *HAPLN1*), and proteoglycan sulfation genes (*SLC26A2, CHST1,* and *CHST3*) in chondroblasts of vertebral body growth plates in patients with grade III–IV idiopathic scoliosis (shown in deep blue) and chondroblasts of the fetal spine (shown in red) are characterized by statistically significant differences ($P < 0.01$); factor coordinates of the variables based on correlations are plotted along the vertical and horizontal axes

tribution to the development of pathological changes in the vertebral body growth plate in patients with idiopathic scoliosis. The unbalanced expression of the *PAX* genes probably disturbs the chondrogenic differentiation of cells, while the unbalanced expression of the *IHH* gene slows down chondrocyte hypertrophy, maintaining the non-hypertrophic proliferative status of the cells. That is the probable reason for the characteristic morphological pattern observed on the concave side of the deformity in patients with idiopathic scoliosis.

Another key aspect related to scoliosis is the disturbance of proteoglycan sulfation in the growth plate. Since recent studies have been emphasizing the significant contribution of the *CHST3* gene to locomotor disorders [28], it might potentially have some influence on scoliosis as well. Since proteoglycan sulfation is regulated by a gene complex, and expression of the *CHST3* and *SLC26A2* genes is disturbed in the samples from patients with scoliosis compared to the control samples, it is fair to assume that the functions of the main sulfation genes are mismatched. As a result, sulfate-containing groups unrelated to hyaluronic acid are synthesized, and proteoglycans eventually have a low polymerization degree.

A comprehensive analysis of 17 genes revealed no disturbances in expression of the genes encoding the synthesis of collagen and proteoglycans in the vertebral body growth plate in patients with idiopathic scoliosis. Meanwhile, genes whose expression was disturbed in patients with scoliosis were also identified. Malfunction of the genes responsible for chondrocyte differentiation (*PAX1, PAX9,* and *IHH*), the genes encoding receptors to transcription factors and growth factor (*SOX9, TGFR1*, and *GHR*), and proteoglycan sulfation genes (*SLC26A2* and *CHST3*) gives rise to characteristic morphological and functional changes in the vertebral body growth plate on the concave side of scoliotic deformity. The detected morphogenesis and growth disturbances may indicate that there are cells having a different phenotype, which are not typical of the tissue under study and cannot respond to differentiation signals. Factor analysis has verified this conclusion by demonstrating that growth plate cells and control chondroblasts belong to separate groups; i.e., they fundamentally differ in terms of expression levels of the genes discussed above (Fig. 6).

Hence, we have revealed the gene expression profile typical of chondrocytes in the vertebral body growth plate in patients having severe idiopathic scoliosis: the imbalance between the genes responsible for chondrogenic differentiation, the genes encoding receptors to growth factors and transcription factors, and the genes involved in proteoglycan sulfation. These findings agree with the morphological and biochemical data and can be a marker of the pathology.

Factor analysis revealed that the phenotype of growth plate chondroblasts differs significantly in patients with idiopathic scoliosis. These cells do not perceive signals from growth factors and growth hormone. It has become necessary to identify the

phenogenotype of these cells. The cells were identified in the cell culture as it is known that cells within the culture undergo the early morphogenesis stages. Cells of vertebral body growth plates in patients with idiopathic scoliosis were identified at the next stage [29].

Ectopic localization of neural crest cells is an etiological factor of scoliosis

The real-world data showed that cells having different phenotypes are identified in vertebral body growth plates in patients with idiopathic scoliosis depending on localization. Cultured cells of the growth plate of the convex side of the deformity were identified to be chondroblasts (Fig. 7). The morphological structure (including the ultrastructural organization (Fig. 8)), synthesis of organ-specific proteoglycans, and expression of growth-related genes (Fig. 9) were the criteria for evaluating the chondrogenic differentiation of cells [30].

The cultured cells isolated from the growth plate on the concave side of spinal deformity in patients with idiopathic scoliosis were identified to be neuroblasts and glioblasts (Fig. 10). Morphologically, neuroblasts are multi-, uni-, bipolar, and pseudounipolar cells that form multiple contacts with cellular processes and cell bodies. The cytoplasm was found to contain the Nissl substance (Fig. 11); neurospecific proteins III-tubulin, NF1 and NF200 were expressed (Fig. 12).

The electron microscopy data demonstrated that the cells under study had a neuronal origin as evidenced by the long neurofilament network, the vesicle-containing sinuses being formed and already formed ones, as well as the typical elongated mitochondria. Numerous axon hillocks containing vesicles were located in the cellular processes and bodies (Fig. 13). Numerous contacts were revealed between the processes and the cells. The second type of cells was round cells containing many contact-forming processes. Glial proteins were expressed in the processes and cells stained using the Gömöry and Cajal's method (Figs. 14 and 15). The morphological and ultrastructural data allowed us to recognize this cell type as glioblasts. The scanning electron microscopy data for the cells derived from the growth plate on the concave side of the deformity showed that cells had an elongated shape and long contact-forming processes. Synapses and a large spinelet between the cells are seen. The cellular cytoplasm and processes contain dense granules 300–500 nm in diameter and numerous spinelets on neuronal processes (the non-sprayed specimen; Figs. 16 and 17).

A question naturally arises: why are the cells having a neuronal origin located in growth plates in patients with idiopathic scoliosis? In order to answer this question, let us consider the early stages of embryogenic development.

The spine is known to be formed from the mesenchyme [31]. Meanwhile, during neural tube formation neural crest cells are differentiated from it and migrate via three paths [32, 33].

One of the paths of neural crest cell migration is the trunk migration pathway running through the anterior (rostral) portion of the sclerotome; these cells eventually form sensory ganglia [34]. The migrating cells undergo epithelial–mesenchymal transition by switching the expression of neural cell adhesion proteins to mesenchymal adhesion proteins [35].

Having become round-shaped, neural crest cells acquire the phenotype of mesenchymal cells and do not differ from the surrounding cells [36]. This process is caused by the subsequent migration of neural crest cells along the mesenchymal substrate lining up the path between the neural tube and the somites. Substrate formation is related to the expression of the *PAX3* gene and synthesis of two versican isoforms (V1 and V0) [37]. The path of motion of neural crest cells is determined by asymmetrical distribution of induction (V0 and V1 versicans) and inhibition factors [38]. Neural crest cell movement is inhibited by aggrecan, a well-polymerized proteoglycan because GAG side chains restrict the dispersion of neural crest cells [39]. Mesenchymal cells in the sclerotome undergo chondrogenic differentiation during the migration of neural crest cells. These data indicate that chondrogenesis and gangliogenesis are interrelated processes [40]. Moreover, sclerotome removal disables the formation of sensory ganglia, while impaired sclerotome segmentation results in formation of "ugly" ganglia [41]. Therefore, the migration of neural crest cells through the sclerotome is a regularity; moreover, time-correlated regulation of morphogenesis of the spine and sensory ganglia is also observed [42].

The migration of neural crest cells through the sclerotome is one of the stages of spine and sensory ganglia formation. Taking into account the tropism of these two structures, it is unclear to what extent the function of sensory ganglia is impaired in patients with idiopathic scoliosis. The answer to this question is yet to be found. The presence of neural crest cell derivatives in the vertebral body growth plate in patients with idiopathic scoliosis is undoubtedly the disruption of a spinal morphogenesis stage.

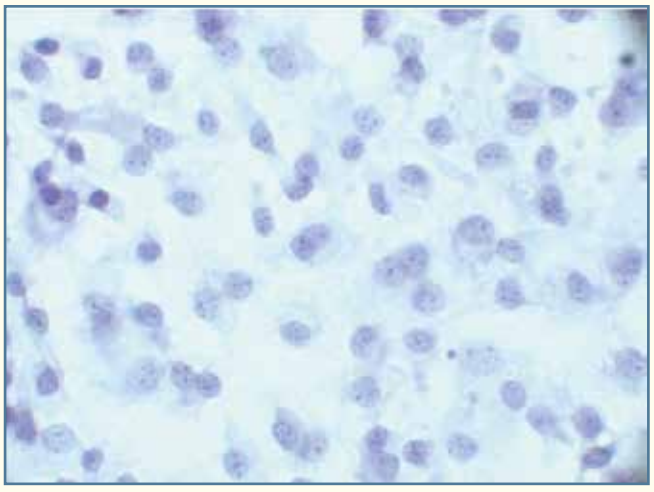

Fig. 7
The monolayer chondroblast culture (the convex side of spine deformity); Hematoxylin and Eosin staining, 10 × 60

One must suppose that some neural crest cells are deposited in the sclerotome as a result of violated spatial and temporal laws of their migration. This might result from a mutation in the *PAX3* gene followed by disrupted versican synthesis along the migration pathway [37]. The study conducted by Krull [42] has confirmed these data. The disruption of secretion and inhibition of versican sulfation halted neural crest cell migration. The interaction between neural crest cells and the interstitial matrix is known to follow the "cell–cell–matrix" principle [43]. Any disruption in synthesis and/or interaction between the receptor (integrin) to neural crest cells and migration substrate molecules may disturb further sclerotome morphogenesis [34].

Inhibition of neural crest cell migration can be associated with many factors. However, the following question arises in this case: why are poorly differentiated chondroblasts detected in the vertebral body growth plates, in the areas of neural crest cell deposition? It is known that at the site to which neural crest cells migrate, they acquire the phenotype of surrounding cells; however, their genotype is not changed [44]. It is quite understandable that neurogenic cells inside the vertebral body growth plates are not genetically determined to the growth process. This fact explains why cells in the growth plate do not undergo chondrogenic differentiation, leading to the asymmetry and local growth disturbance followed by the development of spinal deformity. The disruption of spine morphogenesis that was embryogenically pre-programmed to occur during growth periods eventually develops into scoliosis, with all its clinical and morphological features.

Therefore, an analysis of differentiated culturing of vertebral body growth plate cells from 50 patients with grade III–IV idiopathic scoliosis has revealed the causes of growth disturbance and the development of spinal deformity. In addition, it allowed suggesting that clinical signs of idiopathic scoliosis may vary depending on disturbance severity and morphogenetic processes occurring in the vertebral body growth plate.

Some assumptions regarding the variability of clinical signs of scoliosis can be made on the basis of our findings and the literature data (first of all, about the causes of the progression of spinal deformity and the predominant formation of thoracic curve in patients with idiopathic scoliosis). Since neural crest cells migration occurs at an interval of 10–1 μ and the distance between them is one cell diameter [45], the number of deposited cells may vary. This factor is responsible for the degree of chondrogenesis impairment and growth asymmetry. When the number of deposited cells during intensive growth (growth stage 1) is small, the resulting spine deformity is eventually elim-

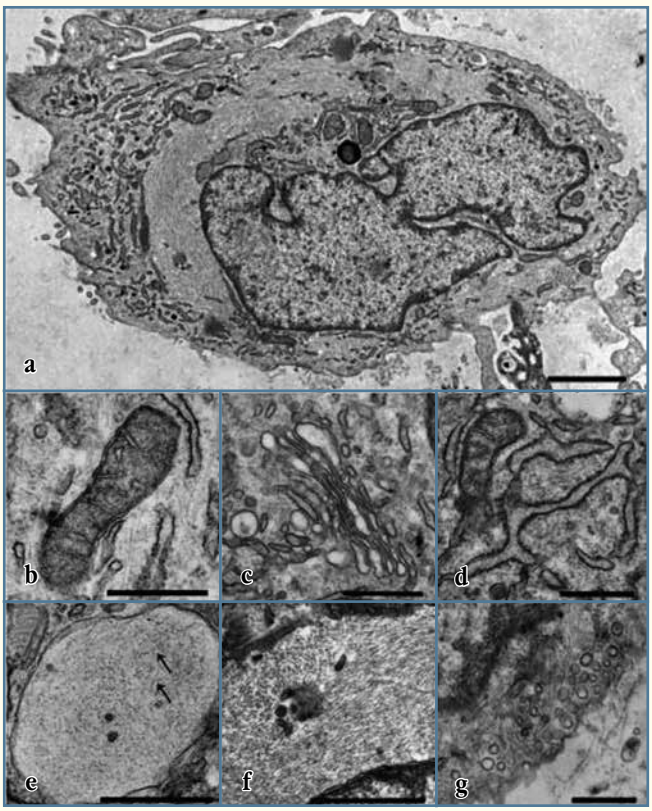

Fig. 8
The ultrastructure of a chondroblast on the convex side of the vertebral body growth plate at the deformity apex in a patient with idiopathic scoliosis: **a** – the general appearance of the cell containing the nucleus with invaginations and significant aggregations of intermediate filaments in the cytoplasm; **b, c** – mitochondria with short transverse cristae, narrow **(b)** and enlarged **(c)** cisterns of the rough endoplasmic reticulum; **d** – dictyosomes within the Golgi apparatus; **e** – a vacuole with short thin filaments (shown with arrows) inside it; **f** – intermediate filaments aggregated near the nucleus; **g** – numerous vesicles near the plasma membrane; scale bar: **a** – 2 μm, **b–g** – 0.5 μm

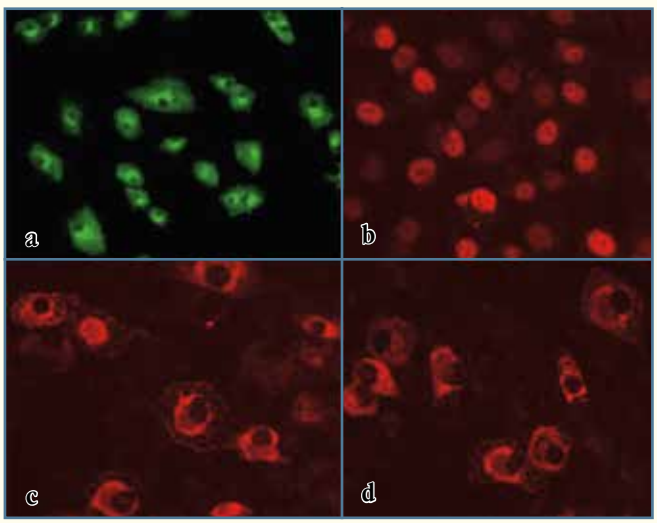

Fig. 9
The immunohistochemical reactions to proteins: **a** – type I collagen (green); **b** – type II collagen (red); **c** – aggrecan (red); **d** – Sox 9 (red)

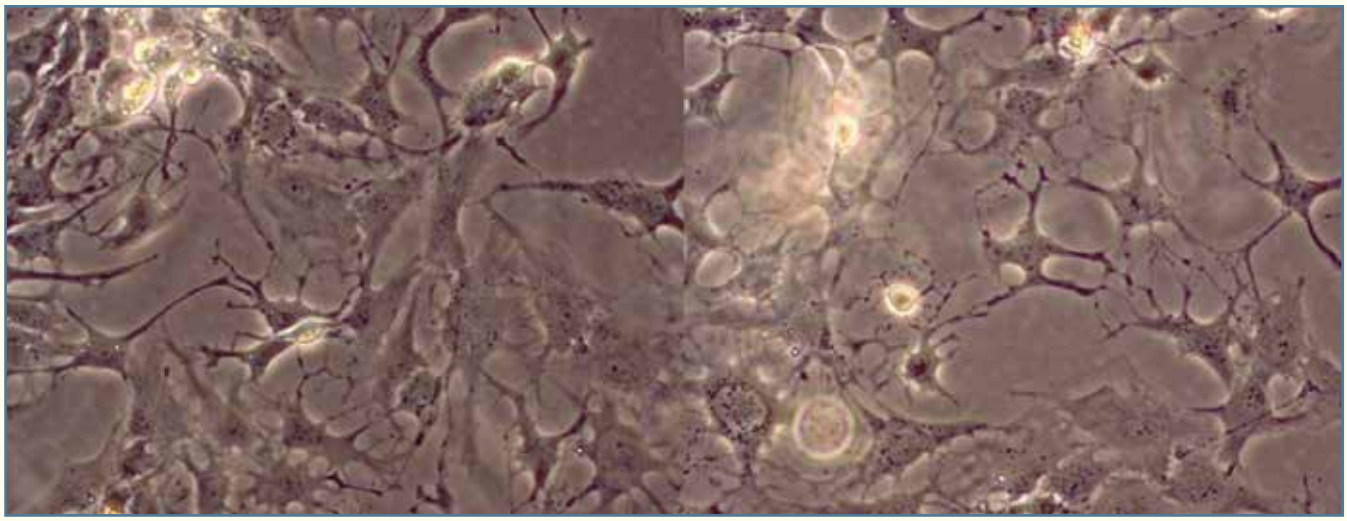

Fig. 10
Cells of neural origin in the cell culture obtained from the concave side of the deformity (a native specimen, 10 × 40)

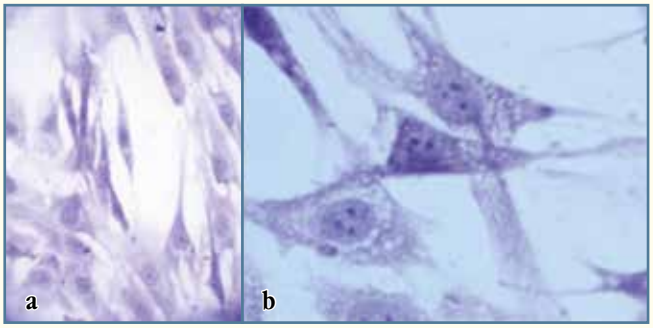

Fig. 11
Cell culture (idiopathic scoliosis, the concave side at the deformity apex); Nissl staining: **a** – 400× magnification; **b** – 200× magnification

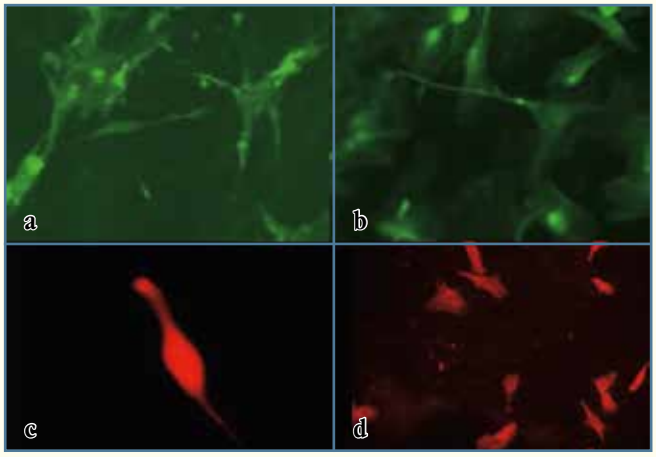

Fig. 12
Immunohistochemical reactions to anti-neuronal antibodies in the cell cultures for the concave side of the spinal deformity (idiopathic scoliosis): **a, b** – *NF1* (green); **c** – IIIβ-tubulin (red); and **d** – *NF200* (red)

inated due to the unchanged growth plate or does not progress and corresponds to the initial stage of development (grade I–II idiopathic scoliosis). These data are based on earlier experimental studies conducted by A.M. Zaydman (data not published). The pinpoint damage to the growth plate in a growing animal resulted in mild deformity, which was then leveled off and did not progress during the subsequent linear growth. If half of the growth plate was damaged, the animal had a severe deformity that was progressing until the end of growth period. One of the questions that have not been answered by spine experts yet is as follows: why is the thoracic type of deformity preferentially formed in patients in idiopathic scoliosis? This question can be answered by analyzing the path of neural crest cell migration. Since the migration of neural crest cells along the trunk path runs only via the thoracic somites, motion disruption and cell deposition only results in growth asymmetry and development of thoracic deformity. Girls are more likely to have idiopathic scoliosis probably because girls mature faster than boys do.

The following conclusions can be drawn:

(1) ectopic localization of neural crest cell derivatives genetically not determined to chondrogenic differentiation and growth is an etiological factor of scoliosis;

(2) local impairment of chondrogenesis in the vertebral body growth plate causes growth asymmetry and spinal deformity development in patients with idiopathic scoliosis;

(3) the degree of structural changes in the spine and the prognosis of deformity progression depend on the severity of

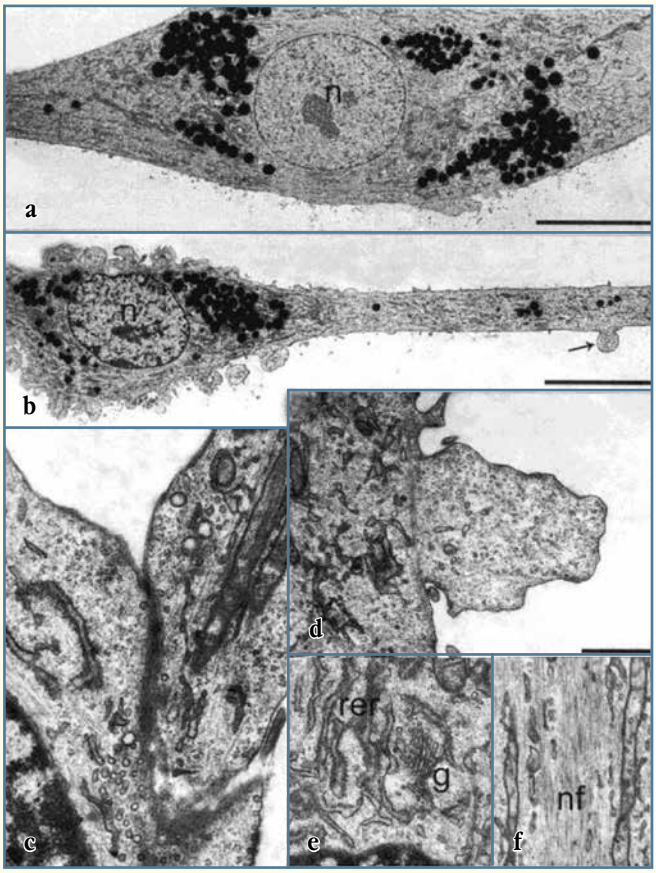

Fig. 13
A microimage of the cultured cells isolated from the vertebral body growth plate (the concave side of the deformity apex) in a patient with idiopathic scoliosis: **a, b** – elongated neuron-like cells having a long process (axon): numerous electron-dense granules around the nuclei (n) and in the cell's cytoplasm, axonal spinelets being formed (shown with an arrow); **c** – cytoplasm fragment for one of the cultured cells with the well developed rough endoplasmic reticulum and the Golgi apparatus (g); **d** – an axonal fragment with an extensive neurofilament network (nf) piercing the cellular processes and elongated mitochondria (shown with arrows); **e** – the contact being formed between the process of one cell and the body of the other cell; numerous vesicles at the contact site (shown with arrows); spinelets formed on the cell body and processes; scale bar: **a, b** – 10 μm; **c–f** – 1 μm

morphogenetic impairments in the vertebral body growth plate pre-programmed during embryogenesis.

The section describing the author's own research reports the data based on which the etiology and pathogenesis of idiopathic scoliosis have been proposed. The following hypotheses have been put forward: about the reasons for spinal deformity progression and about the predominant development and higher rate of thoracic scoliosis in girls compared to boys. Many questions still need to be verified, which has inspired us to continue the research using the developed model of idiopathic scoliosis.

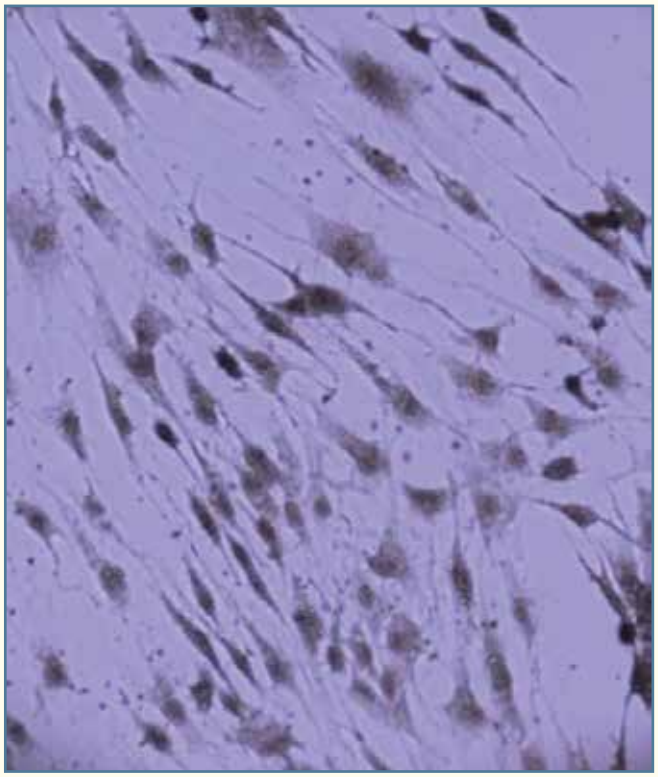

Fig. 14
Cell culture (idiopathic scoliosis, concave side at the deformity apex); Cajal's staining, ×200 magnification

The model of idiopathic scoliosis developed by inhibiting the *PAX3* gene in the chick embryo sclerotome

Ectopic localization of neural crest cell derivatives genetically not determined to chondrogenic differentiation and growth is an etiological factor of idiopathic scoliosis [29]. The disruption of spine morphogenesis during early embryogenesis develops into scoliotic deformity, with several variants of clinical progression. In order to answer the formulated questions, we have developed the chick embryo model of idiopathic scoliosis by inhibiting expression of the *PAX3* gene by small interfering RNA (siRNA) in the sclerotome. siRNA was procured from the Laboratory of Chemistry of the Institute of Chemical Biology and Fundamental Medicine, SB RAS. The experiments were performed using fertilized eggs of Hubbard ISA F15 chicken.

At the first stage, neural crest cells in the chick embryo sclerotome were identified. The fertilized eggs were incubated for 44–48 hrs, and GFP-tagged plasmids were electrochemically injected into the vertebral column of the embryos (Fig. 18).

At the second stage, real-time PCR was used to determine whether it is possible to inhibit *PAX3* expression in chicken embryo fibroblast culture by cholesterol-containing nuclease-

resistant siRNA capable of penetrating into the cells without using a transfection agent (Fig. 19). The siRNA sequence was selected so as to correspond to that of matrix RNA (mRNA) of the *PAX3* gene. siRNA having no significant homology with human or chicken mRNA (antisense RNA) was used as control RNA.

At the third stage, we determined that *PAX3* expression can be inhibited in chick embryos at the stage when neural crest cells are located within the sclerotome. After the fertilized eggs had been incubated for 44 hrs, lipophilic siRNA was injected into the neural tube of chick embryos to inhibit PAX3 expression in the sclerotome (Fig. 20). Antisense siRNA was used in the control experiments.

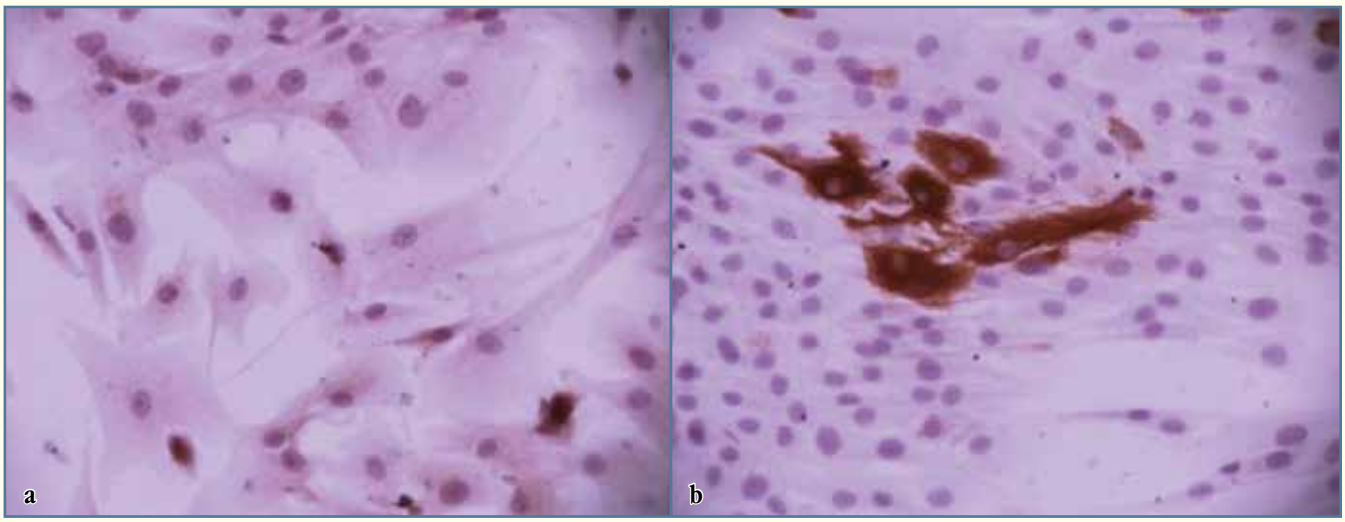

Fig. 15
Immunohistochemical reactions to glial proteins: **a** – S100 astrocytic protein, 200 magnification; **b** – GFAP – glial protein, ×200 magnification

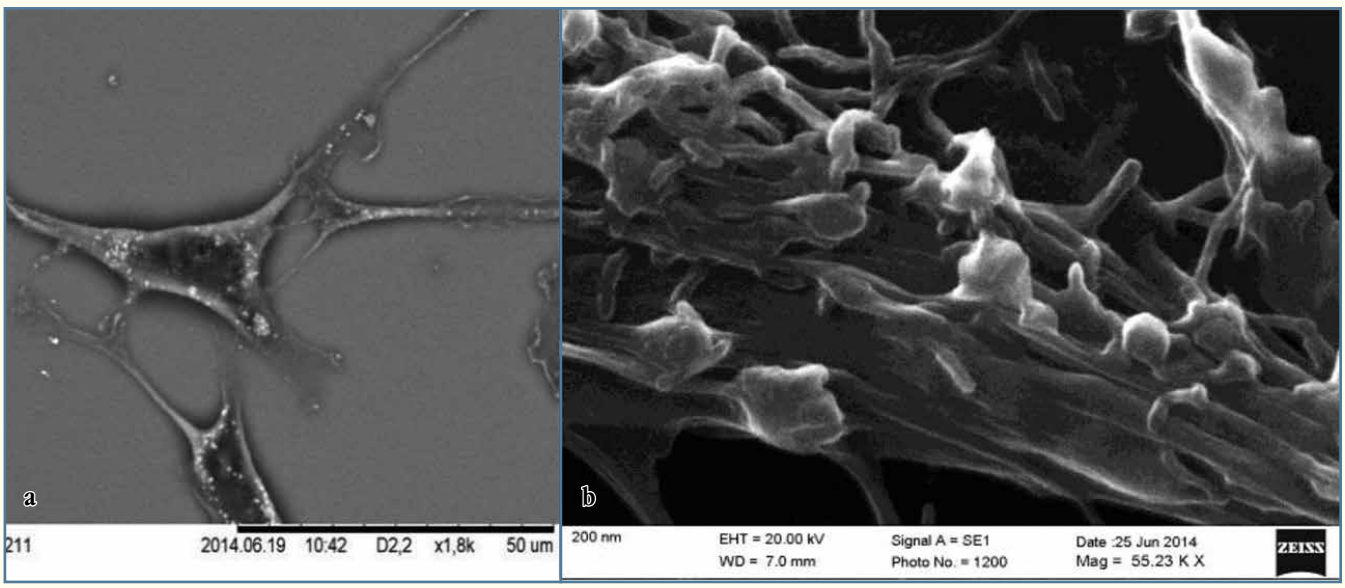

Fig. 16
A scanning microscopy image. Neurons in the cell culture derived from the vertebral body growth plate (the concave side of the deformity apex) in a patient with idiopathic scoliosis (a non-sprayed specimen): the general appearance of the cells at low magnification; the cells are elongated and have long processes; the contacts between two cells; a synapse and a large spinelet can be see; the neuronal cytoplasm and processes contain small dense granules 300–500 nm in diameter; scale bar: 200 μm **(a)** and 50 μm **(b)**

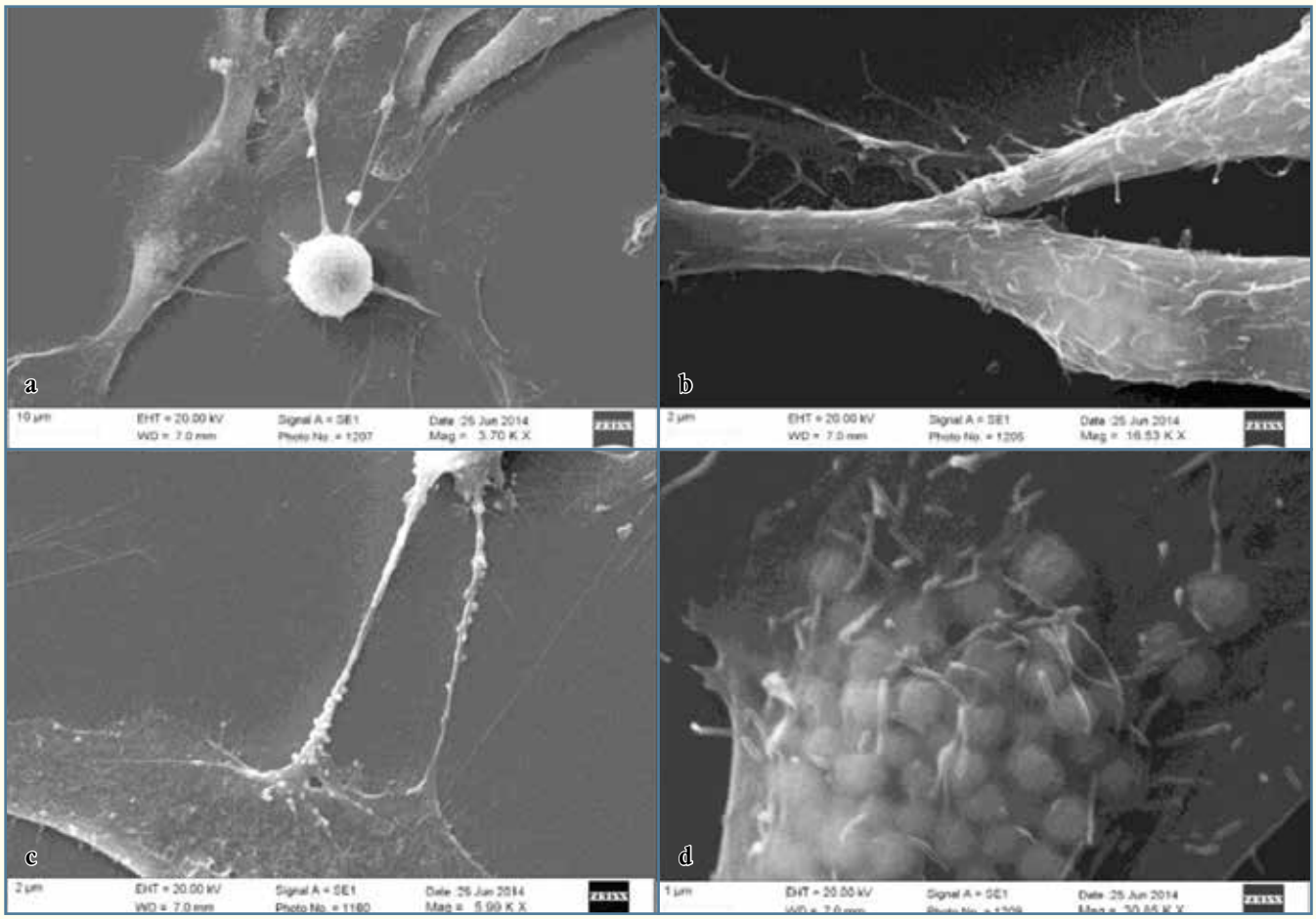

Fig. 17
Scanning microscopy. The SEM image of neurons in the cell culture derived from the vertebral body growth plate (the concave side of the deformity apex) in patient with idiopathic scoliosis (a sprayed specimen): **a** – the general appearance of the cells; **b** – a fragment of neuronal process at high magnification; **c** – a neuronal process (axon) with spinelets; **d** – a neuron fragment at high magnification; the surface contains short processes; granules within the cytoplasm can be seen through the plasma membrane; scale bar = 1 μm **(a)** and 2 μm **(b–d)**

The mechanism of inhibition of *PAX3* expression is as follows: siRNA penetrates into the cell cytoplasm and cleaves mRNA of the target through a series of successive stages. The cleaved mRNA is degraded. As a result, the cell concentration of the target mRNA decreases and *PAX3* protein regulating the neural crest cell migration is no longer synthesized. The migration stops; the neural crest cells are deposited in the sclerotome (the primordial spine). The scoliotic deformity is formed as spine develops.

The eggs were incubated at 38°C and 55% humidity until hatching (21 days). Cervicothoracic spine deformity could be detected by examination and palpation eight days after the hatching (Figs. 21 and 22). S-shaped deformities in the cervicothoracic spine were identified 169 days later (Fig. 23). A wedge-shaped deformity was detected at the level of C2–C3 vertebrae (Fig. 24). Morphological, genetic, and ultrastructural studies are still ongoing.

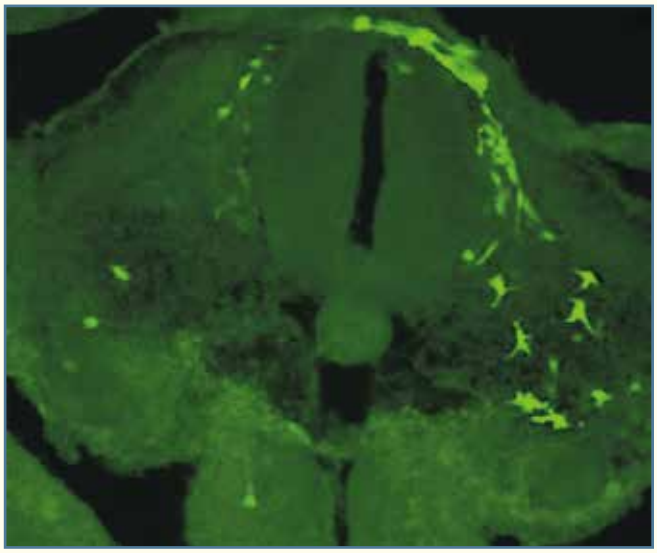

Fig. 18
Neural crest cells with the injected GFP-tagged plasmid in the chick embryo sclerotome (Hamburger–Hamilton stage 11, 42–44 hrs of embryonic development); ×200 magnification

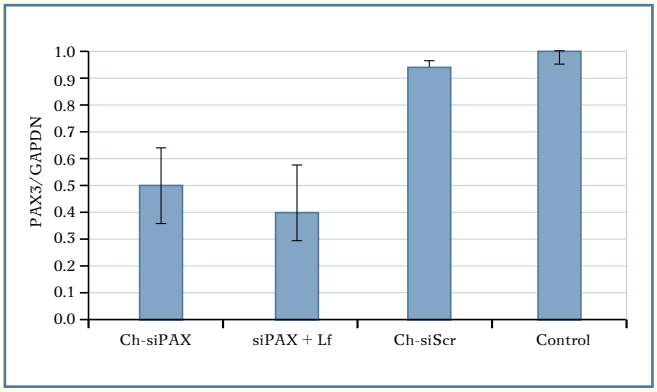

Fig. 19
Inhibition of *PAX3* expression by lipophilic siRNA in the culture of chick embryo fibroblasts by qRT-PCR: Ch-siPAX – siRNA to the *PAX* gene; siPAX + Lf – SiRNA to the gene + Lipofectamine, Ch-siScr – a random siRNA sequence; Control

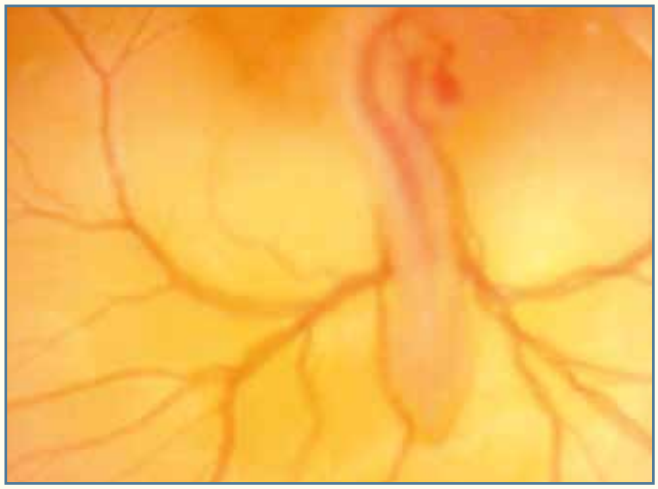

Fig. 20
A chick embryo after incubation for 42–44 hrs (lipophilic siRNA was injected into the neural tube); ×200 magnification

Fig. 21
Appearance of a chick after hatching (study day 21)

Fig. 22
Appearance of the chicks with inhibited *PAX3* expression eight days after hatching

Fig. 23
Thirty-six days after hatching, it was revealed by palpation that chicks had a cervicothoracic deformity

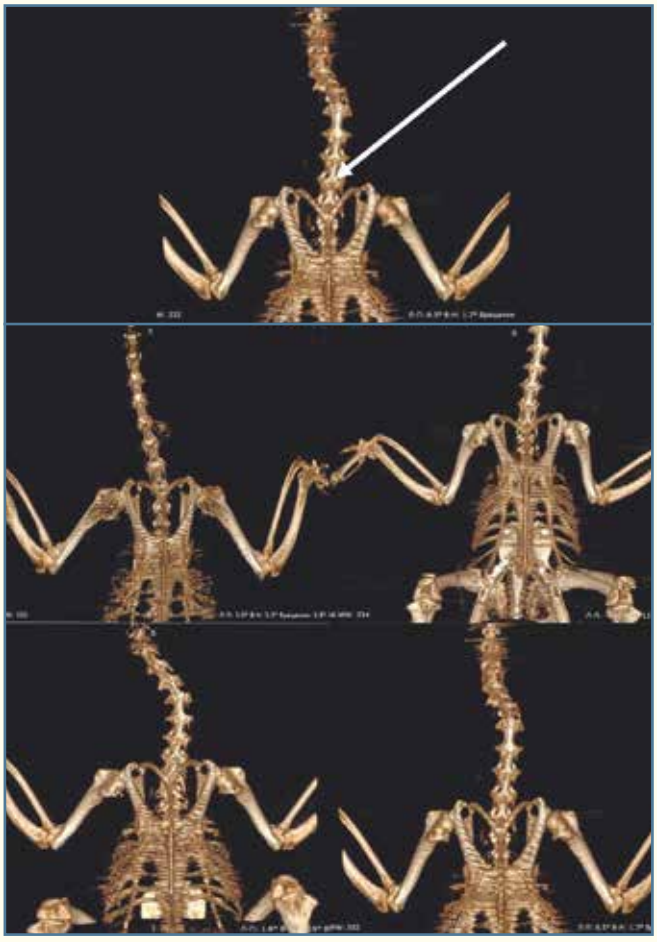

Fig. 24
Computed tomography of a 169-day-old chicken

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