Chapter 5
Animal Models of Idiopathic Scoliosis

Zhaoyang Liu and Ryan Scott Gray

Introduction

The term scoliosis is derived from the Greek skolios (σκολιός), meaning to curve or twist; in human, scoliosis is used to describe any atypical curvatures of the spine greater >10° with rotation of one or more vertebral bodies. In most cases, the term "scoliosis" is commonly used to refer to the so-called (adolescent) idiopathic scoliosis (IS); however, the incidence of abnormal spine curvature can manifest in many human diseases; as of June 2017, there are 774 Online Mendelian Inheritance in Man (OMIM) unique entries with some indication of scoliosis. Given the high incidence of scoliosis among human diseases, it is not surprising that many forms of scoliosis (e.g., congenital, kyphoscoliosis, and IS) are modeled in other vertebrate species. Our focus for this chapter is the review and synthesis of how current animal models of scoliosis inform the pathogenesis of normal spine development, homeostasis, and disease, with particular emphasis on models of scoliosis that develop postnatally without overt vertebral dysplasia. There are detractors of the validity of using animal models to study IS, in some cases declaring that a central tenet of the disease is that it is a strictly "bipedal" or "human" condition. Despite these critiques, we hope to highlight examples where animal models have generated fundamental insights into potential biological origins of IS in humans. Regardless of the ultimate clinical relevance of these models for human IS, we suggest that a deeper mechanistic understanding of spine development, homeostasis, and disease using animal models will broaden the understanding of the molecular genetics of spine development and disease in humans.

Z. Liu · R. S. Gray (✉)
Department of Pediatrics, The University of Texas at Austin Dell Medical School, Austin, TX, USA
e-mail: ryan.gray@austin.utexas.edu

Normal Spine Function Requires the Integration of Multiple Musculoskeletal Tissues

The structural units of the spine – the vertebral bodies and the intervertebral discs – are derived from segmented condensations of cartilaginous anlage that originate from the embryonic somites flanking the notochord, which ultimately fuse at the midline [1, 2]. The notochord is derived from chordamesodermal cells during gastrulation and can function as a primitive spine in free-swimming aquatic larvae, such as zebrafish and frog, prior to skeletogenesis [3–5]. In addition to this structural role, the notochord also has a critical role in both formation and structure of the spine as signals derived from this tissue are absolutely required for the formation of a segmented vertebral column [6] and direct formation of the nucleus pulposus portion of the intervertebral discs [7].

The maturation and homeostasis of a healthy, functional spine requires the integration of several musculoskeletal tissues including the bone, cartilage and connective tissue, muscle, and the peripheral nervous system. It stands to reason that overt defects in one or more of these musculoskeletal components of the spine could yield scoliosis, for instance, severe vertebral dysplasia is commonly associated with the spine curvatures. However, in the case of IS, there have been few indications of underlying structural defects that would explain pathogenesis of IS. Thus, it is reasonable to speculate that more subtle, subclinical defects of one or more musculoskeletal components of the spine may contribute to pathogenesis of IS. Indeed, magnetic resonance imaging studies suggest differences in signal intensity in the apex of the curvature, and postmortem analysis has shown that changes in the typical expression of anabolic markers and known markers of disc degeneration are found in the intervertebral disc (IVD) of IS patients [8]. Finally, the biology of how the spine and its integrating components develop during adolescence and are maintained in adults remains poorly understood. For instance, how are the annulus fibrosis and nucleus pulposus components of the IVD maintained and how might subtle defects in these tissues contribute to instability of the spine in IS patients. Moreover, it has been observed that the outer annulus fibrosis of the IVD is innervated in humans [9] and rats [10], yet it remains to be determined how these innervations are important for normal spine physiology and their pathology might contribute to disease.

Dysfunction of extrinsic neuroendocrine factors, such as melatonin [11], or metabolic hormones, such as incretins [12, 13], has been reported to be associated with IS in humans and animal models. While there has been limited mechanistic insight attributed to these associations in vivo, it is very likely that these or other systemic interactions of extrinsic and intrinsic musculoskeletal components of the spine are important for the development and homeostasis of the spine. For these reasons, it is wise to contemplate more diverse, potentially synergistic pathoetiologies for IS. For example, minor defects of innervations of the paraspinal muscles and intervertebral disc might be sufficient to generate instability of the spine during period of rapid growth, leading to scoliosis, as was recently shown in mouse [14]. Alternatively,

defects in a key neuroendocrine signaling pathway may lead to decreased bone mineral density/osteopenia which could generate weakness in vertebral endplates generating spine instability. Future research into the pathogenesis of IS should begin to address these hypotheses by empirical testing in animal models. The challenge for these models will be to reconcile these mechanistic insights with the known phenotypes of IS in humans and ultimately confirmed in using precious human tissue samples.

Spine Form Follows Dysfunction

The development of the spine begins in utero but undergoes tremendous growth and maturation during adolescence in humans. The same processes of spine development, postnatal refinement, and maturation of the axial skeleton are recapitulated in other vertebrate species including the mouse and zebrafish model systems. There are three broad classifications of scoliosis in humans including congenital, neuromuscular/syndromic, and idiopathic. Congenital scoliosis (CS) is a developmental disorder characterized by overt structural malformation/dysplasia of one or more vertebral units, which can result in focal spine curvatures, present at birth [15]. Defects in somite segmentation or notochord development have been shown to be primary causes of vertebral malformations and CS in animal models. For example, the majority of mutations associated with human CS disrupt Notch signaling components, many of which have been nicely modeled in the mouse [16, 17]. Whereas, the induction of notochord defects via chemical inhibition of lysyl oxidases [18], genetic disruptions of the extracellular matrix components of the notochord sheath [19–21], or by disruptions of lysosomal-dependent vacuolation of the inner most notochord cells [22] lead to vertebral malformations and scoliosis in the zebrafish model. CS can also be modeled by gene-environment interactions in mouse, as was shown by haploinsufficiency of known Notch signaling components in combination with an environmental stressor, hypoxia, in utero [23]. This is particularly important in light of several observations of increased incidence of IS within families of CS patients [24]. Moreover, recent studies in zebrafish suggest that CS and IS may share a common genetic basis [25, 26], where the pathology is altered by changes in gene dosage of *protein tyrosine kinase 7* (*ptk7)* [26] or *ladybird homology domain 1b* (*lbx1b*) [25] during embryonic development. It will be important to determine whether more subtle defects in somite segmentation or disruptions of notochord development during embryonic development can predispose the onset of IS in humans.

Neuromuscular/syndromic scoliosis encompasses spine defects that are thought to be downstream of a general loss of muscle tone via inherent defects of the axial muscles or of their innervations or initiated by severe joint laxity or general weakness of the connective tissues of the axial skeleton. For instance, patients suffering from Duchenne muscular dystrophy [27], neurodegenerative diseases like Rett syndrome [28], or joint hypermobility/connective tissue diseases such as Marfan

syndrome [29] and Ehlers-Danlos syndrome [30] are known to display increased incidence of scoliosis without obvious vertebral malformations in humans. A recent report beautifully illustrates that functional ablation of the TrkC neurons, which provide connections between the proprioceptive mechanoreceptors and the spinal cord, can generate a model of IS in mouse [14]. While animal models exist for a wide-range of neurological and connective tissue disorders, very few examples have been reported to display scoliosis as a phenotype [27, 31, 32]. In our opinion, this may simply be explained by under sampling of the spinal architecture in these models.

In contrast to neurological or syndromic scoliosis, clinical manifestations of IS usually occur during adolescence in otherwise healthy individuals. IS can be viewed as a diagnosis of exclusion, wherein IS patients should not display overt vertebral dysplasia, neurological deficits, or other known diagnoses, although many of these measures of diagnoses are not necessarily tested for in all clinical settings. That said, rare variants in connective genes known to contribute for Marfan and Ehlers-Danlos syndromes are reported to be associated with IS in humans [33, 34], suggesting that some AIS patients could represent subclinical examples of these syndromes. Alternately, the pathogenesis of IS may act via somatic loss of heterozygosity or epigenetic changes in the normal pattern of gene expression of other known CS, neuromuscular, or connective tissue disease genes. Ongoing efforts to utilize modern genomics approaches, coupled with experimental testing in animal models, will be critical to test these complex models of pathogenesis.

What Makes a Good Animal Model of Disease?

The molecular genetics and underlying pathology of IS are not fully understood, despite millennia of clinical investigation [35]. For this reason, it is difficult to understand how progress will be made without well-structured experimental approaches including animal models. In recent years, different animal models displaying characteristics of IS have been characterized. These models represent the most ethical and cost-effective way forward to gain deeper mechanistic understanding of both normal development and disease onset and progression of the spine. An explicit animal model should both model the phenotype(s), underlying genetic causes, and natural history of disease and provide an experimental platform for the development of therapeutic interventions of disease. Unfortunately, there are very few examples of animal models that meet this strict set of criteria for any disease [36]. For these reasons, we see a benefit in considering levels of validity for animal models of human disease [37], both for reviewing the current data and as a way of building toward more relevant animal models of IS in the future. We refrain from making strong assertions of whether an animal model has bona fide relevance to human IS; instead, we will discuss several examples which portray morphological similarities to IS as observed in human; undoubtedly caveats exist. We echo the following metrics of validity to characterize animal models of IS:

Face validity An animal model with similar phenotypic indicators of human IS. At this level, a model should exhibit a postnatal-onset scoliosis, in otherwise healthy individuals, and without obvious vertebral malformations. Indeed, there are multiple examples of animal models of IS induced by surgical resection or tethering of vertebral elements and resection of the pineal gland. Moreover, there are a growing number of heritable, genetic models in mouse and zebrafish that display phenotypes observed in IS (Table 5.1). Undoubtedly, many of these models will fall short of relevance for human IS; however, all of these models provide a foundation for assessing structural principles and biological mechanisms for spine stability.

Construct validity An animal model which displays face validity and also displays a similar underlying molecular genetic basis of IS in humans. At this level, the animal model should be the result of an analogous genetic mutation or relevant cellular pathology that is associated with IS in humans. Thus far, only a few examples of animal models at this level of validity have been reported for IS (Table 5.1). Moving forward the method of genetic engineering utilized to engineer the model should be considered. For instance, a complete loss-of-function, "knockout" model of a candidate IS gene might not be as valid in comparison to an animal model that is engineered to contain a tissue-specific conditional loss-of-function or by engineering a "humanized" allele of a candidate IS mutation. With the advent of modern genome editing and conditional genetic approaches, this level of validity should be the standard for modeling human disease, while less robust genetic approaches such as morpholino "knockdown" or transient transgenics should be avoided.

Predictive validity An animal model that has a similar response to clinically validated therapeutics for IS. Thus far, no animal models of IS have been reported for this level of validity. The development of these "gold-standard" models will be critical for the improvement of current interventions or testing of new therapeutics for IS.

Animal Models

There are four distinct classes of animal models reporting some of the hallmarks of IS: (i) pineal resection models, (ii) mechanical models, (iii) environment models, and (iv) genetic models (Table 5.1). The pineal resection models have been recently, extensively reviewed [11]; for this reason, we will not reassess these models here.

Table 5.1 Animal Models of Idiopathic Scoliosis

Model description	Genetic assay	Induction and specificity of genetic assay	Associated phenotype	Heritable IS model established?	Level of model validity	Association of model with human genetics studies of IS?	Cellular/Tissue defect?	Refs
Guppy	Spontaneous *curveback* mutation	Constitutive	Scoliosis	Yes	F	N/A	N.D.	*Scoliosis*. 2010 Jun 7;5:10.
Medaka	Spontaneous *wavy* mutation	Constitutive	Scoliosis	Yes	F	N/A	Dysplasia of the intervertebral disc	*J Toxicol Pathol* 2016; 29: 115–118
Mouse	Conditional *Gpr126;COL2CRE* mutant	Specific to osteochondroprogenitor cells, constitutive, beginning at E12.5	Late-onset thoracic scoliosis with co-incident rib cage malformation	Yes	F, C	Yes	Increased cell death and misregulation of gene expression in costal rib cartilages and IVD tissues	*Hum Mol Genet.* 2015 Aug 1;24(15):4365–73.
Mouse	Conditional *Slhp2;COL2CRE^ERT2* mutant	Induction in osteochondroprogenitos/chondrocytes at 4 weeks	Thoracic and lumbar scoliosis, kyphosis, with rotation	Yes	F	No	Growth plate defects	*Spine* (Phila PA 1976). 2013 Oct 1;38(21):E1307–12.
Mouse	Heterozygous *Chd2/+* mutant	Constitutive	Lordokyphosis	Yes	F, C	Yes	N.D.	*Am J Med Genet A.* 2008 May 1;146A [9]:1117–27.

Mouse	Double Gdf5/6 mutant	Constitutive	Scoliosis	Yes	F	No	Connective tissue; joint fusion, inner ear defects	*Developmental Biology* 254 (2003) 116–130
Mouse	Conditional transgene Col11a2::NPR2 mutation	Cartilage-specific, constitutive expression, beginning at E11.5	Severe scoliosis	Yes	F	No	Growth plate defects	*PLOS One* vol7, issue 8, Aug 2012
Mouse – Mutant	Fgfr3 −/−	Constitutive	Late-onset progressive scoliosis, kyphosis, with rotation	Yes	F	No	Overgrowth of long bone, osteopenia, inner ear defects	*Frontiers in endocrinology* Gao et al., 2015
Rabbit – Hereditary lordoscoliotic rabbit	Spontaneous mutation	Constitutive	Scoliosis	Yes	F	N/A	Increased melatonin levels were associated with mutants	*Spine* (Phila PA 1976). 2003 Mar 15;28(6):554–8.
Zebrafish – Mutant	ptk7	Zygotic mutant	Scoliosis	Yes	C	Yes	Ependymal cell cilia defects, cerebral spinal fluid flow defects	*Nat Commun.* 2014 Sep 3;5:4777 and *Science.* 2016 Jun 10;352(6291):1341–4.

(continued)

Table 5.1 (continued)

Model description	Genetic assay	Induction and specificity of genetic assay	Associated phenotype	Heritable IS model established?	Level of model validity	Association of model with human genetics studies of IS?	Cellular/Tissue defect?	Refs
Zebrafish – Mutant	*c21orf59*	Temperature sensitive mutation	Scoliosis	Yes	F	No	Ependymal cell cilia defects, cerebral spinal fluid flow defects	*Science*. 2016 Jun 10;352(6291):1341–4.
Zebrafish – Mutants	*ccdc40, ccdc151, dyx1c1*	Constitutive, after RNA injection to rescue embryonic phenotypes	Scoliosis	Yes	F	No	Ependymal cell cilia defects, cerebral spinal fluid flow defects	*Science*. 2016 Jun 10;352(6291):1341–4.
Zebrafish – Mutant	*kif6*	Constitutive	Scoliosis	Yes	F	No	No overt cilia defects in embryos; N.D.	*Dev Dyn*. 2014 Dec;243(12):1646–57.
Zebrafish – Overexpression/knockdown	*poc5*	Morpholino injection, followed by expression of human wild-type or candidate mutations of POC5	Humanized *POC5* expression was unable to rescue morphants	No	C	Yes	N.D.	*J Clin Invest*. 2015 mar 2;125(3):1124–8.

Zebrafish – Overexpression	*lbx1b*	Overexpression of *lbx* genes. Transgenic overexpression of *lbx1b* from the *lbx1b* promoter	Overexpression did not allow for adult viability. Most Tg(GATA2-1b:lbx1b) founder fish displayed CS-like defects, some displayed IS-like spine curvatures	No	F	Yes	N.D.	*PLOS Genetics* January 28, 2016
Zebrafish – Mosaic mutations; F0 CRISPR	*mapk7*	F0, mosaic CRIPSR mutations	Scoliosis	Not reported	C	Yes	N.D.	*Human Mutation.* 2017;1–11.

F face validity, *C* construct validity

Mechanical Models of IS

The majority of IS models reported thus far have been generated by the establishment of a mechanical asymmetry via bracing, tethering, or resection of axial tissues. It is clear that bipedal and quadrupedal animals do not share identical vectors of axial loading and stress; however, all vertebrates experience mechanical loads and torsion on the spine as they move through the environment regardless of whether they are terrestrial or aquatic animals [38]. By systematically altering mechanical properties of the axial column, these studies have provided critical insights of the anatomical components necessary for normal spine stability. Furthermore, the majority of these experimental approaches have been shown to destabilize quadrupedal animals which further supports the notion that these principles are evolutionarily conserved biomechanical properties of spine stability, irrespective of the forces applied to the spine during locomotion.

The first reports of experimental scoliosis by systematic resection analysis were performed in rabbit by Langenskiold and Michelsson [39–41]. In these seminal experiments, the authors provide an anatomical framework for spine stability, concluding that "... unilateral resection of the posterior ends of the sixth to eleventh ribs including the costal parts of costo-ventral joints" provokes a model of progressive scoliosis. This suggests that the normal attachment of the lower rib cage to the vertebral column is a critical biomechanical component for maintaining spine stability. In agreement, unilateral rib osteotomy or nonsurgical rib cage deformity via thoracic restraint prior skeletal maturity was also shown to generate progressive thoracic scoliosis with rotation in mouse [42]. Importantly, the induction of scoliosis in this thoracic restraint model was relieved by bilateral rib neck osteotomy prior to bracing. Together these data support a model where the biomechanical decoupling of the rib cage and spine can lead to onset and progression of scoliosis, as had been previously proposed [43, 44]. For this reason, abnormal interactions of the spine and rib cage should be considered as a principle etiological factor causing progressive thoracic scoliosis in humans. This model is further supported by observations in that IS patients which commonly have compressed, flatten rib cage morphology or disruptions of sternum [35, 45–49]. Longitudinal studies of human IS to assess how changes in the morphology of the rib cage contributes to the onset and progression of IS will be greatly assisted by modern low-dose radiation, 3D imaging platforms [50].

Postnatal spine maturation proceeds via the growth and ossification of several cartilaginous unions (synchondrosis) to include the costovertebral joints – which articulate each rib with an individual vertebral unit – and the neurocentral joints, which join the neural arch to the vertebral body or centrum. Moreover, the vertebral units, which are derived from cartilaginous anlage flanking the notochord, are themselves synchondroses, typically fused prior to birth in humans. Perhaps defects in the maturation and closure of these axial midline synchondroses contribute to the onset of IS. As described above the ablation of the normal mechanical properties of the costovertebral joints can generate robust IS-like scoliosis in rabbit and mouse.

In agreement the hemi-circumferential ablation of the neurocentral joint by electro-coagulation results in mild scoliosis in a growing pig model [51]. Taken together it is clear that the abnormal development and maturation of these axial synchondroses may serve as a source of mechanical asymmetry, contributing to the onset of IS in some cases.

In addition to intrinsic defects of the spine, extrinsic components of the spine such as dysfunctional sensorimotor control, asymmetry of the semicircular canal, and vestibular defects have been proposed to contribute to pathogenesis of IS [52–56]. Resection studies in the frog *Xenopus laevis* show that unilateral resection of the inner ear/vestibular structures results in progressive scoliosis without vertebral dysplasia [57]. The spine curvatures in this model are hypothesized to be due to the introduction of an asymmetric muscle tone and a progressive deformation of the cartilaginous structural elements of the spine prior to ossification [58]. The model born out of these findings suggests that loss of vestibular function may promote a general tonic imbalance in descending pathways of spinal motoroneurons which would contribute to asymmetric torsion on the spine; in particular during development in utero, where weight-supporting limb proprioceptive signals are diminished and the immature skeleton is composed of less ossified structural elements, connective tissues, and synchondrosis. In this manner, even subtle defects of the biomechanical properties of these elements of the spine could be primary to the initiation of scoliosis during periods of rapid growth in adolescence. On the other hand, it may be possible that any imbalance of descending neurological pathways during embryonic and postnatal development, whether peripheral or central, may initiate deformations of the soft spine elements via asymmetric muscle tone. Many disorders affecting the central nervous system in humans also manifest scoliosis; however, several mouse models with defects in the peripheral nervous system do not display scoliosis [59–62]. However, it was recently shown that tissue-specific ablation of *Runx3* from peripheral neurons (*Wnt-1Cre*), which ablates proprioceptive function, generates characteristics of IS with generalized ataxia in mouse [14]. In conclusion, these mechanical animal models of scoliosis provide strong support that both structural and neuromuscular asymmetries during periods of spine development can cause scoliosis that model IS with face validity.

Environmental Models

There is a report of scoliosis in a wild fish population in Belews Lake, North Carolina. These fish displayed curves along both dorsal-ventral and medial-lateral axes, which is phenotypically similar to scoliosis reported in other zebrafish mutants with late-onset scoliosis [63, 64]. The cause of scoliosis in these fish is thought to be due to contaminated wastewater effluent from a coal-fired power plant, found to contain high levels of selenium. Interestingly, after bioremediation efforts which decreased levels of selenium, the incidence of spine deformity was found to be concomitantly decreased in the population [65]. This report suggests that high levels of

selenium have teratogenic effects on normal spine development. The mechanism of this effect has not been determined or recapitulated in other established laboratory animal models.

Genetic/Heritable Models of IS

There are multiple examples of both aquatic and terrestrial animals in the wild which display IS-like scoliosis, including bonobo [66], orangutan [67], dolphins [68, 69], gray whale [70], and sea otter [71], although it is unknown whether the pathogenesis of these curvatures represents environment or genetic factors. Regardless, there are several examples of heritable models that display characteristics of IS reported in guppy, quail, chicken, and rabbit. Unfortunately, most of these animal models are no longer available for modern genetic studies [72–74]. More recently studies using genetically defined laboratory animal models have begun to identify heritable genetic lesions which generate models of IS [26, 62, 63, 75]. The continuation of mechanistic studies in these genetically defined, heritable animal models will greatly aid in the deeper understanding of the genetics of normal spine development and will contribute to our understanding of the pathogenesis of scoliosis in humans.

Aquatic Models of IS

The spontaneous *curveback* mutant guppy (*Poecilia reticulata*) model of IS which displays face validity due to larval-onset scoliosis with a dorsal-ventral sinusoidal curvature, with additional medial-lateral curves and rotation, without the presence of any underlying vertebral malformations [76]. This mutant phenotype was mapped to a qualitative trait locus on the guppy linkage group 14, which was estimated to explain >80% of the genetic variance [77]. The exact nature of the genetic lesion controlling spine instability in the *curveback* mutant has not been reported.

Several other IS models have been reported in the zebrafish (*Danio rerio*) model. The zebrafish model system is one of the premier model organisms for unbiased forward genetic screening of relevant phenotypes as well as for the application of functional testing of genetic associations of human disease using modern gene-editing techniques. Robust techniques have been successfully employed in the zebrafish model to study development and human disease including injection of synthetic RNA constructs, chemical genetic screens, modern transgenic approaches, and the use of antisense morpholino oligonucleotides (morpholinos) to reduced or knockdown gene expression or translation of gene products [78]. With the recent advent of modern reverse genetic approaches such as TALENs or CRISPR-Cas9 [79, 80], it is now feasible to substitute wild-type (WT) alleles with variant, "humanized" alleles found in human genetic studies, making this model a cost-effective

resource for the development of animal model of IS displaying construct validity. Moreover, zebrafish undergo external development and have mostly transparent bodies during the range of skeletal development making them increasingly valuable for studying postembryonic skeletal development and disease [21, 64, 81–86].

For these reasons, recent genome-wide association studies (GWAS) of human IS cohorts have utilized the zebrafish model to test for functional roles of these risk loci. For example, the human variant, rs6570507, located within an intron of the *G protein-coupled receptor 126* (*GPR126*) locus, is associated with human IS [87]. To test a model of loss-of-function for *gpr126* in zebrafish, morpholinos were used to knockdown *gpr126* expression in embryonic zebrafish. This resulted in minor defects of the ossification of the axial skeleton in early larval stages [87]; however, no scoliosis was observed in adult mineralized spines in this fish. At first glance this *gpr126* morpholino-based model appears to have some mechanistic validity in the spine; however, it is important to note that several missense and nonsense alleles of *gpr126* in zebrafish have been described to exhibit defects in myelination due to defects in Schwann cell biology [88] and dysplasia of the cartilaginous semicircular canal of the inner ear [89]; in contrast none of these *gpr126* mutants were reported to display defects in bone mineralization or scoliosis. Perhaps the simplest interpretation is that *gpr126* knockdown reagents are exhibiting non-specific effects on the mineralization of the axial centra, having little to do with the true nature of *gpr126* function in zebrafish development.

The human variant, rs11190870, represents one of the most robust signals for risk of IS in multiple ethnic backgrounds [90–93]. This SNP is located several kilo bases downstream of the *Ladybird Homology Domain 1* (*LBX1*) gene. Lbx proteins are homeodomain DNA-binding nucleoproteins with well-defined roles in transcriptional regulation and cell lineage determination including migration of muscle lineages [94] and specification of neuronal subtypes in the brain and spinal cord [95–97]. Guo et al. found increased binding of nucleoproteins as well as increased transcriptional activity using a region of DNA containing the "risk" rs11190870 T-allele as compared to the same region of DNA with the "non-risk" C-allele [25]. Together these findings suggest a gain-of-function model for the T-allele, perhaps leading to increased or ectopic expression of the *LBX1* gene. However, it should be pointed out that both T- and C-alleles are common in a diverse range of ethnic backgrounds. Moreover, the T/T and T/C haplotypes are the most common in all ethnic backgrounds tested which implicates the T-allele as the ancestral allele. In agreement, we have only observed the T-allele in the mouse and zebrafish models, where we have assayed thus far (unpublished data).

The zebrafish model was utilized to test this gain-of-function model for *LBX1* in the pathogenesis of IS. Zebrafish have three paralogues of *LBX1*, *lbx1a*, *lbx1b*, and *lbx2*, and a *LBX1 antisense RNA 1* (*LBX1-AS1*; *FLJ41350*) gene closely linked to the *lbx1a* locus. Scoliosis or axis defects were not observed in either *lbx1b⁻ᐟ⁻* or *lbx2⁻ᐟ⁻* mutants; however, neither *lbx1a* mutants nor compound mutants were assayed. To test for a gain-of-function of these genes, the authors injected synthetic RNAs of each of the three zebrafish *LBX1*-related genes, human *LBX1*, or *LBX1-AS1* RNA finding that overexpression of all zebrafish and human paralogues induced

embryonic defects including notochord bending and convergent and extension defects of the axial mesoderm. The overexpression of *Lbx* RNAs induced decreased expression of *wnt5b* in the presomitic mesoderm, which is necessary for convergent and extension behaviors during zebrafish gastrulation [98]; however, experiments did not yield viable adult fish for analysis of the spine.

Interestingly, CS-like defects and kinks and bends of the notochord and vertebral malformations and scoliosis in mineralized adult spines were observed in transient transgenic zebrafish engineered to express *lbx1b* under the control of a portion *lbx1b* regulatory region (*Tg[lbx1b:lbx1b]*), which faithfully recapitulates the endogenous embryonic expression pattern of *lbx1b*. As previously discussed, structural defects of the notochord are well-established to contribute to vertebral malformations in zebrafish [20–22, 99, 100], which is very likely to be the antecedent of the vertebral malformations in these F0 *Tg[lbx1b:lbx1b]* transgenic zebrafish. Interestingly, a few F0 *Tg[lbx1b:lbx1b]* transgenic animals displayed scoliosis without vertebral malformations, more reminiscent of IS, presumably due to differences in the efficiency of integration or size of the clones expressing the transgene. Alternately, the locus of integration may regulate the spatial and temporal expression of the transgene in turn regulating the onset and severity of the phenotype.

In general, somatic transgenes (F0 s) are not generally considered a valid method for genetic analysis in the zebrafish community, and thus any conclusions made via this type of transient analysis are difficult to interpret. For instance, these defects could be related to non-specific effects due to the overexpression of *lbx1b*, rather than revealing a relevant function of *lbx* genes during zebrafish spine development. Unfortunately, this approach produced no viable F1 transgenic lines precluding analysis of the mature spine in stable *Tg[lbx1b:lbx1b]* transgenics. These data illustrate that expression of *lbx1b* must be tightly regulated during normal development in zebrafish and that dysregulation of *LBX1* expression may highlight a common genetic basis of CS and IS in humans.

Additional support for a genetic basis for CS and IS was described by loss-of-function studies of the *ptk7* gene in zebrafish [26], which showed that maternal-zygotic *ptk7* mutants (*MZptk7*) – where the eggs are lacking *ptk7* gene product – display vertebral anomalies commonly associated with CS (e.g., hemivertebrae and vertebral fusions). Mechanistically, *MZptk7* mutant embryos display defects in the patterning of known somite segmentation pathway genes and defective Wnt signaling, which is well known to be important during somitogenesis [101]. In contrast, zygotic *ptk7* (*Zptk7*) mutant zebrafish – where the egg contains maternally deposited wild-type *ptk7* gene products but has little or no functional zygotic transcription of *ptk7* – displayed defects more characteristic of IS. Importantly, a single rare variant of *PTK7* (*P545A*) isolated in a patient with IS was shown to generate defects in Wnt signaling. The pathogenicity of this PTK7 coding variant has not been directly tested in an animal model. *Zptk7* mutant zebrafish display strong face validity with several hallmarks of IS observed in humans including postnatal-onset scoliosis and lack of vertebral dysplasia and in animals that display no apparent defects in behavior or viability.

Further analysis demonstrated that *ptk7* expression in *foxj1a*-postive cells or tissues (Tg[*foxj1a::ptk7*]) is sufficient for maintaining spine stability during larval development [75]. *Foxj1* is a master transcriptional regulator of motile cilia [102], and the motile ependymal cell cilia lining the ventricles are thought to help in the establishment of normal function of the ventricular system by helping to facilitate flow of the cerebral spinal fluid (CSF) in the brain. Importantly, a stable *Tg[foxj1a::ptk7]* transgene rescued the loss of ependymal cell cilia and normal CSF flow in *Zptk7* mutant zebrafish. Together these data implicate defects in ependymal cell cilia and CSF flow in the pathogenesis of IS. Similar defects are typically associated with hydrocephalus and postnatal lethality in mouse; it will be interesting to see if milder defects in the ventricular system would affect spine stability. It will be important to determine if defects in the ventricular system of the brain and spinal cord might underlie the association of scoliosis that is secondary to many neurological disorders such as Rett syndrome and cerebral palsy.

The theme of ependymal cell cilia and ventricular defects contributing to scoliosis in zebrafish is recapitulated by several other mutants which disrupt components of primary or motile cilia, CSF flow, or generate primary ciliary dyskinesia in zebrafish [75]. In particular, a temperature-sensitive allele (*tm304*) of the *c21orf59* gene was used to define a temporal window of larval development (from 18 to 30 days' postfertilization (dpf)) when its function is required to maintain normal spine morphology. Presumably, these experiments also define a critical window when the function of these motile cilia components and functional CSF flow is necessary for spine homeostasis; however, the precise link between the ventricular system and spine stability remains to be determined. Interestingly, this period of development in zebrafish corresponds to well-documented periods of rapid growth in zebrafish [26, 103] and may be analogous to periods of rapid growth during adolescence in humans. These mutant zebrafish models of IS exhibit face validity and provide novel models of defective CSF flow in zebrafish with which to investigate how this process may regulate typical spine morphology.

Finally, the relationship between inadequate CSF flow and scoliosis has been observed in other experimental models of syringomyelia, generated by injection of Kaolin (hydrated aluminum silicate) into the subarachnoid space in rabbit and dog [104, 105] and may underlie spine defects humans with Chiari malformations which also display obstructed CSF flow and higher rates of scoliosis [106]. It will be interesting to understand if subclinical deficits in ependymal cell cilia or alterations to CSF flow may contribute to risk of IS in humans.

Other genes predicted to encode proteins that are core components of the cilia have been implicated in IS in zebrafish including the *kinesin family member 6* (*kif6*) gene, a microtubule motor protein, and the centriolar protein homolog *poc5*. In the case of *kif6,* multiple non-complementing frameshift mutations generate late-onset scoliosis without vertebral dysplasia [63], which provides a model of face validity for IS. While no defects in ciliogenesis of either primary or motile cilia were observed in *kif6* mutant embryos up to 5dpf, the *kif6* transcript was shown to be expressed in the brain. It will be interesting to test whether *kif6* has a role in the ventricular system as was shown for *ptk7*. While there are no associations reported

as yet between *KIF6* and IS in humans have been reported thus far, a recent report suggests that a micro RNA, *MIR4300HG*, associated with progression of IS in human may bind *kif6* transcript, presumably to control its regulation [107].

Several familial cohorts containing IS are reported to be associated with rare single nucleotide variants (SNVs) of the *POC5* gene [108]. Using morpholino anti-sense oligonucleotides (MO), the authors observed that knockdown of zebrafish *poc5* generated a curly-tailed phenotype with lethality after 3dpf phenotypes commonly associated with primary cilia dyskinesia in zebrafish [109–112]. A *poc5* MO was used to "knockdown" endogenous *poc5* expression, and human RNAs were co-injected to examine the effects of candidate human *POC5* SNVs during spine development. Embryos expressing mutant *POC5* RNAs were viable but were observed to have mild to severe axial curvatures in larval fish and IS in the fully mineralized adult skeleton, whereas embryos injected with WT human *POC5* RNA did not develop scoliosis, suggesting that the IS-associated SNVs of *POC5* are loss-of-function alleles [108]. The frequency of these SNVs of *POC5* in these human cohorts was 75% and 30% in each of the cohorts, suggesting that these variants of *POC5* are not sufficient to generate pathology in humans, rather there are likely to be additional disease-modifying mutations that co-segregate with effected individuals. It remains to be determined if the generation of endogenous "humanized" alleles of *poc5* would display similar defects as were observed by knockdown and overexpression of exogenous *POC5* variants. The generation of these alleles may provide construct validity and would be a valuable resource for modifier screens to identify IS loci in the zebrafish model.

Zebrafish genetics provide powerful models for validation for human genetic studies. Many important advances in developmental biology have been made by careful use of morpholino oligonucleotide-based gene interference [113]; however, there are many examples where lack of sufficient controls using MOs confounds the interpretation of the results. Along the same lines, overexpression by RNA injection or by transgenesis in zebrafish allows robust functional assays of human genetic findings. However, careful analysis using dose curves for injection of RNA or generation of stable transgenic lines should be the standard for all experimental design in future studies.

Mouse Models

There are several mutant mouse strains that model aspects of IS, with the majority of these being the result of defects in the development or homeostasis of connective tissues and cartilages (Table 5.1). Using the inducible Col2a1CreERt2 deleter strain, postnatal conditional loss of the *protein tyrosine phosphatase, non-receptor-type 11* gene (also known as *Shp2*) in chondrocytes (*Col2a1+* lineages) during juvenile development (4 weeks of age) generates a late-onset scoliosis in about 40% of the *SHP2*-conditional mutant mice by the age of 12 weeks [114]. The spine curvature in this conditional mutant mouse was very severe, noticeable without X-ray, atypical

lordosis in the upper thoracic spine and kyphosis of the lower thoracic to lumbar spine as well as rotation of individual vertebrae. SHP2 is a positive regulator of RAS-MAPK signaling, which is essential for connective tissue growth [115], and this conditional mouse model of *Shp2* suggests this signaling is required for homeostasis of the intervertebral disc. Indeed, histological analysis revealed variable thickness and a disruption of typically aligned columnar chondrocytes in the vertebral growth plate in these conditional mutant mice. Interestingly, when *Shp2* was removed from Col2a1-expressing lineages later in development (8 weeks of age), no spinal deformity or scoliosis was observed up to 16 weeks of age. The analysis of this conditional *Shp2* mutant mouse reveal a prospective window of susceptibility for pathogenesis of scoliosis due to defects of the disc and that homeostasis of cartilage tissues can be a major driver of spine stability.

Additional associations between cartilage homeostasis and spine integrity are underscored cartilage-specific deletion of SOX9 in postnatal mice, using an inducible aggrecan enhancer-driven CRE deleter strain (*Agc1-CreERT2*) [116]. SOX9 is an essential transcription factor for the differentiation of the chondrocyte lineages during embryonic development [117]. In order to circumvent the embryonic requirement for SOX9, recombination of a floxed *Sox9* allele was induced at 6 weeks of age, and by 4 months the mice displayed severe kyphosis of the thoracic spine. This postnatal loss of *Sox9* also resulted in compression and degeneration of the IVDs, depletion of sulfated proteoglycan and aggrecan content in IVD cartilage, and precocious growth plate closure, and the mutant mice were observed to have defects in overall growth, although the size of individual vertebrae in adult mice was not affected. Global RNA transcriptome sequencing (RNA-seq) analysis revealed that depletion of SOX9 in aggrecan-expressing lineages of the IVD remarkably reduced the expression of several genes encoding extracellular matrix proteins, as well as some enzymes responsible for their posttranslational modification. Furthermore, several cytokines, cell-surface receptors, and ion channels were dysregulated, confirming that SOX9 also has a critical role in coordinating the homeostasis of the IVD postnatally and which is likely to contribute to loss of spine stability. Similar testing at 8 weeks was not reported in this model so it is unclear if spine stability is resistant to a loss of *Sox9* expression at later time points as was observed for *Shp2*.

The regulation of spine structure during postnatal development is also controlled by the growth differentiation factors 5 and 6 (*Gdf5* and *Gdf6*) genes, wherein *Gdf5/6* double mutant mice display severe vertebral column defects in adult mice that are not seen in either *Gdf5* or *Gdf6* single mutant animals [118]. While most double mutant mice fail to thrive to adulthood, severe lateral curvatures (Cobb angle between 39 and 67 degrees) of the spine were observed in roughly 30% of the double knockout mice by the age of 3 months, without vertebral dysplasia. *Gdf5* is expressed in the developing joints of the skeleton and is one of the earliest known markers of joint formation [118, 119]. Histological analysis of the spine and IVD in these double mutant mice reveals reduced proteoglycan staining as indicating a degeneration phenotype of the cartilage. More global defects in the skeletal system were also observed, including a reduction in mineralized bone and narrowing or loss joint space in the limbs, which are not common traits of IS in humans. Despite this

these data illustrate that both *Gdf5* and *Gdf6* are candidate risk loci for the pathogenesis and progression of IS.

While these models all display some face validity for understanding IS, the overt nature of other co-occurring pathologies makes these models less than ideal. Regardless it is important to underscore that these mutant mouse models are likely due to strong loss-of-function models, which may not be the case in human IS. Alternatively, it is more likely that hypomorphic mutations, compound heterozygous mutations, or extragenic enhancer mutations that reduce the level or pattern of expression of these genes will provide some better models of IS. For this reason, variants in the genes highlighted above as well as other loci of well-known genes important for cartilage biology, especially those containing genes known to be important for cartilage, connective tissue, or IVD development, should be considered for validation in animal models.

Changes in bone mineral density or osteopenia may contribute to risk of IS in humans [120, 121]. Interestingly, loss-of-function mutant mice of the *Fibroblast growth factor receptor 3* (*Fgfr3*) gene display scoliosis and kyphosis in juvenile *Fgfr3*$^{-/-}$ mice, which is associated with overgrowth of the axial and appendicular skeleton [122, 123]. *Fgfr3*$^{-/-}$ mice have also been characterized to display reduced cortical bone thickness, defective trabecular bone mineralization, premature joint degeneration, and early arthritis [124, 125]. Longitudinal X-ray and Micro-CT analysis of individual *Fgfr3* mutant mice shows that this model displays progressive onset of spine curvature, where rapid progression in the thoracic spine coincided with rapid body growth up to 4 months [126]. Additional analysis of these mutant spines revealed lateral displacement of the spine and axial rotation of the vertebrae in *Fgfr3* mutants. In addition, an overall increase in the length of vertebrae and a significant reduction of height on the concave side of IVD or wedging were also observed. Quantitative analysis of vertebrae, outside of the curve, showed reduced trabecular bone volume. Interestingly, higher measures of bone mineral density were observed at the concave side compared with the convex side vertebrae within the curve. This phenotype may result from an anabolic response to increased biomechanical strain on the concave side [127]; alternatively, this could be the result of a collapse of the disorganized trabecular network under increased strain [128].

Local treatment of the thoracic spine with a bone anabolic agent PTHrP-1-34 inhibited the progression of lateral scoliosis and vertebral wedging phenotypes in *Fgfr3* mutant mice, but this treatment had little impact on the formation of kyphosis in this model. The changes in IVD morphology in these *Fgfr3* mutant mice were also corrected with PTHrP-1-34 treatment. Taken together, these results suggest that FGFR3 plays a critical role in regulating bone and cartilage growth, and PTHrP activity might be complementary to a loss of FGF signaling in the spine. While this *Fgfr3* mutant model displays strong face validity for IS, no associations with this locus have been observed in humans with IS. That said, the mutations uncovered in the human *FGFR3* locus have been dominant mutations and are known to cause a variety of limb malformations or cranial bone dysplasia – with one exception, where a novel pathogenic homozygous missense mutation in *FGFR3* was isolated in a cohort of patients characterized by tall stature and scoliosis. While this is an inter-

esting validation of the potential role of *FGFR3* for pathogenesis of IS, other abnormalities of the appendicular and axial skeletons in these patients preclude this from being clinically classified as IS; instead this condition is labeled as a skeletal overgrowth syndrome [129]. Regardless, these studies illustrate that FGFR3 function is required for postnatal regulation of spine development and stability and further investigation of this locus and of FGFR3 signaling in human IS patients is warranted.

The C-type natriuretic protein (CNP) and its receptor, *natriuretic peptide receptor 2* (*Npr2*), and downstream effectors are involved in long bone growth in mice [130, 131]. Several gain-of-function mutations of *NPR2* have been identified in patients with an overgrowth syndrome characterized by tall stature, macrodactyly, and scoliosis [132, 133]. Interestingly, transgenic mice strain that expresses a gain-of-function allele of *NPR2* (p.Val883Met) from a cartilage-specific *Co11a1* promoter was observed to display kyphoscoliosis, wider growth plates, increased bone length, as well as upregulation of cyclic guanosine monophosphate (cGMP) level in cartilage [133]. While these alleles in human generate a broader spectrum of phenotypes than commonly observed in IS in human, the mechanism of cartilage overgrowth potentially via over production of cGMP and its downstream effectors may be a relevant mechanism of IS to consider. This mouse model highlights a successful approach in using transgenic mouse genetics to test gain-of-function models of human disease, which may be applied to study IS in the future.

Perhaps the most relevant model of IS has been observed as a conditional loss-of-function of *Gpr126* specifically deleting in osteochondroprogenitor cells (using the Collagen Type II Cre (Col2Cre) deleter strain) [62]. It was observed that embryonic loss of *Gpr126* in osteochondroprogenitor cells resulted in postnatal-onset of spine curvature without vertebral dysplasia in ~50% of the *Gpr126* conditional knockout mice by the age of postnatal day 20 and with increased incidence (>85%) by 4 months of age. Because this *Col2Cre;Gpr126* mutant mouse model has many hallmarks of IS including lack of other vertebral dysplasia and postnatal-onset of the pathology (Fig. 5.1), and because the *GPR126* locus is associated with human IS [87], we suggest it should be viewed as a model of IS with construct validity.

GPR126 has been shown to be required in multiple tissues including during endocardium development [134], in Schwan cells for the myelination of peripheral axons [88, 135], and for inner ear development [89]. The exact cellular etiology of the *Col2Cre;Gpr126* mutant mouse has not been established; however, it is clear that osteochondroprogenitor cells give rise to the bone, cartilages and connective tissues, the IVD, and to many of the tendons and ligaments of the spine. Further work using more refined conditional mouse genetics is needed to address which of these tissues is critical for the pathogenesis of IS in this model.

That said histological analysis of the IVDs did not reveal any overt changes in the patterning or differentiation of the IVD tissues a P1 or P20 in *Col2Cre;Gpr126* mutant mice, with the exception of some incidence of acellular clefts at the midline of the annulus fibrosis and growth plate at both P1 and P20. This suggests that *Gpr126* may have a role in the normal closure of the midline axial synchondroses of the vertebral bodies during the transition from notochord to IVD. Moreover, mild
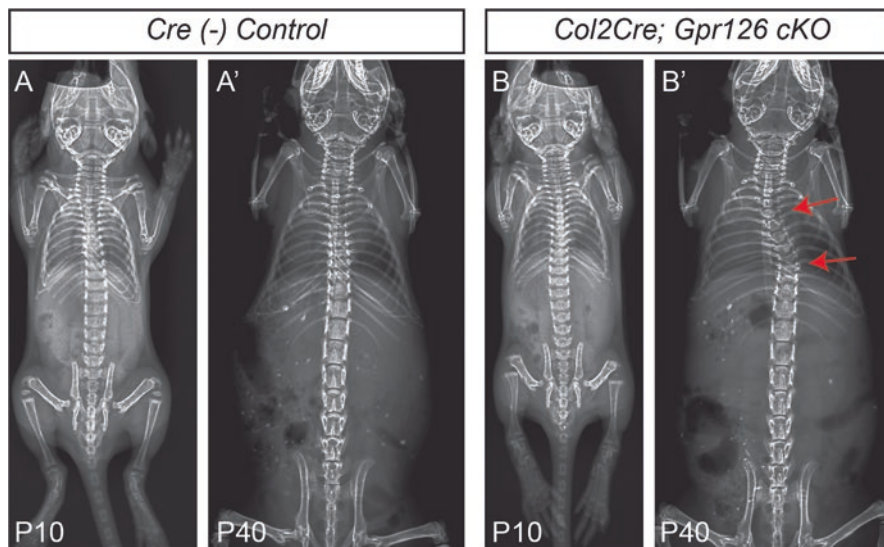
Fig. 5.1 Col2Cre;Gpr126 mutant mice display late-onset scoliosis without vertebral dysplasia. Longitudinal X-ray imaging of (dorsal view) of an individual Cre(-) control (**A-A'**) and *Col2Cre;Gpr126* mutant (**B-B'**) mutant mice. The mice of both genotypes are phenotypically normal without overt vertebral dysplasia at postnatal day 10 (P10); however, by P40 the *Col2Cre;Gpr126* mice display right thoracic spine curvature (red arrows) that are not observed in Cre (-) control mice

increases in cell death visualized by increased TUNEL-positive cells of the vertebral growth plate and IVD were also observed, suggesting a minor role of *Gpr126* in cell survival in these tissues. In contrast, no significant changes in the trabecular bone of the vertebrae or defects in the development or mineralization of the long bones was observed in *Col2Cre;Gpr126* mutant mice, suggesting that *Gpr126* functions in chondrocytes or other connective tissues and not in the bone to maintain the homeostasis of the spine. However, the use of well-characterized lineage-specific CRE transgenic mouse strains will be necessary to support this model. Additionally, further studies should also seek to address if the scoliosis observed in conditional mouse mutants of *Gpr126* is the result of embryonic defects or if its function is required during periods of rapid spine growth.

Interestingly, a significant fraction of these *Gpr126* conditional mutant mice also exhibited dorsal-ward deflections of the sternum, reminiscent of rib cage defects clinically termed *pectus excavatum* (PE) in humans. PE is a common musculoskeletal disorder of the anterior chest wall which has high concomitant incidence with IS in humans [46, 47, 136]. The authors found that loss of *Gpr126* led to upregulated expression of a matrix modifying gene *Galactose-3-O-sulfatransferase* (*Gal3st4*), a gene implicated in human PE [137]. Taken together, these data suggest that *Gpr126* may act as a common genetic cause for the pathogenesis of both IS and

PE, possibly via a misregulation of extracellular matrix gene expression, which was also observed in the inner ear cartilages of *gpr126* mutant zebrafish [89].

Distinct Elements of the Mammalian Spine

While both mouse and zebrafish models are being utilized to study scoliosis, it is important to note distinct differences in the components of the vertebral column between them. For example, the formation of the vertebral units during development and the morphology and composition of the bony vertebrae and of the IVD are quite different. In mouse, the vertebrae form by endochondral ossification of cartilaginous anlagen which fuse at the midline [138]. In contrast, zebrafish vertebrae form by direct mineralization of the notochord sheath to form the perichordal centra which underlie the elaboration of the vertebrate, which does not pass through a cartilaginous stage [139, 140]. Mature mouse vertebrae are columnar-shaped structures that form by endochondral ossification which leaves cavities the interior of the vertebrae, housing the bone marrow. In contrast, mature vertebrae in zebrafish are hourglass-shaped structures, without bone marrow; instead they are filled with vacuolated tissue which is likely derived by the fusion of notochord-derived vacuolated cells [141].

There are also overt differences in the tissue components of the IVD. In mouse, the IVD is a lamellar fibrocartilaginous joint surrounding the nucleus pulposus (NP), characterized by an abundance of hydroscopic proteins (e.g., aggrecan) which act to generate high osmotic pressure, allowing for resilience during compressive strain of the spine [142]. The inner annulus fibrosis (AF) layers of the IVD are composed of strands of fibrocartilage attached to the cartilaginous endplate (CEP). These strands of fibrocartilage in composite form the inner and outer AF lamellar layers that radially and circumferentially surround the NP tissue (Fig. 5.2A, A'), providing structural integrity and containment for the NP [6].

The IVD of zebrafish contains notochord-derived [141] vacuolated cells embedded in a fibrocartilaginous matrix (Fig. 5.2B, B') which highlights a common lineage of the innermost portion of the IVD in both mouse and zebrafish. Despite the common origin of the inner disc tissue (NP), zebrafish do not display some of the molecular hallmarks of NP or AF tissues observed in mouse and human. For instance, Safranin-O staining – which stains mature, healthy AF and NP of sectioned IVD tissue in mouse – does not stain the IVD of adult zebrafish (Fig. 5.2B, B'), in contrast to the cartilage-rich NP of the mouse (Fig. 5.2A, A'). Indeed the formation of glycosaminoglycan-rich NP tissue appears to be a hallmark of mammals [143]. The analogous structure to the AF in zebrafish appears to be a small acellular intervertebral ligament (IVL) that encircles the IVD, originally described in medaka fish (*Oryzias latipes*) [144], and in contrast to the robust fibrocartilaginous strands of cells which make up the AF in mouse (Fig. 5.2A, A'). Interestingly, Safranin-O staining of zebrafish IVD is seen just interior, adjacent to the IVL (Fig. 5.2B', D); however, the function of this group of cells remains unclear.
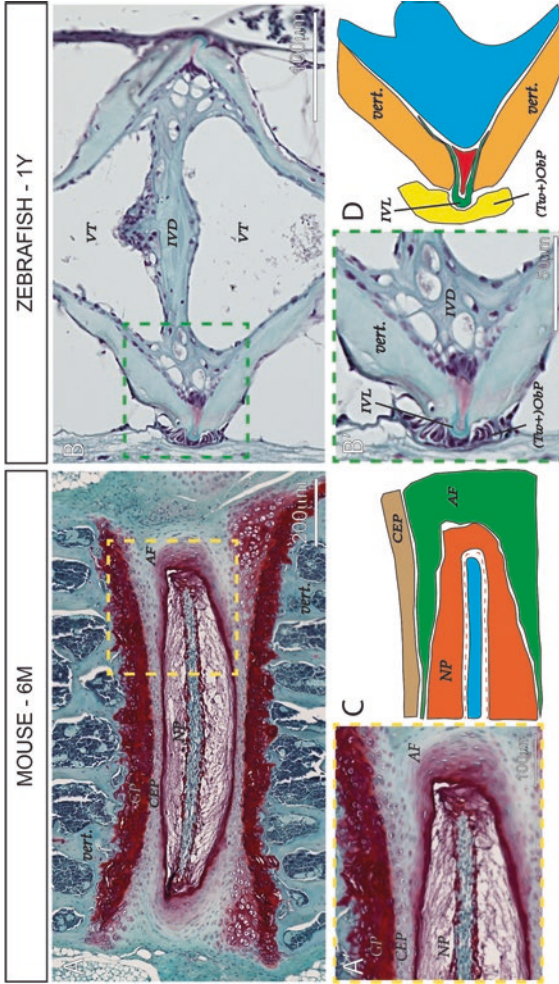
Fig. 5.2 Key differences of the intervertebral disc (IVD) between mouse and zebrafish. Safranin-O/fast green staining of a midline section of a mature intervertebral region in mouse (**A-A'**) and zebrafish (**B-B'**) shows high levels of proteoglycan-rich cartilage in mouse IVD, compared to very little zebrafish (**D**). The mouse IVD displays a large AF which connects two flanking vertebrae at the level of the CEP. The AF in mouse surrounds the NP structure. The NP appears to be composed of three distinct layers: an outer tissue layer which stains for Safranin-O (orange in (**C**)), inner cell layer (dotted red line in (**C**), and an inner tissue layer that does not stain well for Safranin-O (blue in (**C**). In contrast, the zebrafish IVD has only weak Safranin-O staining (red in (**B'**, **D**)) in an interior region adjacent to the intervertebral ligament (IVL) (**B'**, green in (**D**)). The zebrafish does not display a true cartilaginous NP tissue, rather the IVD is composed of vacuolated cells and fibrocartilaginous matrix. In contrast to mouse vertebrae which contains bone marrow and trabecular bone filling the vertebrae, zebrafish vertebrae contain bone-shaped vacuolated tissue (VT). The presence of twist-positive osteoblast progenitor cells ((*Tw+*)ObP) are observed adjacent to the IVL. GP growth plate, CEP cartilaginous endplate, NP nucleus pulposus, AF annulus fibrosis, IVL intervertebral ligament, (Tw+)ObP twist-positive osteoblast progenitors, vert. Vertebrae

Zebrafish IVDs are also observed to host a collection of cells adjacent to the IVL on the exterior side, presumably these are *twist*-positive as shown in the medaka fish, hypothesized to be osteoblast progenitors [144], and may also be important for maintaining the IVL tissue. Regardless of these unambiguous differences in the structure and protein composition of mouse and zebrafish IVD, genetic studies that illustrate defects in these tissues, which are correlated to scoliosis, may have conserved mechanisms in humans in some cases. In the future, careful analysis of these tissues in mouse and zebrafish mutants that display scoliosis should be considered. Case in point, a recent report of a *wavy* mutant medaka, which displays characteristics of IS [145], may be due to dysplasia of the intervertebral disc region. This phenotype may be reminiscent of work showing that defective fusion of notochord vacuolated cells during larval development is sufficient to generate scoliosis and vertebral fusions in zebrafish [22]. It will be interesting to address these issues in mouse models as well as the functional role of the inner most cellular/tissue layer of the NP are likewise unknown (Fig. 5.2A', C). One could imagine that if these cells have a role in growth in homeostasis of the IVD, even subtle defects in their function could contribute to a loss of spine stability. In conclusion, clear structural differences in the mouse and zebrafish spine exist; undoubtedly caveats exist for all animal models of human disease. For this reason, the use of animal models to study IS should take care to reflect on anatomical differences and be cognizant of other potential caveats for each model system.

Considerations of Analysis of Spinal Curvatures in Mouse and Zebrafish

When working with mouse models, it is imperative to ensure careful alignment and placement of mice for X-ray analysis of the spine. In particular, we find that a "scoliosis" can be falsely observed in mice that are improperly placed in the X-ray scanner, and when readjusted, with hips and shoulders carefully aligned and adjusted along the AP axis, these same mice do not display spinal curvature (Fig. 5.3). This imaging artifact is likely due to the normal kyphosis of the mouse that is best seen on lateral views. When imaged appropriately using a lateral view, extreme kyphosis in mice may also be abnormal. Indeed, similar spinal pathology is observed in human scoliosis representing a spectrum of rotational deformities that often includes kyphosis (in combination, this deformity may be referred to as kyphoscoliosis). For example, humans with Marfan syndrome have increased incidence of both scoliotic and kyphotic defects of the spine. Mouse model of Marfan syndrome has often been described as having extreme kyphosis [146], with no description of scoliosis, although it is unclear whether scoliosis was even evaluated in this study.

Our personal experience with zebrafish models of IS has not uncovered this susceptibility to positioning affects. Indeed, mutant zebrafish with scoliosis are uniquely identified in swimming fish; however, we have observed some mutant
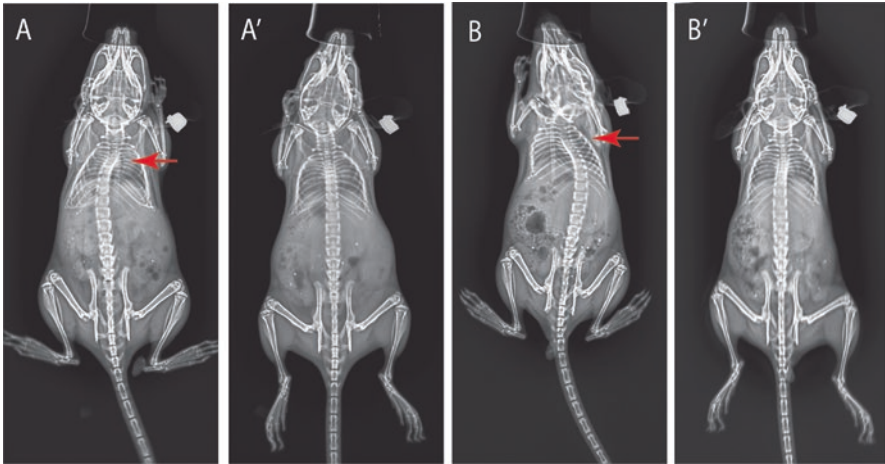
Fig. 5.3 Positioning of mice is critical for accurate determination of spine curvatures. Two wild-type C57BL/6J(JAX) mice at P40 imaged live under isoflurane anesthesia (**A** and **B** panels). In some cases, mice that imaged without proper care for placement will show an obvious spine curvature (red arrows) (**A**, **B**). However, after manipulation and lengthning of the spine and alignment of the shoulders and hips by gentle traction with the thumb and fingers you can balance the mouse on its ribcage, which allow for imageing of normal spine alignment (**A'**, **B'**).

zebrafish that display very mild scoliosis that are not readily noticeable without X-ray or skeletal preparation analysis (unpublished observations). This is in stark contrast to our observations in mouse where even severe thoracic scoliosis is not readily noticeable with X-ray analysis. Importantly this suggests that many more mouse mutant models may exist that have never been reported to have spine defects simply due to lack of observation, this is recently been supported by clear indications of scoliosis from high-throughput analysis of over 3000 novel mouse mutant strains as part of the International Mouse Phenotyping Project [147, 148].

Conclusions

While no single animal model can replicate the pathophysiology of the human spine clearly, animal models including teleost and mouse can be useful in studying the molecular genetics and mechanics of spine development and disease. While genetic studies in human IS are certain to become more powerful ways of advancing its molecular genetics, we argue that validation of these findings in animal models is critical to ensuring actionable biomedical research whether in the model or in human-derived in vitro culture models. Moreover, the discovery of the cellular pathogenesis of IS in these animals is important for advancing approval for studies of resected tissue from human IS and nonpathogenic patient sources.

Moving forward, it is worthwhile to consider the caveats of each model and start to hold future study of IS models to a higher standard, for instance: (i) in particular for zebrafish, we suggest that analysis must be done in a relatively mature spine, in contrast making conclusions of relevance to IS based on kinking body plans in embryonic zebrafish; (ii) in particular for mouse, care should be taken to ensure accurate alignment of the spine prior to analysis of the spine curvature by X-ray as false positives are easily observed; (iii) we suggest levels of validity that might help to address the distinctive hallmarks of human IS for individual models; (iv) where possible we suggest that analysis of IS models should be done using reproducible stable transgenic or heritable genetic animals or highly reproducible mechanically induced models.

References

1. Smith LJ, Nerurkar NL, Choi KS, Harfe BD, Elliott DM. Degeneration and regeneration of the intervertebral disc: lessons from development. Dis Model Mech. 2011;4(1):31–41.
2. Eckalbar WL, Fisher RE, Rawls A, Kusumi K. Scoliosis and segmentation defects of the vertebrae. Wiley Interdiscip Rev Dev Biol. 2012;1(3):401–23.
3. Koehl MA, Quillin KJ, Pell CA. Mechanical design of fiber-wound hydraulic skeletons: the stiffening and straightening of embryonic notochords. Am Zool. 2000;40:28–41.
4. Adams DS, Keller R, Koehl MA. The mechanics of notochord elongation, straightening and stiffening in the embryo of Xenopus laevis. Development. 1990;110(1):115–30.
5. Glickman NS, Kimmel CB, Jones MA, Adams RJ. Shaping the zebrafish notochord. Development. 2003;130(5):873–87.
6. Shapiro IM, Risbud MV. Introduction to the structure, function, and comparative anatomy of the vertebrae and the intervertebral disc. In: Shapiro IM, Risbud MV, editors. The intervertebral disc: molecular and structural studies of the disc in health and disease. Vienna: Springer; 2014. p. 3–15.
7. Choi KS, Cohn MJ, Harfe BD. Identification of nucleus pulposus precursor cells and notochordal remnants in the mouse: implications for disk degeneration and chordoma formation. Dev Dyn. 2008;237(12):3953–8.
8. Newton Ede MM, Jones SW. Adolescent idiopathic scoliosis: evidence for intrinsic factors driving aetiology and progression. Int Orthop. 2016;40(10):2075–80.
9. Jackson HC 2nd, Winkelmann RK, Bickel WH. Nerve endings in the human lumbar spinal column and related structures. J Bone Joint Surg Am. 1966;48(7):1272–81.
10. Kojima Y, Maeda T, Arai R, Shichikawa K. Nerve supply to the posterior longitudinal ligament and the intervertebral disc of the rat vertebral column as studied by acetylcholinesterase histochemistry. I. Distribution in the lumbar region. J Anat. 1990;169:237–46.
11. Man GC, Wang WW, Yim AP, Wong JH, Ng TB, Lam TP, et al. A review of pinealectomy-induced melatonin-deficient animal models for the study of etiopathogenesis of adolescent idiopathic scoliosis. Int J Mol Sci. 2014;15(9):16484–99.
12. Lombardi G, Akoume MY, Colombini A, Moreau A, Banfi G. Biochemistry of adolescent idiopathic scoliosis. Adv Clin Chem. 2011;54:165–82.

13. Normand E, Franco A, Moreau A, Marcil V. Dipeptidyl Peptidase-4 and adolescent idiopathic scoliosis: expression in osteoblasts. Sci Rep. 2017;7(1):3173.
14. Blecher R, Krief S, Galili T, Biton IE, Stern T, Assaraf E, et al. The proprioceptive system masterminds spinal alignment: insight into the mechanism of scoliosis. Dev Cell. 2017;42(4):388–99. e3
15. Pourquie O. Vertebrate segmentation: from cyclic gene networks to scoliosis. Cell. 2011;145(5):650–63.
16. Giampietro PF, Dunwoodie SL, Kusumi K, Pourquie O, Tassy O, Offiah AC, et al. Progress in the understanding of the genetic etiology of vertebral segmentation disorders in humans. Ann N Y Acad Sci. 2009;1151:38–67.
17. Sparrow DB, Chapman G, Dunwoodie SL. The mouse notches up another success: understanding the causes of human vertebral malformation. Mamm Genome. 2011;22(7–8):362–76.
18. Gansner JM, Mendelsohn BA, Hultman KA, Johnson SL, Gitlin JD. Essential role of lysyl oxidases in notochord development. Dev Biol. 2007;307(2):202–13.
19. Gansner JM, Gitlin JD. Essential role for the alpha 1 chain of type VIII collagen in zebrafish notochord formation. Dev Dyn. 2008;237(12):3715–26.
20. Christiansen HE, Lang MR, Pace JM, Parichy DM. Critical early roles for col27a1a and col27a1b in zebrafish notochord morphogenesis, vertebral mineralization and post-embryonic axial growth. PLoS One. 2009;4(12):e8481.
21. Gray RS, Wilm TP, Smith J, Bagnat M, Dale RM, Topczewski J, et al. Loss of col8a1a function during zebrafish embryogenesis results in congenital vertebral malformations. Dev Biol. 2014;386(1):72–85.
22. Ellis K, Bagwell J, Bagnat M. Notochord vacuoles are lysosome-related organelles that function in axis and spine morphogenesis. J Cell Biol. 2013;200(5):667–79.
23. Sparrow DB, Chapman G, Smith AJ, Mattar MZ, Major JA, O'Reilly VC, et al. A mechanism for gene-environment interaction in the etiology of congenital scoliosis. Cell. 2012;149(2):295–306.
24. Purkiss SB, Driscoll B, Cole WG, Alman B. Idiopathic scoliosis in families of children with congenital scoliosis. Clin Orthop Relat Res. 2002;401:27–31.
25. Guo L, Yamashita H, Kou I, Takimoto A, Meguro-Horike M, Horike S, et al. Functional investigation of a non-coding variant associated with adolescent idiopathic scoliosis in zebrafish: elevated expression of the ladybird Homeobox gene causes body Axis deformation. PLoS Genet. 2016;12(1):e1005802.
26. Hayes M, Gao X, Yu LX, Paria N, Henkelman RM, Wise CA, et al. ptk7 mutant zebrafish models of congenital and idiopathic scoliosis implicate dysregulated Wnt signalling in disease. Nat Commun. 2014;5:4777.
27. McGreevy JW, Hakim CH, McIntosh MA, Duan D. Animal models of Duchenne muscular dystrophy: from basic mechanisms to gene therapy. Dis Model Mech. 2015;8(3):195–213.
28. Harrison DJ, Webb PJ. Scoliosis in the Rett syndrome: natural history and treatment. Brain and Development. 1990;12(1):154–6.
29. Taylor LJ. Severe spondylolisthesis and scoliosis in association with Marfan's syndrome. Case report and review of the literature. Clin Orthop Relat Res. 1987;221:207–11.
30. Shirley ED, Demaio M, Bodurtha J. Ehlers-danlos syndrome in orthopaedics: etiology, diagnosis, and treatment implications. Sports Health. 2012;4(5):394–403.
31. Blanco G, Coulton GR, Biggin A, Grainge C, Moss J, Barrett M, et al. The kyphoscoliosis (ky) mouse is deficient in hypertrophic responses and is caused by a mutation in a novel muscle-specific protein. Hum Mol Genet. 2001;10(1):9–16.
32. Chen F, Guo R, Itoh S, Moreno L, Rosenthal E, Zappitelli T, et al. First mouse model for combined osteogenesis imperfecta and Ehlers-Danlos syndrome. J Bone Miner Res. 2014;29(6):1412–23.
33. Haller G, Alvarado D, McCall K, Yang P, Cruchaga C, Harms M, et al. A polygenic burden of rare variants across extracellular matrix genes among individuals with adolescent idiopathic scoliosis. Hum Mol Genet. 2016;25(1):202–9.

34. Buchan JG, Alvarado DM, Haller GE, Cruchaga C, Harms MB, Zhang T, et al. Rare variants in FBN1 and FBN2 are associated with severe adolescent idiopathic scoliosis. Hum Mol Genet. 2014;23(19):5271–82.

35. Cheng JC, Castelein RM, Chu WC, Danielsson AJ, Dobbs MB, Grivas TB, et al. Adolescent idiopathic scoliosis. Nat Rev Dis Primers. 2015;1:15030.

36. van der Worp HB, Howells DW, Sena ES, Porritt MJ, Rewell S, O'Collins V, et al. Can animal models of disease reliably inform human studies? PLoS Med. 2010;7(3):e1000245.

37. McGonigle P, Ruggeri B. Animal models of human disease: challenges in enabling translation. Biochem Pharmacol. 2014;87(1):162–71.

38. Boszczyk BM, Boszczyk AA, Putz R. Comparative and functional anatomy of the mammalian lumbar spine. Anat Rec. 2001;264(2):157–68.

39. Langenskiold A, Michelsson JE. Experimental progressive scoliosis in the rabbit. J Bone Joint Surg Br. 1961;43-B:116–20.

40. Langenskiold A, Michelsson JE. Experimental scoliosis. Acta Orthop Scand. 1959;29:158–9.

41. Langenskiold A, Michelsson JE. The pathogenesis of experimental progressive scoliosis. Acta Orthop Scand Suppl. 1962;59:1–26.

42. Kubota K, Doi T, Murata M, Kobayakawa K, Matsumoto Y, Harimaya K, et al. Disturbance of rib cage development causes progressive thoracic scoliosis: the creation of a nonsurgical structural scoliosis model in mice. J Bone Joint Surg Am. 2013;95(18):e130.

43. Stokes IA, Laible JP. Three-dimensional osseo-ligamentous model of the thorax representing initiation of scoliosis by asymmetric growth. J Biomech. 1990;23(6):589–95.

44. Andriacchi T, Schultz A, Belytschko T, Galante J. A model for studies of mechanical interactions between the human spine and rib cage. J Biomech. 1974;7(6):497–507.

45. Grivas TB, Burwell RG, Purdue M, Webb JK, Moulton A. A segmental analysis of thoracic shape in chest radiographs of children. Changes related to spinal level, age, sex, side and significance for lung growth and scoliosis. J Anat. 1991;178:21–38.

46. Gurnett CA, Alaee F, Bowcock A, Kruse L, Lenke LG, Bridwell KH, et al. Genetic linkage localizes an adolescent idiopathic scoliosis and pectus excavatum gene to chromosome 18 q. Spine. 2009;34(2):E94–100.

47. Hong JY, Suh SW, Park HJ, Kim YH, Park JH, Park SY. Correlations of adolescent idiopathic scoliosis and pectus excavatum. J Pediatr Orthop. 2011;31(8):870–4.

48. Dubousset J, Wicart P, Pomero V, Barois A, Estournet B. Spinal penetration index: new three-dimensional quantified reference for lordoscoliosis and other spinal deformities. J Orthop Sci. 2003;8(1):41–9.

49. Doi T, Harimaya K, Matsumoto Y, Iwamoto Y. Aortic location and flat chest in scoliosis: a prospective study. Fukuoka Igaku Zasshi. 2011;102(1):14–9.

50. Ilharreborde B, Dubousset J, Le Huec JC. Use of EOS imaging for the assessment of scoliosis deformities: application to postoperative 3D quantitative analysis of the trunk. Eur Spine J. 2014;23(Suppl 4):S397–405.

51. Caballero A, Barrios C, Burgos J, Hevia E, Correa C. Vertebral growth modulation by hemicircumferential electrocoagulation: an experimental study in pigs. Eur Spine J. 2011;20(Suppl 3):367–75.

52. Catanzariti JF, Agnani O, Guyot MA, Wlodyka-Demaille S, Khenioui H, Donze C. Does adolescent idiopathic scoliosis relate to vestibular disorders? A systematic review. Ann Phys Rehabil Med. 2014;57(6–7):465–79.

53. Hawasli AH, Hullar TE, Dorward IG. Idiopathic scoliosis and the vestibular system. Eur Spine J. 2015;24(2):227–33.

54. Hitier M, Hamon M, Denise P, Lacoudre J, Thenint MA, Mallet JF, et al. Lateral Semicircular Canal asymmetry in idiopathic scoliosis: an early link between biomechanical, hormonal and neurosensory theories? PLoS One. 2015;10(7):e0131120.

55. Noshchenko A, Hoffecker L, Lindley EM, Burger EL, Cain CM, Patel VV, et al. Predictors of spine deformity progression in adolescent idiopathic scoliosis: a systematic review with meta-analysis. World J Orthop. 2015;6(7):537–58.

56. Pialasse JP, Mercier P, Descarreaux M, Simoneau M. Sensorimotor control impairment in young adults with idiopathic scoliosis compared with healthy controls. J Manip Physiol Ther. 2016;39(7):473–9.

57. Lambert FM, Malinvaud D, Glaunes J, Bergot C, Straka H, Vidal PP. Vestibular asymmetry as the cause of idiopathic scoliosis: a possible answer from Xenopus. J Neurosci. 2009;29(40):12477–83.

58. Lambert FM, Malinvaud D, Gratacap M, Straka H, Vidal PP. Restricted neural plasticity in vestibulospinal pathways after unilateral labyrinthectomy as the origin for scoliotic deformations. J Neurosci. 2013;33(16):6845–56.

59. Dahlhoff M, Emrich D, Wolf E, Schneider MR. Increased activation of the epidermal growth factor receptor in transgenic mice overexpressing epigen causes peripheral neuropathy. Biochim Biophys Acta. 2013;1832(12):2068–76.

60. Smit JJ, Baas F, Hoogendijk JE, Jansen GH, van der Valk MA, Schinkel AH, et al. Peripheral neuropathy in mice transgenic for a human MDR3 P-glycoprotein mini-gene. J Neurosci. 1996;16(20):6386–93.

61. Mogha A, Benesh AE, Patra C, Engel FB, Schoneberg T, Liebscher I, et al. Gpr126 functions in Schwann cells to control differentiation and myelination via G-protein activation. J Neurosci. 2013;33(46):17976–85.

62. Karner CM, Long F, Solnica-Krezel L, Monk KR, Gray RS. Gpr126/Adgrg6 deletion in cartilage models idiopathic scoliosis and pectus excavatum in mice. Hum Mol Genet. 2015;24(15):4365–73.

63. Buchan JG, Gray RS, Gansner JM, Alvarado DM, Burgert L, Gitlin JD, et al. Kinesin family member 6 (kif6) is necessary for spine development in zebrafish. Dev Dyn. 2014;243(12):1646–57.

64. Boswell CW, Ciruna B. Understanding idiopathic scoliosis: a new zebrafish School of Thought. Trends Genet. 2017;33(3):183–96.

65. Yang Z, Xie Y, Chen J, Zhang D, Yang C, Li M. High selenium may be a risk factor of adolescent idiopathic scoliosis. Med Hypotheses. 2010;75(1):126–7.

66. Lloyd HMS, Kirchhoff CA. Case study: scoliosis in a bonobo (Pan paniscus). J Med Primatol. 2018 Apr;47(2):114–116. doi: 10.1111/jmp.12325. Epub 2017 Nov 29.

67. Naique SB, Porter R, Cunningham AA, Hughes SP, Sanghera B, Amis AA. Scoliosis in an orangutan. Spine. 2003;28(7):E143–5.

68. Berghan J, VIsser IN. Vertebral column malformations in New Zealand delphinids with a review of cases world wide. Aquat Mamm. 2000;26(1):17–25.

69. Ambert AM, Samuelson MM, Pitchford JL, Solangi M. Visually detectable vertebral malformations of a bottlenose dolphin (Tursiops truncatus) in the Mississippi sound. Aquat Mamm. 2017;43(4):6.

70. Andrews B, Davis W, Parham D. Corporate response and facilitation of the rehabilitation of a California gray whale calf. Acad Radiol. 2001;Aquatic Mammals 273:209–11.

71. Ellis Giddens W, Ryland M, Casson CJ. Idiopathic scoliosis in a Newborn Sea otter, Enhydra lutris (L.). J Wildl Dis. 1984;20(3):248–50.

72. Mochida J, Benson DR, Abbott U, Rucker RB. Neuromorphometric changes in the ventral spinal roots in a scoliotic animal. Spine. 1993;18(3):350–5.

73. Nakai S. Histological and histochemical changes in the neck muscles of spontaneously occurring scoliosis in a special strain of Japanese quail, SQOHM. Nihon Seikeigeka Gakkai Zasshi. 1990;64(4):229–39.

74. Sobajima S, Kin A, Baba I, Kanbara K, Semoto Y, Abe M. Implication for melatonin and its receptor in the spinal deformities of hereditary Lordoscoliotic rabbits. Spine. 2003;28(6):554–8.

75. Grimes DT, Boswell CW, Morante NF, Henkelman RM, Burdine RD, Ciruna B. Zebrafish models of idiopathic scoliosis link cerebrospinal fluid flow defects to spine curvature. Science. 2016;352(6291):1341–4.

76. Gorman KF, Tredwell SJ, Breden F. The mutant guppy syndrome curveback as a model for human heritable spinal curvature. Spine. 2007;32(7):735–41.
77. Gorman KF, Christians JK, Parent J, Ahmadi R, Weigel D, Dreyer C, et al. A major QTL controls susceptibility to spinal curvature in the curveback guppy. BMC Genet. 2011;12(1):16.
78. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 2007;8(5):353–67.
79. Hwang WY, Fu Y, Reyon D, Maeder ML, Kaini P, Sander JD, et al. Heritable and precise zebrafish genome editing using a CRISPR-Cas system. PLoS One. 2013;8(7):e68708.
80. Auer TO, Duroure K, De Cian A, Concordet JP, Del Bene F. Highly efficient CRISPR/Cas9-mediated knock-in in zebrafish by homology-independent DNA repair. Genome Res. 2014;24(1):142–53.
81. Luderman LN, Unlu G, Knapik EW. Zebrafish developmental models of skeletal diseases. Curr Top Dev Biol. 2017;124:81–124.
82. Fisher S, Jagadeeswaran P, Halpern ME. Radiographic analysis of zebrafish skeletal defects. Dev Biol. 2003;264(1):64–76.
83. Henke K, Daane JM, Hawkins MB, Dooley CM, Busch-Nentwich EM, Stemple DL, et al. Genetic screen for postembryonic development in the zebrafish (Danio rerio): dominant mutations affecting adult form. Genetics. 2017;207(2):609–23.
84. Paul S, Schindler S, Giovannone D, de Millo Terrazzani A, Mariani FV, Crump JG. Ihha induces hybrid cartilage-bone cells during zebrafish jawbone regeneration. Development. 2016;143(12):2066–76.
85. Huitema LF, Apschner A, Logister I, Spoorendonk KM, Bussmann J, Hammond CL, et al. Entpd5 is essential for skeletal mineralization and regulates phosphate homeostasis in zebrafish. Proc Natl Acad Sci U S A. 2012;109(52):21372–7.
86. Mackay EW, Apschner A, Schulte-Merker S. Vitamin K reduces hypermineralisation in zebrafish models of PXE and GACI. Development. 2015;142(6):1095–101.
87. Kou I, Takahashi Y, Johnson TA, Takahashi A, Guo L, Dai J, et al. Genetic variants in GPR126 are associated with adolescent idiopathic scoliosis. Nat Genet. 2013;45(6):676–9.
88. Monk KR, Naylor SG, Glenn TD, Mercurio S, Perlin JR, Dominguez C, et al. A G protein-coupled receptor is essential for Schwann cells to initiate myelination. Science. 2009;325(5946):1402–5.
89. Geng FS, Abbas L, Baxendale S, Holdsworth CJ, Swanson AG, Slanchev K, et al. Semicircular canal morphogenesis in the zebrafish inner ear requires the function of gpr126 (lauscher), an adhesion class G protein-coupled receptor gene. Development. 2013;140(21):4362–74.
90. Takahashi Y, Kou I, Takahashi A, Johnson TA, Kono K, Kawakami N, et al. A genome-wide association study identifies common variants near LBX1 associated with adolescent idiopathic scoliosis. Nat Genet. 2011;43(12):1237–40.
91. Cao Y, Min J, Zhang Q, Li H, Li H. Associations of LBX1 gene and adolescent idiopathic scoliosis susceptibility: a meta-analysis based on 34,626 subjects. BMC Musculoskelet Disord. 2016;17:309.
92. Chettier R, Nelson L, Ogilvie JW, Albertsen HM, Ward K. Haplotypes at LBX1 have distinct inheritance patterns with opposite effects in adolescent idiopathic scoliosis. PLoS One. 2015;10(2):e0117708.
93. Londono D, Kou I, Johnson TA, Sharma S, Ogura Y, Tsunoda T, et al. A meta-analysis identifies adolescent idiopathic scoliosis association with LBX1 locus in multiple ethnic groups. J Med Genet. 2014;51(6):401–6.
94. Brohmann H, Jagla K, Birchmeier C. The role of Lbx1 in migration of muscle precursor cells. Development. 2000;127(2):437–45.
95. Kruger M, Schafer K, Braun T. The homeobox containing gene Lbx1 is required for correct dorsal-ventral patterning of the neural tube. J Neurochem. 2002;82(4):774–82.
96. Muller T, Brohmann H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, et al. The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron. 2002;34(4):551–62.

97. Sieber MA, Storm R, Martinez-de-la-Torre M, Muller T, Wende H, Reuter K, et al. Lbx1 acts as a selector gene in the fate determination of somatosensory and viscerosensory relay neurons in the hindbrain. J Neurosci. 2007;27(18):4902–9.

98. Kilian B, Mansukoski H, Barbosa FC, Ulrich F, Tada M, Heisenberg CP. The role of Ppt/Wnt5 in regulating cell shape and movement during zebrafish gastrulation. Mech Dev. 2003;120(4):467–76.

99. Madsen EC, Gitlin JD. Zebrafish mutants calamity and catastrophe define critical pathways of gene-nutrient interactions in developmental copper metabolism. PLoS Genet. 2008;4(11):e1000261.

100. Mendelsohn BA, Yin C, Johnson SL, Wilm TP, Solnica-Krezel L, Gitlin JD. Atp7a determines a hierarchy of copper metabolism essential for notochord development. Cell Metab. 2006;4(2):155–62.

101. Hubaud A, Pourquie O. Signalling dynamics in vertebrate segmentation. Nat Rev Mol Cell Biol. 2014;15(11):709–21.

102. Yu X, Ng CP, Habacher H, Roy S. Foxj1 transcription factors are master regulators of the motile ciliogenic program. Nat Genet. 2008;40(12):1445–53.

103. Parichy DM, Elizondo MR, Mills MG, Gordon TN, Engeszer RE. Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. Dev Dyn. 2009;238(12):2975–3015.

104. Turgut M, Cullu E, Uysal A, Yurtseven ME, Alparslan B. Chronic changes in cerebrospinal fluid pathways produced by subarachnoid kaolin injection and experimental spinal cord trauma in the rabbit: their relationship with the development of spinal deformity. An electron microscopic study and magnetic resonance imaging evaluation. Neurosurg Rev. 2005;28(4):289–97.

105. Chuma A, Kitahara H, Minami S, Goto S, Takaso M, Moriya H. Structural scoliosis model in dogs with experimentally induced syringomyelia. Spine. 1997;22(6):589–94. discussion 95

106. Godzik J, Dardas A, Kelly MP, Holekamp TF, Lenke LG, Smyth MD, et al. Comparison of spinal deformity in children with Chiari I malformation with and without syringomyelia: matched cohort study. Eur Spine J. 2016;25(2):619–26.

107. Ogura Y, Kou I, Takahashi Y, Takeda K, Minami S, Kawakami N, et al. A functional variant in MIR4300HG, the host gene of microRNA MIR4300 is associated with progression of adolescent idiopathic scoliosis. Hum Mol Genet. 2017;26(20):4086–92.

108. Patten SA, Margaritte-Jeannin P, Bernard JC, Alix E, Labalme A, Besson A, et al. Functional variants of POC5 identified in patients with idiopathic scoliosis. J Clin Invest. 2015;125(3):1124–8.

109. Becker-Heck A, Zohn IE, Okabe N, Pollock A, Lenhart KB, Sullivan-Brown J, et al. The coiled-coil domain containing protein CCDC40 is essential for motile cilia function and left-right axis formation. Nat Genet. 2011;43(1):79–84.

110. Jaffe KM, Grimes DT, Schottenfeld-Roames J, Werner ME, Ku TS, Kim SK, et al. c21orf59/kurly controls both cilia motility and polarization. Cell Rep. 2016;14(8):1841–9.

111. Serluca FC, Xu B, Okabe N, Baker K, Lin SY, Sullivan-Brown J, et al. Mutations in zebrafish leucine-rich repeat-containing six-like affect cilia motility and result in pronephric cysts, but have variable effects on left-right patterning. Development. 2009;136(10):1621–31.

112. Sullivan-Brown J, Schottenfeld J, Okabe N, Hostetter CL, Serluca FC, Thiberge SY, et al. Zebrafish mutations affecting cilia motility share similar cystic phenotypes and suggest a mechanism of cyst formation that differs from pkd2 morphants. Dev Biol. 2008;314(2):261–75.

113. Eisen JS, Smith JC. Controlling morpholino experiments: don't stop making antisense. Development. 2008;135(10):1735–43.

114. Kim HK, Aruwajoye O, Sucato D, Richards BS, Feng GS, Chen D, et al. Induction of SHP2 deficiency in chondrocytes causes severe scoliosis and kyphosis in mice. Spine. 2013;38(21):E1307–12.

115. Tidyman WE, Rauen KA. The RASopathies: developmental syndromes of Ras/MAPK pathway dysregulation. Curr Opin Genet Dev. 2009;19(3):230–6.

116. Henry SP, Liang S, Akdemir KC, de Crombrugghe B. The postnatal role of Sox9 in cartilage. J Bone Miner Res. 2012;27(12):2511–25.
117. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrugghe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev. 2002;16(21):2813–28.
118. Settle SH Jr, Rountree RB, Sinha A, Thacker A, Higgins K, Kingsley DM. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. Dev Biol. 2003;254(1):116–30.
119. Hartmann C, Tabin CJ. Wnt-14 plays a pivotal role in inducing synovial joint formation in the developing appendicular skeleton. Cell. 2001;104(3):341–51.
120. Lee WT, Cheung CS, Tse YK, Guo X, Qin L, Lam TP, et al. Association of osteopenia with curve severity in adolescent idiopathic scoliosis: a study of 919 girls. Osteoporos Int: a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2005;16(12):1924–32.
121. Hung VW, Qin L, Cheung CS, Lam TP, Ng BK, Tse YK, et al. Osteopenia: a new prognostic factor of curve progression in adolescent idiopathic scoliosis. J Bone Joint Surg Am. 2005;87(12):2709–16.
122. Colvin JS, Bohne BA, Harding GW, McEwen DG, Ornitz DM. Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat Genet. 1996;12(4):390–7.
123. Deng C, Wynshaw-Boris A, Zhou F, Kuo A, Leder P. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. Cell. 1996;84(6):911–21.
124. Valverde-Franco G, Liu H, Davidson D, Chai S, Valderrama-Carvajal H, Goltzman D, et al. Defective bone mineralization and osteopenia in young adult FGFR3−/− mice. Hum Mol Genet. 2004;13(3):271–84.
125. Valverde-Franco G, Binette JS, Li W, Wang H, Chai S, Laflamme F, et al. Defects in articular cartilage metabolism and early arthritis in fibroblast growth factor receptor 3 deficient mice. Hum Mol Genet. 2006;15(11):1783–92.
126. Gao C, Chen BP, Sullivan MB, Hui J, Ouellet JA, Henderson JE, et al. Micro CT analysis of spine architecture in a mouse model of scoliosis. Front Endocrinol (Lausanne). 2015;6:38.
127. Clin J, Aubin CE, Parent S, Labelle H. A biomechanical study of the Charleston brace for the treatment of scoliosis. Spine. 2010;35(19):E940–7.
128. MacIntyre NJ, Recknor CP, Grant SL, Recknor JC. Scores on the safe functional motion test predict incident vertebral compression fracture. Osteoporos Int : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 2014;25(2):543–50.
129. Makrythanasis P, Temtamy S, Aglan MS, Otaify GA, Hamamy H, Antonarakis SE. A novel homozygous mutation in FGFR3 causes tall stature, severe lateral tibial deviation, scoliosis, hearing impairment, camptodactyly, and arachnodactyly. Hum Mutat. 2014;35(8):959–63.
130. Komatsu Y, Chusho H, Tamura N, Yasoda A, Miyazawa T, Suda M, et al. Significance of C-type natriuretic peptide (CNP) in endochondral ossification: analysis of CNP knockout mice. J Bone Miner Metab. 2002;20(6):331–6.
131. Tsuji T, Kunieda T. A loss-of-function mutation in natriuretic peptide receptor 2 (Npr2) gene is responsible for disproportionate dwarfism in cn/cn mouse. J Biol Chem. 2005;280(14):14288–92.
132. Miura K, Kim OH, Lee HR, Namba N, Michigami T, Yoo WJ, et al. Overgrowth syndrome associated with a gain-of-function mutation of the natriuretic peptide receptor 2 (NPR2) gene. Am J Med Genet A. 2014;164A(1):156–63.
133. Miura K, Namba N, Fujiwara M, Ohata Y, Ishida H, Kitaoka T, et al. An overgrowth disorder associated with excessive production of cGMP due to a gain-of-function mutation of the natriuretic peptide receptor 2 gene. PLoS One. 2012;7(8):e42180.
134. Waller-Evans H, Promel S, Langenhan T, Dixon J, Zahn D, Colledge WH, et al. The orphan adhesion-GPCR GPR126 is required for embryonic development in the mouse. PLoS One. 2010;5(11):e14047.

135. Monk KR, Oshima K, Jors S, Heller S, Talbot WS. Gpr126 is essential for peripheral nerve development and myelination in mammals. Development. 2011;138(13):2673–80.

136. Brochhausen C, Turial S, Muller FK, Schmitt VH, Coerdt W, Wihlm JM, et al. Pectus excavatum: history, hypotheses and treatment options. Interact Cardiovasc Thorac Surg. 2012;14(6):801–6.

137. Wu S, Sun X, Zhu W, Huang Y, Mou L, Liu M, et al. Evidence for GAL3ST4 mutation as the potential cause of pectus excavatum. Cell Res. 2012;22(12):1712–5.

138. Lefebvre V, Bhattaram P. Vertebrate skeletogenesis. Curr Top Dev Biol. 2010;90:291–317.

139. Fleming A, Keynes R, Tannahill D. A central role for the notochord in vertebral patterning. Development. 2004;131(4):873–80.

140. Grotmol S, Kryvi H, Nordvik K, Totland GK. Notochord segmentation may lay down the pathway for the development of the vertebral bodies in the Atlantic salmon. Anat Embryol (Berl). 2003;207(4–5):263–72.

141. Haga Y, Dominique VJ 3rd, Du SJ. Analyzing notochord segmentation and intervertebral disc formation using the twhh: gfp transgenic zebrafish model. Transgenic Res. 2009;18(5):669–83.

142. Cortes DH, Elliott DM. The intervertebral disc: overview of disc mechanics. In: Shapiro IM, Risbud MV, editors. The intervertebral disc: molecular and structural studies of the disc in health and disease. Vienna: Springer; 2014. p. 17–31.

143. Bruggeman BJ, Maier JA, Mohiuddin YS, Powers R, Lo Y, Guimaraes-Camboa N, et al. Avian intervertebral disc arises from rostral sclerotome and lacks a nucleus pulposus: implications for evolution of the vertebrate disc. Dev Dyn. 2012;241(4):675–83.

144. Inohaya K, Takano Y, Kudo A. The teleost intervertebral region acts as a growth center of the centrum: in vivo visualization of osteoblasts and their progenitors in transgenic fish. Dev Dyn. 2007;236(11):3031–46.

145. Irie K, Kuroda Y, Mimori N, Hayashi S, Abe M, Tsuji N, et al. Histopathology of a wavy medaka. J Toxicol Pathol. 2016;29(2):115–8.

146. Pereira L, Lee SY, Gayraud B, Andrikopoulos K, Shapiro SD, Bunton T, et al. Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1. Proc Natl Acad Sci U S A. 1999;96(7):3819–23.

147. Dickinson ME, Flenniken AM, Ji X, Teboul L, Wong MD, White JK, et al. High-throughput discovery of novel developmental phenotypes. Nature. 2016;537(7621):508–14.

148. Meehan TF, Conte N, West DB, Jacobsen JO, Mason J, Warren J, et al. Disease model discovery from 3,328 gene knockouts by the international mouse phenotyping consortium. Nat Genet. 2017;49(8):1231–8.