Congenital kyphoscoliosis: Analysis of vertebral abnormalities using model animals (Review)

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Abstract. The normal structure of the spinal vertebrae is important for maintaining posture and the normal function of the thoracoabdominal organs and nervous system. Kyphoscoliosis occurs when the spinal vertebrae curve excessively beyond their physiological curvature to the back and side. Congenital kyphoscoliosis, a type of kyphoscoliosis, develops in the fetal period and is present in early childhood. However, neither the mechanism of pathogenesis nor the responsible gene has been identified. The lack of established animal models is a significant hurdle that limits the study of congenital kyphoscoliosis. Over the past 15 years, we have been accumulating data on this issue using rat models, based on the idea that the development of congenital kyphoscoliosis is caused by the abnormal expression of genes involved in normal bone formation. We hypothesize that analysis of an animal model of congenital kyphoscoliosis will provide a basis for the treatment of this disease in humans. The present review aimed to introduce molecules and mechanisms associated with the pathogenesis of kyphoscoliosis and to discuss the usefulness of studying this disease using model rats that develop kyphoscoliosis.

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Abbreviations: AIS, adolescent idiopathic scoliosis; atRA, all-*trans*-retinoic acid; BDNF, brain-derived neurotrophic factor; BMP-2, bone morphogenetic protein-2; CaSR, Ca^{2+}-sensing receptor; Col I, type I collagen; COL1A1, alpha-1 Col I; Cy, Cyanine; EDS, Ehlers-Danlos syndrome; GWAS, genome-wide association studies; IS, Ishibashi; JNK1, c-Jun N-terminal kinase 1; LBX1, Ladybird homeobox 1; LFNG, O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase; Nell-1, neural EGFL-like 1; NGF, nerve growth factor; NT3, neurotrophin-3; Pai-1, plasminogen activator inhibitor-1; PTH, parathyroid hormone; Rarα, retinoic acid receptor α; SNP, single nucleotide polymorphism; TBX6, T-box transcription factor 6; Trk, tropomyosin receptor kinase

Key words: congenital kyphoscoliosis, kyphoscoliosis model rats, DNA microarrays, microRNA array

Contents

1. Introduction

The spinal vertebrae (spinal column) consist of a sequence of vertebrae, each separated and united by an intervertebral disc. The intervertebral disc is elastic and acts as a cushion between vertebrae. This multilayer structure supports the body trunk and provides movement for the trunk, such as bending forward, backward and to the side, and rotation (1). Furthermore, the spinal cord and central nervous system are protected from physical impact because they travel through the vertebral foramen (spinal canal). The spine consists of 7 cervical vertebrae, 12 thoracic vertebrae, 5 lumbar vertebrae, and 5 sacral vertebrae that are fused together to form the sacrum, and several caudal vertebrae that are fused together to form the coccyx (2).

The spinal vertebrae are originally vertical on the coronal (frontal) plane. However, on the sagittal plane, the cervical and lumbar spines have lordosis and the thoracic and sacral spines have kyphosis. Furthermore, the pelvis was tilted forward by approximately 30 degrees. These physiological curvatures arose during the evolution of humans to two-legged locomotion (bipedalism). This physiological curvature results in a 10-fold increase in the resistance to pressure applied in the vertical direction relative to a straight spinal column (3).

On the other hand, scoliosis is a condition in which the spinal vertebrae are curved laterally in the frontal plane (4). Scoliosis can be divided into functional scoliosis and structural

scoliosis. Functional scoliosis (not featured in this review) includes painful scoliosis, which occurs reflexively and defensively in response to pain, and compensatory scoliosis, which is caused by lateral inclination due to a difference in the length of the left and right legs. Functional scoliosis is characterized by a mild degree of spinal curvature that disappears when the cause is removed.

There are 3 main types of structural scoliosis: congenital, idiopathic, and symptomatic secondary to another condition (e.g., cerebral palsy, acute poliomyelitis, or spinal muscular atrophy) (5). Idiopathic scoliosis accounts for 65% of all scoliosis cases (6). Depending on the age of onset, scoliosis can be divided into infantile, juvenile, and adolescent scoliosis. Adolescent idiopathic scoliosis (AIS) occurs in adolescents over 11 years of age and affects the majority of patients. Most cases occur in young females. On the other hand, congenital scoliosis is not a rare disease; rather, it is a common condition that occurs in 0.5 to 1 in 1,000 births worldwide (7,8). Congenital scoliosis is difficult to detect in its early stages because the curvature is almost painless. Currently, surgery is the only treatment option available for scoliosis. If the pathogenic mechanism is analyzed in detail and a genetic diagnosis enables the early detection of the disease, it may prevent the disease from progressing, reduce the pain and burden on the patient, and enable surgery to be avoided.

In this review, we summarize the current state of kyphoscoliosis research and discuss the results of a comprehensive analysis of genes that may be associated with spinal malformations using a rat model of congenital kyphoscoliosis. Four-legged locomotion, such as in laboratory rodents, requires less rotation of the spine, whereas two-legged locomotion places a greater load of gravity and motion on the spine. Therefore, the incidence of scoliosis was higher in 2 locomotion than in 4-legged locomotion. Considering the anatomical structure and the load placed on the spine, human samples are desirable but extremely difficult to obtain for research, so research is being conducted using animals with similar spinal structures (9). This review adds new findings and insights to the mini-review article on kyphosis that was previously published (10).

2. Current situation of kyphoscoliosis research

In recent years, the analysis of genes associated with AIS has progressed. Genetic testing for AIS was initiated in 2009 and is ongoing (11). Neurotrophin 3 (NT3) (12), transforming growth factor β1 (13), and basonuclein 2 (14) have been reported as candidate genes for AIS. A single nucleotide polymorphism (SNP) rs 11190870, which is strongly correlated with AIS, was discovered in ladybird homeobox 1 (LBX1) by a genome-wide association study (GWAS) (15). The LBX1 gene is located in the vicinity of rs 11190870. The genomic region containing rs11190870 interacts with the promoter region of the human LBX1 gene and causes the overexpression of LBX1, which has been shown to be involved in the pathogenesis of AIS (16). Moreover, a GWAS identified SNPs (rs6570507) in the genes encoding G protein-coupled receptor 126 (GPR126), which is reported to be associated with AIS (17). The knockdown of gpr126 in zebrafish (*Danio rerio*) caused delayed ossification (calcification of soft tissue into bonelike tissue) of the developing spinal vertebrae. Thus, significant progress has been made in the analysis of genes associated with the pathogenesis of AIS. However, the exact pathogenesis of AIS remains unknown and no clear genetic factors directly related to AIS have been identified. It has only been reported that 9 genes have been implicated in the pathogenesis of AIS and that the incidence of the disease is higher in families in which at least one other first-degree relative is affected (18). Genetic studies on AIS provide direction for the diagnosis and treatment of this disease and for future research. However, currently, the only curative treatment is surgery. One limitation is the lack of strategies to approach the causative gene itself, such as genome editing.

Recently, an interesting study on genes associated with the development of congenital scoliosis was reported in China and Japan. Polymorphisms (19) and heterozygous null mutations (20) of the T-box transcription factor 6 (TBX6) gene have been identified in Han Chinese patients with congenital scoliosis. Takeda *et al* (21) examined 94 Japanese patients with congenital scoliosis for genetic abnormalities in TBX6 and found deletions in 5 cases and severe mutations in 3 cases. Otomo *et al* (22) screened the mutations in the TBX6 gene in 196 Japanese patients with congenital scoliosis. An *in vitro* functional analysis of novel and known missense mutations revealed that most of the mutations cause abnormal subcellular localization of TBX6 protein. Congenital scoliosis associated with TBX6 in these Japanese cohorts accounted for approximately 10% of the total incidence of the disease, which is comparable to the aforementioned Chinese report. The Tbx6 gene encodes a T-box transcription factor that is expressed in cells that pass through the striae (primitive streak, a ridge seen on the midline of the caudal end of the blastoderm and gives a head-tail and a left-right axis to the developing embryo) and induces the differentiation of chondrocytes, skeletal muscle, and cardiac muscle from the mesoderm (23,24). In Tbx6-deficient mouse embryos, the neural tube is ectopically formed caudally from the neck in the region that normally differentiates into the somitic mesoderm that differentiates into bone and muscle (25,26). The decreased expression of this gene due to mutations or deletions may inhibit differentiation into chondrocytes and consequently prevent the formation of normal vertebrae.

Spondylostal dysostosis is a congenital disorder that causes severe deformities of the axial skeleton and is diagnosed based on radiographic features such as a combination of multiple segmental defects of the vertebrae and abnormalities of the ribs. In addition, most patients present with mild to severe scoliosis. The disease is caused by pathogenic mutations in delta-like protein 3, mesoderm posterior protein 2, O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase (*LFNG*), Hes family BHLH transcription factor 7, and Ripply Transcriptional Repressor 2. These mutations are inherited in an autosomal recessive fashion. Abnormalities in these genes have been reported to be strongly involved in the development of congenital scoliosis (27,28). In fact, a missense mutation in LFNG was identified in a patient with congenital scoliosis. *LFNG* encodes an N-acetylglucosamine-transferase, and this mutant was shown to have lost its enzymatic function (27). Takeda *et al* (27) experimentally demonstrated-for the first time-that *LFNG* is one of the leading candidate causative genes

of congenital scoliosis. Since all of these genes, including *LFNG*, are involved in the signal transduction of Notch1, their analysis may shed light on the pathogenesis of this disease (28). However, as Takeda *et al* (27) stated, 'the current list of known disease genes could explain only a small fraction of genetic cause' and it is still difficult to understand the full picture.

Thus, there are only a few interesting reports on the analysis of the causative genes of congenital kyphoscoliosis. However, this research has not progressed quickly and its expansion has been limited. Studies using animal models are needed to advance research quickly and widely. Oda *et al* (29) performed spinal fusion surgery on sheep as a model animal of spinal kyphosis. While this may be useful for analyzing the effects of kyphotic deformities on adjacent motor segments. However, it is not appropriate for studies to determine the pathogenesis of kyphosis. To accelerate this research, we analyzed rats with spontaneous lumbar kyphoscoliosis as an animal model of congenital kyphoscoliosis.

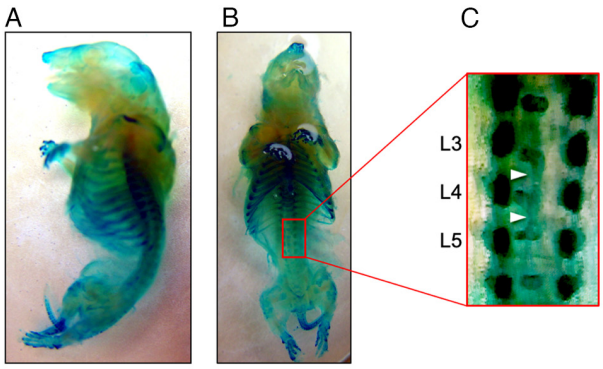

Figure 1. Skeletal abnormalities of fetal IS rats. An analysis of the axial skeletal phenotype in the lumbar region of IS rats at 16.5 days after pregnancy. The fetal skeletons were stained with Alcian blue and Alizarin red. (A) The lateral view, (B) ventral view and (C) magnified subregions indicated by the rectangle of B in skeletal specimens (magnification, 5x). The IS rats showed unilateral fusion of the ventral primary ossification centers in the lumbar vertebrae (between L3-L5, white arrowheads). These images are unpublished pictures of the results reported by Seki *et al* (34).

3. Ishibashi rats, an animal model of kyphoscoliosis

There is no need to mention that studies using animal models are useful for the rapid and efficient progression of human disease research. Recently, transgenic or-deficient mice showing the development of kyphosis have been reported. Chae *et al* (30) reported that mice deficient in the mitochondrial enzyme isocitrate dehydrogenase 2 gene exhibit spinal kyphosis. More recently, the transgenic LmnaG609G progeric mouse has been developed as an animal model for studying human Hutchinson-Gilford progeria syndrome, which is caused by mutations in the LMNA (lamin A/C) gene, showing severe kyphosis (31). Furthermore, thoracic kyphosis also appears in mice homozygous for a humpback mutation in *Notch3* (32).

Ishibashi (IS) rats were used in the present study. This rat strain was maintained at The National Bio Resource Project (NBRP) for the Rat in Japan (strain No. 0008). IS rats were provided by the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University (Kyoto, Japan), and all researchers can use this rat (http://www.anim.med.kyoto-u.ac.jp/NBR/strains/Strains_d.aspx?StrainID=5&s_Strain name=is).

IS rats have an agouti coat color. We believe that this rat has good reproductive performance, but a somewhat rough temperament. This rat is characterized by malformation of the lumbar spine, leading to kyphoscoliosis with restricted spinal canals and spinal cord. IS rats were established by Ishibashi (Azabu University School of Veterinary Medicine, Kanagawa, Japan) in 1968 as an inbred strain from a male wild-type and female Wistar rat (33). Wistar rats are medium-sized albinos that originated from the Wistar Institute in Philadelphia (PA, USA). Spinal abnormalities are spontaneous and are not caused by artificial genetic modifications. As reported by Seki *et al* (34), IS rats exhibit typical and pronounced kyphoscoliosis of the lumbar vertebrae. In addition to kyphoscoliosis, homeotic transformation was observed only in the lumbosacral transitional areas of adult IS rats. In the fetus, unilateral unions of ventral primary ossification centers were observed in the lumbar vertebrae (Fig. 1). Almost all homozygous individuals crossed between male and female IS rats have vertebral anomalies (34). IS rats are generally regarded as an animal model of human spinal malformation in which bone deformities are restricted to the spinal vertebrae. Furthermore, the number of clinical cases of human scoliosis is relatively small, making it difficult to compare scoliosis sites with the normal areas of the spine in the same individual. Therefore, we considered that an analysis of IS in rats might lead to a new understanding of human scoliosis.

In contrast, a mouse with kyphoscoliosis was identified in the offspring of mice treated with a chemical mutagen (N-ethyl-N-nitrosourea) (35). The mice showed an autosomal dominant mode of inheritance and were named hereditary vertebral fusion (HVF) mice. The phenotype of HVF mice was similar to that of IS rats. They mainly have lumbar segmental defects, narrowing of the intervertebral space, irregularity of the adjacent ends of the vertebral bodies, and kyphosis with wedging and complete spinal fusion of the adjacent vertebral bodies. However, no specific genetic mutations or expression abnormalities have been reported in the HVF mice.

Thus far, with the exception of our studies, only 6 studies have investigated malformations of the spinal vertebrae in IS rats. The main findings of these studies are as follows: i) The administration of the sex hormones estrogen and testosterone inhibited the progression of kyphosis in male IS rats (36,37); ii) Plasma alkaline phosphatase activity is low in IS rats, but this is not related to the malformation of the spinal vertebrae (38); iii) The phenotypic spinal abnormality in IS rats is not due to a single gene, but may be due to multiple genes (39); iv) IS-Tlk/Kyo, a rat mutant strain derived from the IS strain, exhibits abnormalities of the sacral and tail vertebrae, in addition to a congenital malformation of the lumbar spine, which is a significant phenotype of IS rats (40,41). These studies were interesting but sporadic and did not focus on the pathogenesis of kyphoscoliosis.

4. Comprehensive analysis of the gene expression in the flexed lumbar spine using IS rats

When we first analyzed congenital kyphoscoliosis in IS rats, we thought that because congenital scoliosis is a skeletal

Table I. List of genes/proteins with abnormal expression in the lumbar spine of Ishibashi.

Classification by function	Symbol	Description	Array	mRNA	Protein	(Refs.)
Vertebral formation	*Hox a11*	Homeobox a11	-	↓ 0.09	-	(34)
	Hox c10	Homeobox c10	↓ 0.86	↓ 0.10	-	(34)
	Hox d10	Homeobox d10	-	↓ 0.38	-	(34)
	Hox d11	Homeobox d11	-	↓ 0.19	-	(34)
NGF receptor	*Ntrk1*	trkA; High affinity nerve growth factor receptor	↓ 0.10	↓ 0.45	↓ 0.34	(46)
	Ntrk2	trkB; BDNF/NT-3 growth factors receptor	↓ 0.11	↓ 0.32	↓ 0.53	(46)
	Ntrk3	trkC; NT-3 growth factor receptor	↓ 0.09	↓ 0.54	↓ 0.27	(46)
Retinol metabolism	*Adh1*	Alcohol dehydrogenase 1	↓ 0.37	↓ 0.55	↓ 0.58	(47)
	Aldh1a2	Aldehyde dehydrogenase family 1 member A2	↓ 0.31	↓ 0.33	↓ 0.40	(47)
	Rara	Retinoic acid receptor α	↓ 0.83	↓ 0.69	↓ 0.58	(47)
	Stra6	Stimulated by retinoic acid 6	↓ 0.33	-	-	(47)
Osteogenesis	*BMP-2*	Bone morphogenetic protein 2	↓ 0.83	↓ 0.71	↓ 0.35	(47)
	Col1A1	Collagen α-1	↓ 0.45	-	↓ 0.44	(48)
RNA interference	*miR-224-5p*	rat-miRNA-224-5p	2.82	3.46	-	(48)
Bone loss	*Pai-1*	Plasminogen activator inhibitor-1	↓ 0.58	2.20	2.48	(48)
	Serbp1	Pai-1 RNA binding protein 1	1.43	-	-	(48)
Ca^{2+} signaling	*CaSR*	Calcium-sensing receptor	↓ 0.14	-	↓ 0.27	(75)
	Trpv1	Transient receptor potential vanilloid 1	↓ 0.10	-	↓ 0.14	(75)
	Nell-1	Neural EGFL-like 1	↓ 0.17	↓ 0.56	-	(75)
	Jnk1	c-Jun N-terminal kinase 1	↓ 0.31	↓ 0.62	-	(75)

↓, downregulated gene/protein; ↑, upregulated gene/protein. The number in columns indicates the results of expression in IS rats relative to that in WT rats in each category (array, mRNA, protein).

abnormality, the cause of the disease could be determined by analyzing the abnormalities in homeotic genes that determine the composition of the skeleton. For example, in *Hox*A10 knockout mice, the first lumbar vertebra is altered to look like the 13th thoracic vertebra just anterior to it (on the head side), resulting in ribs that do not arise normally (42,43). In addition, for *Hox*A11 and *Hox*D11, no significant changes were observed with the knockout of each gene alone ($Hox A11^{-/-}/Hox D11^{+/+}$ or $Hox A11^{+/+}/Hox D11^{-/-}$); however, in the double knockout ($Hox A11^{-/-}/Hox D11^{-/-}$), a pronounced trait change appeared in the form of bone loss in the forearm (44).

Based on these reports, the third to fifth lumbar spine segments (L3-L5), the segments that are the most common area of deformity in IS rats (36), were analyzed to determine the *Hox* gene expression levels (34). The expression of *Hox* a10 and c11 in the vertebrae of IS and heterozygotes was not significantly different from that of Wistar (control) rats. However, the expression levels of *Hox* 10 and 11 paralogs (c10, d10, a11, and d11) in the vertebrae of both IS rats and heterozygotes were significantly lower than those in Wistar rats (Table I). In knockout and transgenic mice with Hox 10 and 11, malformations of the vertebrae and homeotic transformation in the lumbosacral vertebrae were observed (45). As mentioned in the previous section. In addition to kyphoscoliosis, homeotic transformation in adult IS rats was only observed in the lumbosacral transitional areas (34). Taken together, the partial reduction of the Hox 10 and 11 expression may cause spinal deformities and homeotic transformation of the 1st sacral vertebra into the 7th lumbar vertebra.

Thus, it is worth examining each scoliosis-related gene that may be associated with the development of scoliosis. In contrast, spinal abnormalities, such as spinal curvature, homeotic transformation, and fusion/division of primary ossification, in IS rats are extremely diverse. We therefore changed our strategy from analyzing individual genes that might be involved in the development of kyphoscoliosis to conducting a comprehensive genetic analysis. For this purpose, we performed a DNA microarray (DNA chip) analysis. We are the first to apply this method in a comprehensive analysis of the gene expression in congenital kyphoscoliosis (46-48). A DNA microarray, also known as a DNA chip, is an analytical tool in which a large number of DNA fragments are densely arranged on a substrate, such as resin or glass, to analyze changes in the gene expression levels of specimens (49,50). This method enables a comprehensive analysis of the gene expression in various organisms with a small number of specimens in a relatively short time. It is currently used in various research

fields. Protein arrays that use antibodies and peptides instead of nucleic acids have also been developed (51).

For the DNA microarray analysis, total RNA was extracted from L3-L5 of male rats on postnatal day 4 (when ossification is less advanced). RNA samples from IS and wild-type (Wistar, Wt) rats were labeled with Cyanine3 (Cy3) and Cyanine5 (Cy5) fluorescent dyes, respectively. The rat gene expression was analyzed using the 3D-Gene Rat Oligo chip 20k (approximately 20,000 distinct genes; Toray Industries, Tokyo, Japan). In the lumbar spine, 194 genes showed altered expression levels in IS rats in comparison to WT rats. Among these, 90 genes were upregulated (Cy3/Cy5 ratio ≥8.0-fold) and 104 genes were downregulated (Cy3/Cy5 ratio ≤0.125) in IS rats in comparison to WT rats (46). The experiments and results presented in the five original papers that have been published to date analyzed specimens of similar severity (i.e., the degree of lumbar spine curvature) in the pathogenesis of kyphoscoliosis. Therefore, the same conclusion was reached regardless of who interpreted it, and the reproducibility of the experiment is assured. According to the results of a gene clustering analysis for genome-wide expression data, the genes significantly downregulated in IS rats were classified into several functional groups.

i) Neurotrophin receptor family signaling. We first focused on the receptors for nerve growth factors, since there have been no previous reports of their relevance to the pathogenesis of scoliosis and kyphoscoliosis. Significant decreases in the expression levels of neurotrophin receptors (a tropomyosin receptor kinases (Trks): TrkA, TrkB and TrkC) were observed in the spines of IS rats (Table I) (46). In all Trks, a significant decrease was confirmed in the mRNA and protein expression as well as in the DNA microarray results. Furthermore, an immunohistochemical analysis showed that Trk-immunopositive cells were reduced by 50-70% in IS rats relative to WT rats. TrkA is the receptor for nerve growth factor (NGF), TrkB is the receptor for brain-derived neurotrophic factor (BDNF) and neurotrophin (NT) -4/5, and TrkC is the receptor for NT 3 (52). It has recently been reported that neuronal factors and their receptors play critical roles in bone formation. For example, in the developing mouse skeleton, TrkA-expressing sensory neurons innervate the cartilage of the developing femur. NGF serves as a skeletal neurotrophin and NGF-TrkA signal is required for the differentiation and mineralization of bone progenitor cells at sites of NGF production (53). During recovery from ulnar stress fracture in mice, the injection of TrkA catalytic inhibitor (1 NMPP1) reduced the numbers of sensory fibers, and then delayed ossification of the fracture callus (54). Recently, Rivera *et al* (55) reported that the endogenous expression of NGF and TrkA during tibial fracture repair peak during the cartilaginous phase, and that NGF injections to the fracture site at that time can increase the bone formation by decreasing cartilage tissue. On the other hand, TrkB mRNA is expressed in the developing spine of rats on embryonic day 17.5 (56). TrkB is also widely expressed in bone tissues, such as chondrocytes and osteoblasts (57). Hutchison reported that BDNF-TrkB signals promote growth plate chondrocytes differentiation (58) and mice lacking TrkB showed dwarfism and delayed hypertrophic differentiation (59). Genetic testing for AIS reported that NT-3, the ligand for TrkC, is a candidate causative gene (12). Our previous analysis of congenital kyphoscoliosis also found that TrkC, a receptor for NT3, is a pivotal gene/molecule in this disease (46). NT3-TrkC signaling appears to be deeply involved in the pathogenesis of scoliosis. On the other hand, during the repair of rib fracture in mice, NT-3 and TrkC is observed in osteoblast-like cells and hypertrophic chondrocytes (60). Moreover, NT-3-TrkC signal promotes the repair of injured growth plate cartilage and bone in the tibia of rats through bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor activation (61). Thus, evidence is accumulating to support that Trks are essential for normal bone formation. The gene transfer of Trks in early embryos to IS rats, in which the gene expression of all Trks is severely reduced, may help prevent the development of congenital kyphoscoliosis.

ii) Retinol-retinoic acid metabolic pathway. Next, we analyzed the retinol-retinoic acid metabolic pathway because retinol (vitamin A_1) and its metabolite, all-*trans*-retinoic acid (atRA), are morphogens involved in various developmental phenomena. In particular, this pathway plays an important role in osteogenesis, anterior-posterior patterning, and left-right asymmetry of axis formation (62). Excess or insufficient retinol/atRA causes congenital anomalies and bone malformation. Vitamin A-deficient rats show hypoplasia of the skull, ectopic bone at the dorsal root of the C1 spinal nerve, and malformation of the sternum and pelvic region (63). Furthermore, congenital spinal deformity is caused in the fetuses of pregnant rats given a vitamin A-deficient diet (64). In our DNA microarray analysis, we showed that the expression levels of the 2 rate-limiting enzymes of retinol metabolism, alcohol dehydrogenase I and aldehyde dehydrogenase 1 family member A2, and retinoic acid receptor α (Rarα) are significantly decreased in the lumbar spine of IS rats (Table I) (47). Furthermore, the mRNA level of stimulated by retinoic acid 6 (Stra6), which encodes the transmembrane receptor that mediates the cellular uptake of blood retinol (65), was also low (Table I) (47). Interestingly, however, the serum retinol levels in IS rats were higher than those of WT rats (47). These findings seem to reflect impaired retinol utilization and metabolism through the downregulation of the retinol metabolic pathway in IS rats (Fig. 2). Regarding the involvement of retinol/atRA in spinal malformation in IS rats, there are two possibilities based on the previous reports described above and our results. First, metabolic abnormalities in RA in IS rats may induce spinal deformities. Retinoic acid regulates the expression of the Hox gene that define the pattern of the body plan along the head-tail axis of an embryo (66,67), especially Hox 10 and 11 regulate the formation of the lumbar and sacral vertebrae (68). In a previous study, we also found that the expression levels of Hox10 and 11 paralogs were extremely low in the lumbosacral transition of IS rats in comparison to WT rats (34). These findings suggest that kyphoscoliosis in IS rats may be caused, at least in part, by the reduced expression of the Hox genes due to impaired retinol utilization and metabolism. Second, the low expression of Rarα may induce abnormal spine formation in IS rats by reducing the expression of TrkC via BMP-2 (Fig. 2). *In vitro* experiments have shown that Rarα-specific agonists and atRAs induce the expression of BMP-2 via Rarα (69) and that BMP-2 induces

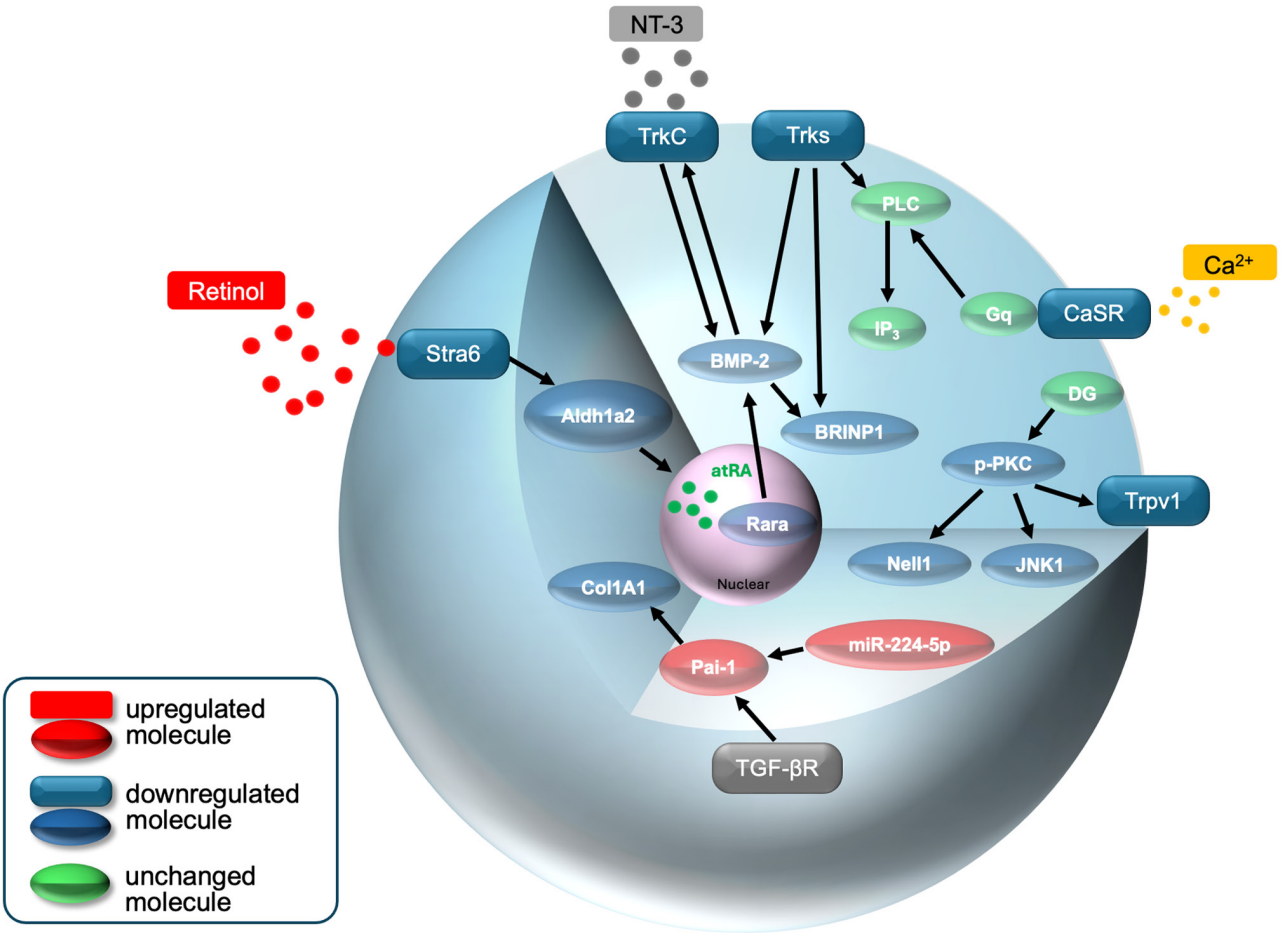

Figure 2. A schematic model of Trks, retinol, Ca^{2+} and Pai-1 (via miR-224-5p) signaling in osteocytes of the curved lumbar spine of IS rats. Details of the intensity of the expression of each molecule and its role in the development of kyphoscoliosis are described in the text. These combined disorders may be at least partly responsible for kyphoscoliosis. Stra6, stimulated by retinoic acid 6; Aldh1a2, aldehyde dehydrogenase 1 family member A2; atRA, all-trans-retinoic acid; Rarα, retinoic acid receptor α; BMP-2, bone morphogenetic protein-2; TrkC, tropomyosin receptor kinase 3; NT3, neurotrophin-3; BRINP1, BMP/retinoic acid inducible neural specific 1; TGF-βr, transforming growth factor-beta receptor; Pai-1, plasminogen activator inhibitor-1; Col1A1, type 1 collagen α1; N, nucleus,

the expression of TrkC (70). Asaumi *et al* (60) reported that NT-3 and its receptor TrkC promote osteogenesis in rat bone marrow stromal cells and enhance the expression of BMP-2 as an osteogenic factor located upstream of BMP-2. In our previous study, the mRNA and protein expression levels of TrkC in IS rats were 54 and 27% lower, respectively, in comparison to WT rats (46). Similarly, the expression levels of BMP-2 mRNA and protein were 71 and 35%, respectively, in comparison to WT rats (47). Taken together, it is strongly suggested that the breakdown of BMP-2-mediated crosstalk between RA-Rarα and NT3-TrkC signals causes kyphoscoliosis (Fig. 2). Thus, this is an extremely attractive scenario. However, for this to be convincing, further studies are needed to examine in detail the interrelationship between the retinol-retinoic acid metabolic pathway involved in osteogenesis and BMP-2 and TrkC.

iii) Ca^{2+} sensing/signaling. Bone contains 99% of the calcium in the body and buffers fluctuations in blood calcium. While maintaining calcium homeostasis, the bone also functions to maintain itself through a process called bone remodeling. When blood calcium is low, the mobilization of calcium from the bone to the blood, that is, bone resorption, is accelerated (71). Maintaining calcium homeostasis *in vivo* is crucial for normal bone formation and metabolism. Calcium homeostasis involves a balance between calcium deposition and bone absorption, the uptake of calcium by the gastrointestinal tract, and calcium excretion by the kidneys. The maintenance of this homeostasis is controlled by3 calcium-regulating hormones (parathyroid hormone; PTH, calcitonin, and activated vitamin D_3) (72). On the other hand, a Ca^{2+}-sensing receptor (CaSR) was identified on the cell surface of chief cells in the parathyroid glands that sense the Ca^{2+} concentration in the blood (73). This receptor is activated by elevated blood Ca^{2+} levels and inhibits the secretion of PTH. Conversely, a decrease in blood Ca^{2+} levels promotes PTH secretion. Functional CaSR is also expressed in osteoblasts and osteoclasts, so that these cells are able to sense changes in the extracellular Ca^{2+} and as a result control intracellular Ca^{2+} signaling (74). A DNA microarray analysis revealed that the expression of CaSR was very low in the deformed lumbar spines of IS rats (Cy3/Cy5 ratio: 0.14) (Table I) (75). The protein expression is also significantly lower than that of WT rats (0.27). Accordingly, the expression of PTH (Cy3/Cy5 ratio: 2.99) and its receptor (PTHR) (Cy3/Cy5 ratio: 2.48) was markedly upregulated.

The Trpv1 channel, also known as a capsaicin receptor, is a member of the Ca^{2+}-permeable cation channel subfamily (76). The channel is widely expressed in tissues/cells, including osteoblasts and osteoclasts (77). Interestingly, Ca^{2+} transport through Trpv channels is required for bone remodeling (77,78). Idris *et al* (79) reported that the antagonist (capsazepine) of Trpv1 inhibits alkaline phosphatase activity and bone nodule formation in osteoblasts. Furthermore, TRPV1 deficient mice had reduced intracellular Ca^{2+} concentrations and decreased calcium deposition in osteoclast precursor cells during fracture healing (80). Therefore, the appropriate signaling of Ca^{2+} in osteoblasts and osteoclasts by Trpv1 is required for normal osteogenesis. Our previous studies have shown that the Trpv1 gene expression, as well as the number of Trpv1-expressing cells, is lower in the lumbar spine of IS rats than in WT rats. In fact, the calcium content in the lumbar spine of IS rats was 73.1% of that in WT rats. Therefore, we believe that the cause of kyphoscoliosis in IS rats involves, at least in part, the impairment of calcium sensing and uptake due to the decreased expression of CaSR and Trpv1.

Based on these findings, we performed a pathway analysis and found that the 2 critical molecules that regulate normal bone formation downstream of protein kinase C were decreased. One is neural EGFL-like 1 (Nell-1), and the other is c-Jun N-terminal kinase 1 (JNK1). Nell-1 accelerates osteogenic differentiation *in vitro* and the bone formation of the top part of the skull (calvaria) *in vivo* (81). Moreover, a study using Nell-1-deficient mice revealed that Nell-1 serves an indispensable function in the development and growth of normal craniofacial and skeletal structures (82). On the other hand, Xu *et al* (83) reported that JNK1-deficient mice are severely osteopenic due to impaired phosphorylation of molecules downstream of JNK1 signaling in osteoblasts. They concluded that JNK1 is a critical mediator of the osteoblast function. Furthermore, the inhibition of JNK1 in osteoclasts increases apoptosis. This indicates that JNK1 is involved in an autophagic mechanism underlying the regulation of osteoclastogenesis (84). Thus, Nell-1 and JNK1-mediated signaling initiated by CaSR and/or Trpv1 in osteocytes may provide a novel pathway for osteogenesis.

In contrast, Slc39a13/ZIP13, an intracellular Zn^{2+} transporter localized in the Golgi apparatus, is responsible for zinc transport from the Golgi apparatus to the cytoplasm. Mice deficient in this gene exhibit growth failure of bone tissue and kyphosis (85). In addition to intracellular Ca^{2+} signaling, the development of scoliosis due to impaired Zn^{2+} signaling should also be considered.

iv) Impairment of osteoblast differentiation by the upregulation of Pai-1 via miR-224-5p. We attempted to comprehensively analyze gene expression in the kyphoscoliotic region in the spines of IS rats using a microRNA (miRNA) array in addition to the DNA microarray. miRNAs are short (~22 nucleotides) and functional RNA molecules that bind complementarily to the 3' untranslated region of specific mRNA and regulate gene expression (86). Recent accumulating evidence indicates that miRNA-based regulation of the differentiation and proliferation of bone and chondrocytes is essential for the normal function of the bone (87). For example, miR-21 (88) and miR-2861 (89) play a protective role against osteoporosis by suppressing the expression of programmed cell death 4 and histone deacetylase 5, respectively. More than 10 miRNAs are able to suppress osteocyte differentiation using runt-related transcription factor 2 as a target gene (90). In addition, miR-224-5p suppresses osteoblast differentiation (91). We reported that the expression of miR-224-5p is significantly increased in the lumbar spines of IS rats in comparison to WT rats (Table I) (48). A pathway analysis revealed that miR-224-5p is linked to the expression of plasminogen activator inhibitor-1 (Pai-1). Two reports support the involvement of Pai-1 in the formation of osteoblasts through collagen synthesis. First, the lack of Pai-1 is associated with the upregulation of collagen synthesis by Smad and non-Smad (ERK1/2 MAPK) signaling (92). Second, Pai-1 deficiency increased the level of type I collagen (Col I) mRNA in the femoral bone of female mice (93). This means that Pai-1 regulates normal bone formation through the expression of Col I, which is a marker of osteoblast differentiation. Indeed, we confirmed that the protein expression of Col I is reduced in the lumbar spine of IS rats (Table I) (48). These findings suggest that congenital kyphoscoliosis may be caused by impaired osteoblast differentiation due to the miR-224-5p-mediated overexpression of Pai-1 (Fig. 2).

Ehlers-Danlos syndrome (EDS) is an inherited disorder characterized by systemic connective tissue fragility, including fragility of the skin, joints, and blood vessels. EDS also includes symptoms of bone formation abnormalities, such as kyphoscoliosis and spondylodysplasia (94). Alpha-1 Col I, (COL1A1), which encodes the major component of ColI, is one of the genes responsible for EDS (95). Whether or not miR-224-5p or Pai-1 is involved in the aberrant expression of COL1A1 in EDS is expected to be the focus of future studies.

5. Conclusions and future perspectives

A comprehensive analysis of the RNA/miRNA expression in IS rats has identified several genes that are likely to be responsible for the development of kyphoscoliosis. In the future, the analysis of these pathways in the lumbar spine of IS rats will provide new insights into the causes of congenital kyphoscoliosis. However, the results of genetic analysis of rat lumbar spine may not be directly applicable to the causative genes of human congenital kyphoscoliosis. Furthermore, our series of studies did not consider the influence of epigenetics, a mechanism of gene expression regulation that does not involve changes in the DNA base sequences. Therefore, further studies are warranted. Moreover, there are considerable limitations to faithfully reproducing human diseases in animal models. In the future, we need to shift our focus to studies using human-derived tissues and cells, including specimens from patients with kyphoscoliosis.

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Authors' contributions

TS and NS conceptualized the study. TS, IT, YW and NS made a substantial contribution to data interpretation and analysis. TS and NS wrote and prepared the draft of the manuscript. DY provided critical revisions. All authors contributed to manuscript revision, and have read and approved the final version of the manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

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