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Morphological Features of Adult Rats of IS/Kyo and IS-*Tlk*/Kyo Strains with Lumbar and Caudal Vertebral Anomalies

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Abstract: IS-*Tlk/*Kyo, a mutant derived from IS*/*Kyo strain, exhibits a kinked and/or short tail, in addition to the congenital lumbar vertebral anomaly. Homozygotes of *Tlk* dominant gene are known to die during embryonic development. We previously reported the morphological features of the skeleton in IS*/*Kyo and IS-*Tlk/*Kyo fetuses and of the heart in IS/Kyo fetuses [19]. This study was conducted to clarify the morphological features of the skeleton in both adult rats and of the heart in adult IS/Kyo rats. Ventricular septal defect (VSD) was observed in 3 out of 10 IS/Kyo rats. Neither splitting of lumbar vertebra and supernumerary rib (in both strains) nor fused or absent caudal cartilage (in IS-*Tlk/*Kyo strain) was detected in adult rats. Fusion of lumbar vertebrae was observed in almost all specimens together with lumbarization of sacral vertebrae in a few specimens in both adult rats as well as fusion of sacral and caudal vertebrae only in adult IS-*Tlk*/Kyo rats. In addition, a severe reduction in the ossified sacral and caudal vertebrae was noted in adult IS-*Tlk/*Kyo rats (mean number: 20.6) and IS*/*Kyo rats (31.8), and the difference was similar to that in the length of sacral and caudal vertebrae. These results suggest that the *Tlk* gene may be involved in both the congenital and acquired abnormal formation of the lower vertebral centra as well as the persistent occurrence of VSD by the background gene in IS/Kyo strain.

Key words: IS rat, lumbar vertebral anomalies, sacral and caudal vertebrae, *Tlk* gene, ventricular septal defect

Introduction

Almost all IS/Kyo (hereinafter referred to as IS) rats express vertebral anomalies, mainly of the lumbar vertebrae [10, 17, 21]. Lumbar vertebral anomalies in IS rats might be caused by multiple genetic determinants [21], in which the Hox10 gene may be partially involved in the pathogenesis [17]. IS-*Tlk*//Kyo (hereinafter referred to as IS/T) rats are derived from IS rats and have characteristic tail vertebral anomalies in addition to the lumbar vertebrae [13]. The tail anomaly is inherited as autosomal dominant. Thus, the gene responsible for the tail anomaly in IS/T rats is called 'tail anomaly lethal Kyoto' (*Tlk*). In addition, the ratio of fetuses showing the tail anomaly is about 70–80%, suggesting that wild-types (IS rat) are lethal to embryos due to severe ventral septal defects.

Previously, we investigated the morphological features of IS/T rat fetuses in comparison with those of IS rat fetuses [19], and the outline of the morphological features obtained was as follows: 1) a high incidence of tail vertebral anomalies in IS/T rats (81.6% versus 0% in IS rats), 2) retarded ossification of 5th sternebra and sacral and caudal vertebrae in IS/T rats (incidence in 5th sternebra:

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23.8% versus 70.5% in IS rats, mean values in sacral and caudal vertebrae: 5.81 versus 6.24 in IS rats) and 3) a low incidence of fetuses with ventral septal defects in IS/T rats (0% versus 54.4% in IS rats). From these results, we suggested that *Tlk* gene might be involved in the formation of the vertebral centra and the ventral septum when it expresses on the genetic background of the IS rats.

Although many morphological features such as VSD and vertebral anomalies have been reported as postnatal changes following exposure to chemical substances [5–7, 15, 18, 22], there are only a few reports of morphological features of the skeleton and heart in adult mutant rats [3, 14].

This study was performed to clarify the morphological changes in adult IS and IS/T mutant rats and to consider how well these rats may serve as models of human congenital pathology. In addition, to reveal a characteristic of short tail in IS/T rats, we examined the difference in the length of sacral and caudal vertebrae between two strains using skeletal specimens obtained from the previous study [19] and adult rats.

Materials and Methods

Animals

Ten IS strain female rats were provided from the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Kyoto, Japan at 11 weeks of age. The animals were housed in an animal room of Bozo Research Center Inc., where temperature was maintained at 23 ± 3°C, relative humidity at 50 ± 20%, air ventilation at 10 to 15 times per hour, and a light cycle at 12-light:12-dark (lighting from 07:00 to 19:00). The animals were housed individually in cages (W 254 × D 350 × H 170 mm: Lead Engineering Co., Ltd.., Tokyo, Japan). Ten female rats of the Crl:CD (SD) strain (hereinafter referred to as SD) at 15 to 19 weeks of age were also used as a reference rat strain.

After an acclimation period of 1 week, animals were sacrificed by exsanguination from the abdominal aorta and *vena cava* under deep anesthesia by isoflurane and subjected to autopsy. After macroscopic examination of the main organs and tissues in the thoracic and abdominal cavities, the heart was excised and fixed in Bouin's solution. After that, visceral abnormalities were examined by the microdissection method. In addition, hematoxylin and eosin (HE)-stained paraffin sections of the heart were subjected to histopathological examination. The carcasses of female rats of IS strain were subjected to alizarin red S staining to prepare skeletal specimens. Skeletal specimens of adult rats of IS/T strain were obtained from the previous study [19]. The length of total sacral and caudal vertebrae was measured in rat fetal skeletons prepared in the previous study [19] by a Digital Microscope (VHX-2000, KEYENCE CORPORATION) and in adult rat skeleton by a vernier micrometer.

Skeletal examinations were done on the specimens stained with alizarin red S. In addition, HE-stained paraffin sections of the femur were prepared following decalcification according to the routine method, and then subjected to histopathological examination.

All experimental procedures involving animals were done in accordance with the animal welfare guidelines of Bozo Research Center Inc.

Statistical analysis

The incidence of occurrence or mean with standard deviation was calculated for each strain. For incidence data in adult rats of strains, the significance of differences between the two strains was tested by Fisher's exact test. For the remainder in adult rats of strains, the significance of differences between the two strains was tested after analysis of the data for homogeneity of variance by F-test and compared by Student's *t*-test or Aspirin-Welch *t*-test. For the length of sacral and caudal vertebrae in rat fetuses of strains, litter mean was the unit of analysis and compared in the same manner as described above.

Results

VSD was observed in 3 out of 10 IS adult rats (30.0%) while it was not detected in 10 SD adult rats. The size of VSD was very small and detected only by the passage of hair from the right ventricle as shown in Fig. 1.

As shown in Table 1, the main type of skeletal abnormalities observed in adult rats of IS and IS/T strains were fusion of the vertebrae. The number of rats with fused cervical, lumbar, sacral, caudal and terminal caudal vertebrae were 2, 10, 4, 8 and 4 out of 10 IS/T adult rats, respectively, while fusion was observed only in lumbar vertebrae in 8 out of 10 IS adult rats. Cervical rib was observed in 1 IS adult rat, and lumbarization of sacral vertebrae was found in 3 IS adult rats and 2 IS/T adult rats. Supraoccipital bone, sternebrae and proximal phalanges in right forepaw were completely ossified in all

adult rats of IS and IS/T strains. In addition, significantly retarded ossification in the sacral and caudal vertebrae was observed in IS/T adult rats (20.6 in mean number of ossification) as compared with that in IS adult rats (31.8).

As shown in Table 2, high incidences of fused lumbar vertebrae, complete fusion of splitting of lumbar vertebrae and normally ossified supernumerary ribs were noted in IS adult rats. Cervical rib was observed in only 1 IS adult rat. In addition, all parts were completely ossified in IS adult rats.

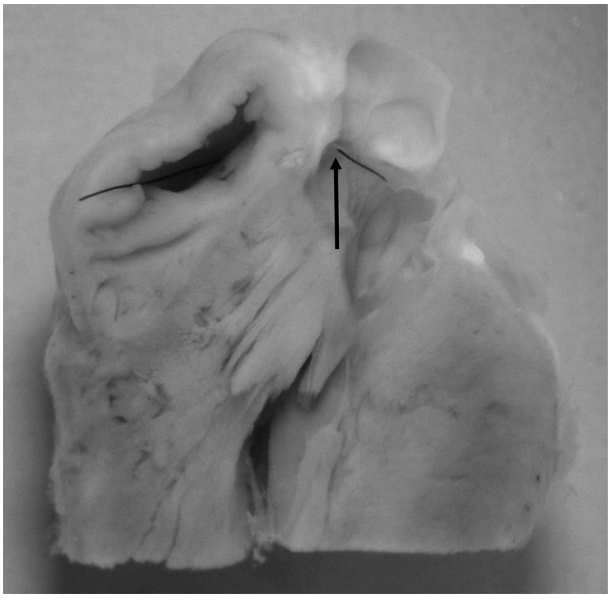

Fig. 1. A transverse view of the heart in IS/Kyo adult rat. Note the size of ventricular septal defect (arrow).

As shown in Table 3 and Figs. 2–5, high incidences of fused lumbar, sacral, caudal and terminal caudal vertebrae, complete fusion of splitting of lumbar vertebrae, completely ossified supernumerary ribs and disappearance of fused and absent caudal cartilage were observed in IS/T adult rats. In addition, all parts were completely ossified.

IS/T rat fetuses exhibited significantly low value in the length of sacral and caudal vertebrae compared with that in the IS rat fetuses and the difference was about two thirds (No. of dams and fetuses examined, mean ± SD; IS/T/IS strain: 11 and 39, 10,821 ± 607 μm/7 and 10, 15,551 ± 2,769 μm). Similar tendency was observed between adult rat strains (No. of rats examined, mean ± SD; IS/T/IS strain: 10, 164 ± 17 mm/10, 232 ± 12 mm).

There were no abnormal histopathological changes in the heart or femur in any rats examined.

Discussion

In IS rat strain, very small-sized VSD was recorded in 30% of adult rats while VSD with larger size was detected in 60% of fetal rats (Total No. of fetuses with VSD/Total No. of fetuses examined: 21/35, [19]), indicating reduction in the incidence and size of VSD during postnatal development. It was reported that mild VSD induced by the critical exposure of fetuses to teratogens tended to disappear during neonatal development due to

Table 1. Skeletal finding of IS/Kyo and IS-*Tlk*/Kyo (Tail vertebral anomalies) adult rats

Observation	IS/Kyo		IS-*Tlk*/Kyo (Tail vertebral anomalies)	
No. of rats examined	10		10	
Main region of fusions[†]				
Cervical vertebrae	0	0	2	20
Lumbar vertebrae	8	80	10	100
Sacral vertebrae	0	0	4	40
Caudal vertebrae	0	0	8	80.0[#]
Terminal caudal vertebrae	0	0	4	40
Cervical rib	1	10	0	0
Lumbarization of sacral vertebrae	3	30	2	20
Progress of ossification[‡]				
Supraoccipital bone		5 ± 0		5 ± 0
Sternebrae		6 ± 0		6 ± 0
Proximal phalanges in right forepaw		5 ± 0		5 ± 0
No. of ossified sacral and caudal vertebrae		31.8 ± 1.0		20.6 ± 3.3*

[†]: Values represent No. of rats with skeletal abnormalities and percentage. [‡]: Values of ossification represent mean ± SD. [#]: $P<0.05$, significantly different from IS/Kyo rats (Fisher's exact test). *: $P<0.05$, significantly different from IS/Kyo rat (Aspin-Welch's *t*-test).

Table 2. Skeletal findings of fetuses and adults in IS/Kyo rats

Observation	Fetuses†		Adults	
No. of pregnant dams or rats examined	11		10	
No. of litters with fetuses or rats having abnormal findings	11		9	
Total no. of fetuses examined	39		-	
Main type of abnormalities (%, mean ± SD)‡				
Cervical rib	0	0 ± 0	1	10
Fusion of lumbar vertebra	12	36.4 ± 34.6	8	80
Splitting of lumbar vertebra	6	11.8 ± 19.3	0	0
Supernumerary rib	39	100 ± 0	0	0
Lumbarization of sacral vertebra	9	15.9 ± 17.7	3	30
Progress of ossification				
Supraoccipital bone (mean ± SD)¶		2.3 ± 0.8		5 ± 0
Region of sternebrae (%, mean ± SD)¶¶				
1st		100 ± 0		100
2nd		60.8 ± 35.3		100
3rd		100 ± 0		100
4th		100 ± 0		100
5th		70.5 ± 39.3		100
6th		98.2 ± 6.0		100
Proximal phalanges in right forepaw (mean ± SD)¶		0 ± 0		5 ± 0
No. of ossified sacral and caudal vertebrae (mean ± SD)¶		6.24 ± 0.41		31.8 ± 1.0

†: Cited from the previous report [15]. ‡: Values represent No. of fetuses with skeletal abnoramlities and mean pecentage ± SD or pecentage of rats with skeletal abnormalities. ‡: Values represent No. of fetuses with skeletal abnoramlities and mean pecentage ± SD or pecentage of rats with skeletal abnormalities.

Table 3. Skeletal findings of fetuses and adults in IS-*Tlk*/Kyo (Tail vertebral anomalies) rats

Observation	Fetuses†		Adults	
No. of pregnant dams or rats examined	7		10	
No. of litters with fetuses or rats having abnormal findings	7		10	
Total no. of fetuses examined	10		-	
Main type of abnormalities (%, mean ± SD)‡				
Cervical rib	0	0 ± 0	2	20
Fusion of lumbar vertebrae	5	45.2 ± 45.9	10	100
Splitting of lumbar vertebrae	2	21.4 ± 39.3	0	0
Supernumerary rib	10	100 ± 0	0	0
Lumbarization of sacral vertebrae	2	19.0 ± 37.8	2	20
Fusion of sacral vertebrae	0	0 ± 0	4	40
Fusion of caudal vertebrae	0	0 ± 0	8	80
Fusion of terminal caudal vertebrae	0	0 ± 0	4	40
Fusion of caudal cartilage	8	71.4 ± 48.8	0	0
Absent caudal cartilage	2	28.6 ± 48.8	0	0
Progress of ossification				
Supraoccipital bone (mean ± SD)¶		0.4 ± 0.4		5 ± 0
Region of sternebrae (%, mean ± SD)¶¶				
1st		95.2 ± 12.6		100
2nd		66.7 ± 47.1		100
3rd		100 ± 0		100
4th		100 ± 0		100
5th		23.8 ± 41.89		100
6th		78.6 ± 39.3		100
Proximal phalanges in right forepaw (mean ± SD)¶		0 ± 0		5 ± 0
No. of ossified sacral and caudal vertebrae (mean ± SD)¶		5.81 ± 0.38		20.6 ± 3.3

†: Cited from the previous report [15]. ‡: Values represent No. of fetuses with skeletal abnoramlities and mean pecentage ± SD or pecentage of rats with skeletal abnormalities. ¶: Values represent mean no. of ossification ± SD. ¶¶: Values represent mean pecentage of fetuses with ossification ± SD or pecentage of rats with ossification.

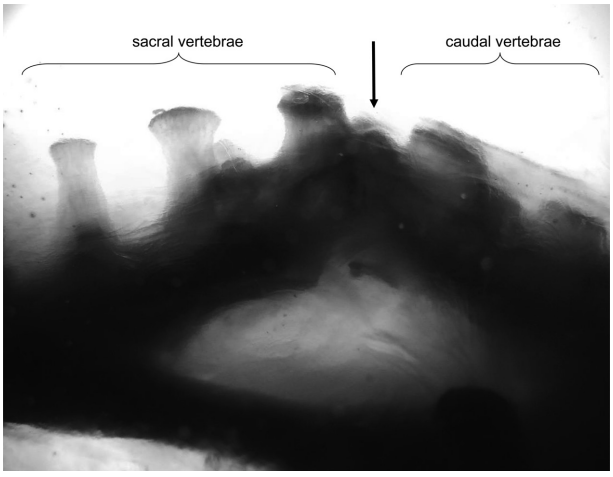

Fig. 2. Fused sacral and caudal vertebrae in IS-*Tlk/*Kyo adult rat (arrow).

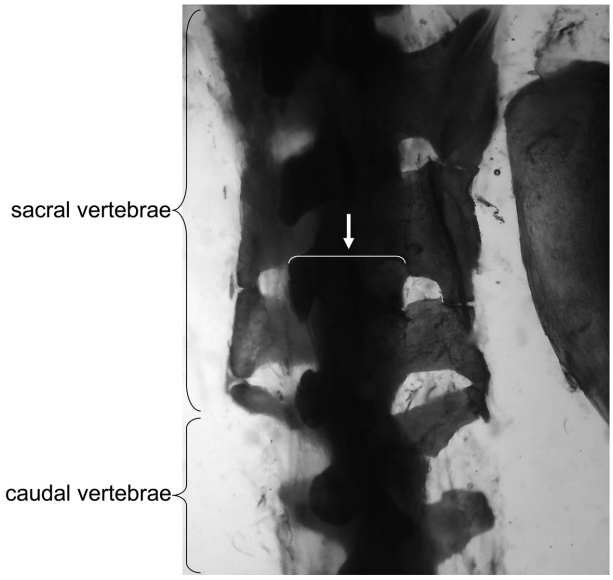

Fig. 3. Fused sacral vertebrae in IS-*Tlk/*Kyo adult rat (arrow).

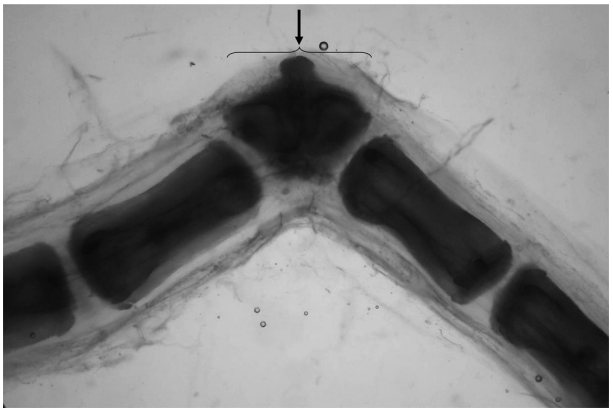

Fig. 4. Fused caudal vertebrae in IS-*Tlk/*Kyo adult rat (arrow).

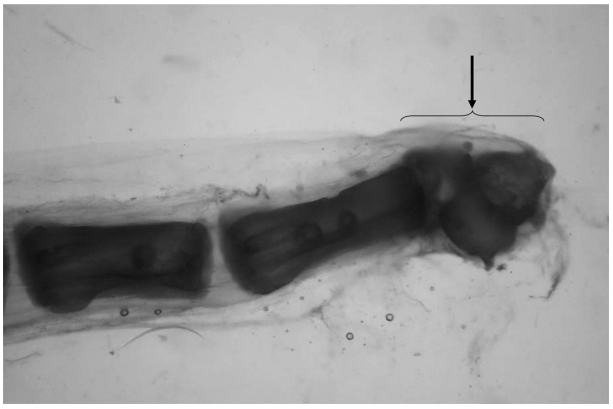

Fig. 5. Fused terminal caudal vertebrae in IS-*Tlk/*Kyo adult rat (arrow).

natural closure of VSD [18] and that fetuses with severe VSD died after birth [6]. In IS adult rats, the reduction in the incidence of VSD may also be due to natural closure of mild VSD as well as to death of pups with severe VSD. In this connection, it was reported that VSD was spontaneously found in 25% of newborns of Olson–Goss rats derived from Long–Evans rats [8, 9]. To date, however, there have been no reports of the occurrence of spontaneous VSD in adult rats. In addition, transforming growth factor β1 or *Tbx5* has been shown to contribute to the recovery appearance of defective vasculogenesis in KO mice or chicks, respectively [4, 11, 20]. Therefore, *Tlk* gene might not be ruled out as recovery factor of VSD. We are conducting an additional experiment on the postnatal closure of membranous VSD for submitting to journals elsewhere in near future.

Out of the phenome data obtained from the project at NBRP-Rat [13], systemic blood pressure in IS rats (95 mmHg) is the lowest among rats of 207 strains, and it is 42.2% of the maximum value (225 mmHg) in M-SHRSP/T rats. This may suggest a possible relationship between the lowest systemic blood pressure indicating abnormal hemodynamic kinetics and the existence of VSD in IS rats, although there is no direct evidence of such relationship. In this connection, it was reported that inhalation of NO decreased pulmonary artery pressure in a dose-dependent manner in Yucatan minipigs with VSD [12].

The skeletal morphological features commonly observed in IS and IS/T rats might be ascribed to genetic factors, which have not yet been identified but certainly

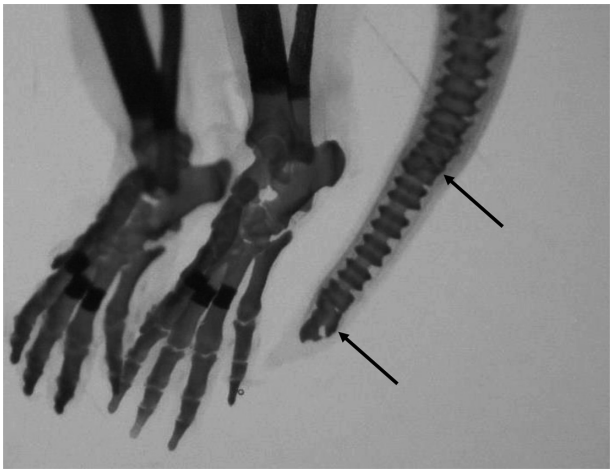

Fig. 6. Fused caudal cartilage in IS-*Tlk*/Kyo fetus (arrow).

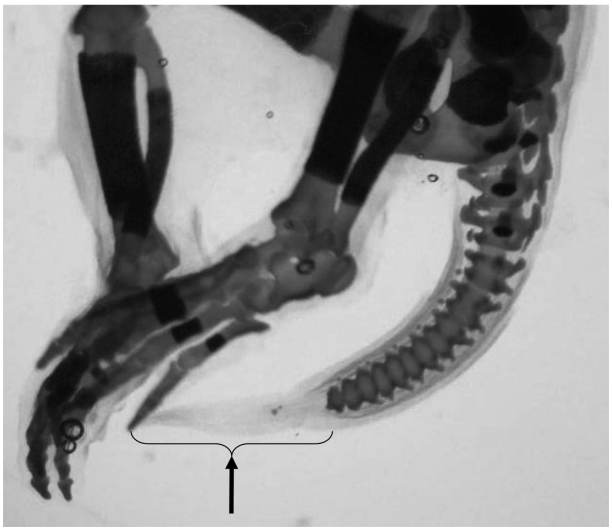

Fig. 7. Absent caudal cartilage in IS-*Tlk*/Kyo fetus (arrow).

been located on the genetic background of IS rats. However, the fusion of lower position such as sacral and caudal vertebrae was noted only in IS/T rats. *Tlk* gene may be involved in the formation of the sacral and caudal vertebrae. As to skeletal ossification, the number and length of ossified sacral and caudal vertebrae were smaller in IS/T rats (mean number in adult: 20.6; length in fetus and adult: 10,821 μm and 164 mm) than in IS rats (mean number in adult: 31.8; length in fetus and adult: 15,551 μm and 232 mm).

Adult rats of IS strain were considered to gain normal skeletal ossification because the number of ossified sacral and caudal vertebrae revealed normal morphological pattern reported in normal adult rats of non-mutant strains. On the other hand, all fetuses of IS/T strain showed the tail vertebral anomalies such as fused or absent caudal cartilage as shown in Figs. 6 and 7 [19], and this probably brings about the frequent occurrence of fused bone in adult rats of this strain. Disturbance of tail growth is thought to be due to complete ossification of fused caudal cartilage, and the difference in tail growth between IS/T and IS strains reflected well the differences in the length of sacral and caudal vertebrae in fetal and adults rats and in the number of ossified sacral and caudal vertebrae in adult rats between IS/T to IS strains. Further studies are needed to clarify the formation of fused sacral and caudal vertebrae on a step by step basis during the postnatal period.

As a decrease in fused lumbar vertebrae was commonly observed in adult rats of both IS and IS/T strains, this anomaly may be induced as latent phenotype by the genetic background of the IS rat. In IS/T strain, fused or absent caudal cartilage was found in fetal rats [19] and fused sacral and caudal vertebrae were noted in adult rats, suggesting that the *Tlk* gene may be involved in both the congenital and acquired abnormal formation of the lower vertebral centra.

In both IS and IS/T strains, splitting of lumbar vertebrae or supernumerary ribs was observed in fetal rats [19] but not in adult rats. Splitting of lumbar vertebrae was regarded as a transiently retarded growth in skeletal maturation. The coexistence of additional presacral vertebrae primarily with extra ribs suggests that both kinds of supernumerary ribs (rudimentary and extra) could be considered separately in developmental toxicology studies [7]. Retardation of skeletal ossification in IS/T fetuses [19] was considered to reflect a transient delay in prenatal growth because all skeletal sites except for caudal vertebrae were completely ossified during postnatal development. In addition, fused and/or absent sacral and caudal cartilage or bone in IS/T rat fetuses [19] were considered to bring about short tail in IS/T adult rats.

The phenotypes of vertebral morphological abnormalities as well as lower vertebral ossification in IS/T rats seem to be locally controlled by the *Tlk* gene. This coincides with the expression of Hoxed-10 to Hoxed-13 within vertebral axis field [16], indicating the similarity of genetic function on skeletal development. More studies should be required in order to reveal the role of *Tlk*-gene on the verterbral ossification. On the other hand, no abnormal findings were found in heart or femur histology in IS adult rats.

In developmental toxicity evaluation of COX inhibitors in rats, prenatal administration caused a significantly higher incidence of developmental variations such as skeletal variations and VSD [1, 2]. These findings suggest a common mechanism for the formation of membranous septum and vertebral centra in IS strain rat.

In conclusion, VSD was observed even in adult rats of IS strain at low incidence. Adult rats of both IS and IS/T strains shared common lumbar vertebral anomalies, and IS/T rats also showed tail anomalies as well as lower vertebral abnormalities. IS and IS/T rats seem to be useful as animal models for VSD and vertebral anomalies such as skeletal developmental disturbance of sacral and caudal vertebrae.

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