

# Development of sidedness of asymmetric body structures in vertebrates

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## Introduction

We live in the three dimensional world. Thus, our body structure has three axes; a dorsal-ventral (DV) axis, an anterior-posterior (AP) axis and a left-right (LR) axis. Although some might argue, Gardner (1990) proposed a simple paradigm for the development of these three axes. The first life on the earth, a single cell organism floating in the ocean, was probably a spherically symmetric structure. When the organism began to live on the bottom of the ocean or on the earth, the difference between "up" and "down" (or "dorsal" and "ventral") became clear because of gravity. Other environmental factors, such as light or contact, could also be the causal factor (Sumner and Huestis, 1921). Subsequently, when the animal began to move around, a distinction between "head" and "tail" (or "anterior" and "posterior") arose. The former is defined as the end which moves forward and has a mouth, the principal sense organs and various organs of offense and defense. When two body axes were determined, another axis, the LR axis, was automatically determined.

Gardner (1990) has suggested that although some degree of functional and structural differentiation may occur between the two sides of the body, such differentiation could usually undergo complete reversal without affecting the welfare of the organism, thus, symmetry across the AP axis is the basic structure of animals on the earth. However, there are many asymmetric structures in our body, e.g., heart, great vessels, lung, liver, spleen, gallbladder, pancreas, stomach and intestine. Furthermore, even bilateral organs show asymmetry in anatomical location, e.g., testes and kidneys. Sidedness of these structures is strongly biased in normal individuals, and failure to establish proper sidedness of organs such as the heart can lead to considerable pathology. How the LR axis is established or how abnormal sidedness (situs inversus) is induced during development have been always questions of great interest in the history of modern science. This manuscript seeks to review what we have learned about these issues in vertebrates. It is beyond the scope of this review to cover other related issues, e.g., development of the LR axis or asymmetry in invertebrates and plants, functional asymmetry such as handedness, asymmetric cell division, or molecular asymmetry.

## LR axis terminology

When discussing LR axis issues, some inconsistencies in terminology should be clarified first. Investigators use terms, such as "sidedness", "handedness", "laterality" and "chirality", in different ways which can be confusing. In this manuscript "sidedness" is mainly used unless citing the literature.

Also important is the definition of "situs inversus", which has not been agreed upon by investigators and was the subject of a recent debate (Lander and Brown, 1994b; Flynn *et al.*, 1994). While some investigators use the term "situs inversus" to describe any type of inversion of asymmetric body structures either complete or partial,

others believe that "situs inversus" should be reserved only for complete inversion (Fig. 1). At the present time, there does not appear to be any standardized usage of the term "situs inversus" in the literature, as discussed by Flynn *et al.* (1994). In this manuscript, the term "situs inversus" describes any abnormal sidedness of asymmetric body structures unless specified as "partial" or "total". If the asymmetries are completely inverted and mirror image, the condition is termed "total situs inversus". There are two types of total situs inversus; one accompanied with other malformations, and another without any malformations. A mixture of normal and inverted asymmetric structures is termed "partial situs inversus". There are two types of partial situs inversus: One in which both sides of the body are mirror images (symmetric) across the AP axis, either two right sided (dextroisomerism) or two left sided (levoisomerism), the other is a mixed type, i.e., some structures are two right sided but others are two left sided. The term "situs solitus" is used to indicate normal body sidedness.

"Heterotaxia" is another term which has been used by investigators with various meanings. While some investigators equate this term with "situs inversus", others use it to indicate only "partial situs inversus" (Fig. 1). Furthermore, in a series of treatises by Layton and colleagues on *iv* mutant mouse (described later), "heterotaxia" was used to indicate "venous heterotaxia", i.e., abnormal development of large veins. In this manuscript, the term "heterotaxia" is avoided as much as possible unless citing the literature. One should also keep in mind that some investigators use the terms "situs ambiguus" or "laterality sequences" to indicate "partial situs inversus".

The terminology for description of heart and great vessel sidedness is even more complicated and confusing (reviewed elsewhere, e.g., by Van Praagh *et al.*, 1964; Campbell and Deuchar, 1965, 1967; Wilkinson and Acerete, 1973; Stanger *et al.*, 1977; Rao, 1981; Van Praagh and Van Praagh, 1990). For example, the terms, "dextrocardia (right-sided heart)", "mesocardia (midline heart)" and "levocardia (left-sided heart)" simply indicate the position of the heart, usually determined by X-ray, and do not give any indication of structural or anatomical sidedness. The term "cardiac malposition" indicates that the position of the heart relative to other organs is abnormal; thus, it is not used for cases of total situs inversus.

Finally, many other synonymous terms have been used to describe cases of "situs inversus", particularly in the old literature, such as, "situs viscerum transposus", "situs transversus", "reversed asymmetry", "lateral transposition", etc. This author could not determine when the term "situs inversus" came into common usage.

## Staging system

### Definition of "embryo"

The term "embryo" in humans usually refers to the developing organism during the period starting after formation of the bilaminar embryonic disc (epiblast and hypoblast), i.e., the 2nd week of gestation, until the end of organogenesis when most of the major organs have been established, i.e., the end of the 8th week (Moore,

1988). Most investigators studying mammals follow this concept. However, the term "embryo" is often used by investigators of non-mammalian animals to refer to the organism at any time period between fertilization and birth (Gilbert, 1994). In this manuscript, the term "embryo" is used in the context of the human definition. Furthermore, the term "fetus" is employed to describe the organism after the end of organogenesis and before birth.

**Staging systems in mouse/rat**

Most investigators use commonly accepted staging systems for embryos of human (O'Rahilly and Müller, 1987) and experimental animals, e.g., chick (Hamburger and Hamilton, 1951), *Xenopus laevis* (Nieuwkoop and Faber, 1994), and zebrafish (Hisaoka and Battle, 1958; Kimmel *et al.*, 1995). In contrast, investigators use various staging systems for embryos of mouse and rat, e.g., those proposed by Nicholas and Rudnick (1938), Witschi (1962), Theiler (1972), Beddington (1987), and Downs and Davies (1993). In this review, a modified Theiler's system proposed by Fujinaga *et al.* (1992b) is used. When citing the literature using other staging systems, their observations have been adapted into this system taking into account any inconsistencies in the definition of each stage. In addition, gestational day (GD) is used in some instances to refer to the approximate time of development or when GD is the only staging information available. In such cases, GD 0 is defined as the day when a copulatory plug was observed (Kalter, 1968). However, one should keep in mind that variation in development among embryos and fetuses within a litter and among litters varies more than a half day in the mouse and rat over the whole gestation including the early postimplantation period (Allen and MacDowell, 1940; Otis and Brent, 1954; Fujinaga *et al.*, 1990b; Fujinaga and Baden, 1992; Downs and Davies, 1993). This fact emphasizes the importance of sorting embryos by a staging system rather than by timing based on explantation or examination.

**Comparison of staging systems among human, mouse/rat, chick, *Xenopus laevis* and zebrafish**

The sequences of developmental events among different species are not completely consistent, although there are many

similarities. Although it may be overly ambitious, it is convenient for this review to compare commonly used staging systems for experimental animals and humans (Fig. 2). The modified Theiler's staging system for mouse and rat, as cited above, has been used as the basis for comparison; other animal staging systems have been aligned analogously. Before somite formation the development of the primitive streak and neural plate are used as landmarks. After the first somite pair appears the number of somite pairs is simply used for comparison. For example, a stage described as stage 12/s1, indicates that the embryo is stage 12 with 1 pair of visible somites.

**Development of early asymmetric body structures**

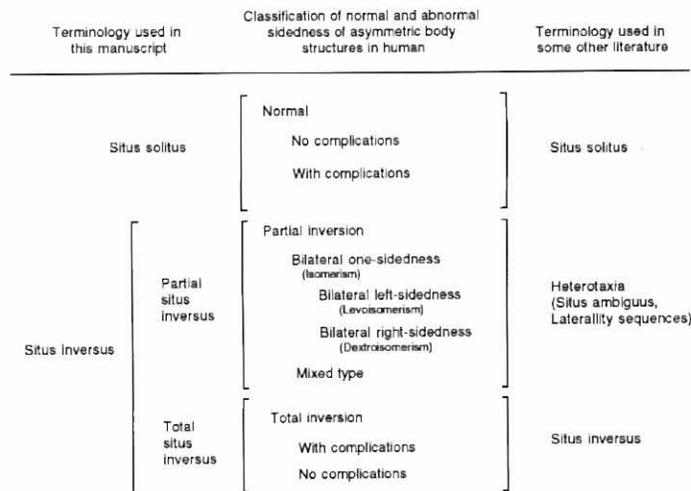
**Node/primitive streak/neural plate**

In chick, Hensen's node is defined as the area lying immediately around the primitive pit, although there is some confusion in its definition in the literature (discussed by Hara, 1978). Hensen's node is known to be asymmetric at the primitive streak stage (Wetzel, 1929) (Fig. 3). The shortening process of the primitive streak is also reported to be asymmetric at the "primitive streak stage", occurring slightly earlier on the left side than on the right leading to the deeper neural furrow on the left side and shifting of the bottom of the primitive groove toward the right (Lepori, 1966a,b). Lepori (1967) reported, that even at an earlier stage, so-called "convergence movements" accompanying primitive streak formation take place in an asymmetric fashion, and they are more marked on the left side of blastoderm than on the right side, most noticeably in the "marginal zone". Cooke (1995) reported also that thickness and form of the neural plate and the sheet of emerging mesoderm on either side of the Hensen's node are asymmetric.

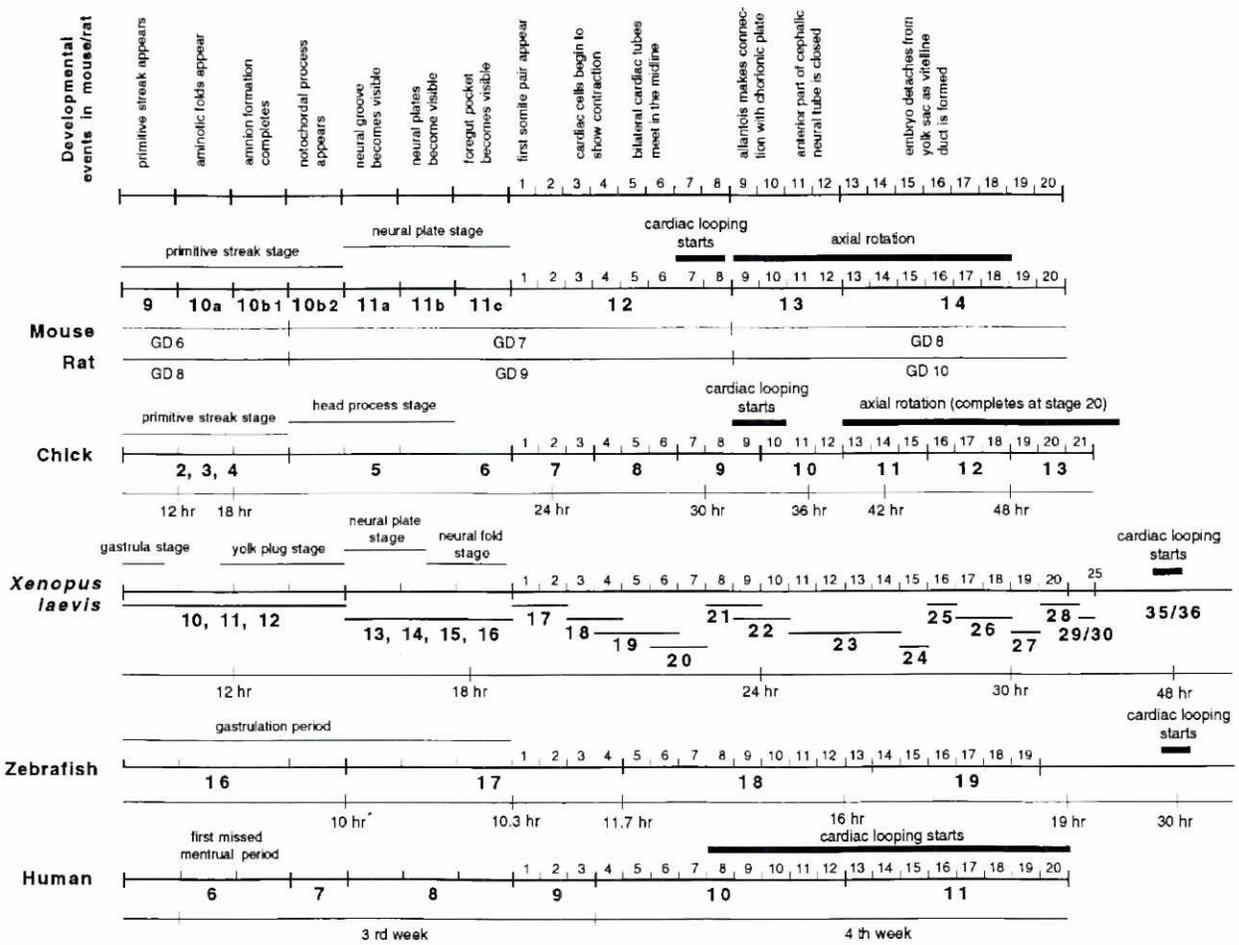
In rat, Long and Burlingame (1938) reported asymmetry in the embryonic disc, with the anterior and posterior ends of the neural fold inclined slightly towards the right at stage 11. Deuchar and Parker (1975) have claimed that they did not observe such asymmetry in several hundred embryos that they examined, and suggested that the observation made by Long and Burlingame might have been artifact that occurred during fixation. However, they discussed the possibility that if such asymmetry existed, this might imply some asymmetry in the mechanical properties of the tissues.

**Heart**

Most investigators agree that cardiac looping is the first gross morphological sign of body asymmetry in vertebrates. In most vertebrates, the primitive heart originally develops as bilateral cardiac tubes, which gradually approach the midline and fuse to form a single cardiac tube. The cardiac tube then begins normally to show "dextral looping" (Fig. 4). According to the literature, there are records of investigations of the development of cardiac looping since the late 17th century. However, the first systematic observations of cardiac looping were performed by Patten, in 1922, in chick embryos using plastic and wax-plate reconstruction models. Various hypotheses on the mechanism of cardiac looping have been proposed; nevertheless, our understanding is still incomplete (reviewed by, e.g., Stalsberg, 1969, 1970; Manasek, 1976; Manasek *et al.*, 1984; Taber *et al.*, 1995). Cardiac looping is certainly a process intrinsic to the heart that is independent of function or boundary conditions, and cytoskeletal microfilaments appear to be important structural elements involved in cardiac looping (e.g.,



**Fig. 1. Classification and terminology of abnormal sidedness of asymmetric body structures that are used in this manuscript.** The term "complications" indicates malformations not related to the LR asymmetry.



**Fig. 2. Comparison of staging systems among several different vertebrates.** The staging system for the mouse/rat, a modified Theiler's system (Fujinaga *et al.*, 1992b), is used as the basis for comparison. Bold numerals indicate "stages", and small numerals above the bar indicate "the number of somite pairs". For the mouse and the rat, the day when a copulatory plug is observed is defined as gestational day (GD) 0. For human and other animals, the age indicates weeks or hours after fertilization. See the text for references of staging systems for human and other animals.

Manasek *et al.*, 1978; Itasaki *et al.*, 1989, 1991; Manning and McLachlan, 1990; Price *et al.*, 1996b).

In human development, the earliest sign of the heart is the appearance of paired endothelial cords, so-called cardiogenic cords, during the middle of the third week, which become canalized to form bilateral cardiac tubes (cited from Moore, 1988). Although the timing in the fusion of cardiac tubes and the start of cardiac looping have been reported to have wide variations, they seem to be similar to those of mouse and rat. Detailed descriptions of human heart development are made elsewhere, e.g., Davis (1927) and Patten (1960).

In mouse and rat, the splitting of mesoderm in the cardiogenic areas of the embryonic disc occurs around stage 11c (Kaufman and Navaratnam, 1981). Bilateral cardiac tubes meet in the midline and fuse at stage 12/s5-6, and cardiac looping begins around stage 12/s7-8 (Long and Burlingame, 1938; Burlingame and Long, 1939). The first heart beat is observed also during this stage (Goss, 1938; Patten, 1949).

In chick, cardiac primordia appear at stage 5, and cardiac looping begins about stage 9-10 (Romanoff, 1960; Castro-Quezada *et al.*, 1972). Lepori (1967, 1969) reported that the fusion of two cardiac tubes occurs on the right side of midline instead of in the

midline resulting from asymmetric closing of the foregut pocket. This investigator assumed that asymmetric closing of the foregut pocket was a consequence of the faster movement of the left splanchnopleure as compared to the right one, but this asymmetry itself did not relate to sidedness of cardiac looping. On the contrary, Castro-Quezada *et al.* (1972) reported that the fusion of the two cardiac tubes occurs in the mid-line in the chick embryo.

In amphibia, "The two presumptive *heart anlagen*, located in the anterior, latero-ventral mesoderm at stage 15, begin to fuse at stage 16", and "At stage 33/34, the *heart* becomes twisted both in a sagittal and a frontal plane, so that the heart anlage shows the characteristic S shape at stage 35/36." (Nieuwkoop and Faber, 1994). By stage 45 the final form of the heart is recognized (Fig. 5).

In zebrafish, the heart consists of four chambers in series, i.e., sinus venosus, atrium, ventricle, and bulbus arteriosus (Santer, 1985). According to Stainier *et al.* (1993), bilateral tubular cardiac primordia are formed in the lateral plate mesoderm at the 15 somite stage. Fusion of bilateral cardiac tubes occurs at the 21 somite stage leading to the formation of the definitive heart tube by 24 h post-fertilization. Cardiac looping begins by 30 h post-fertilization and becomes clearly visible by 36 h post-fertilization.

### Axial rotation in mouse/rat

In mouse and rat embryos, the embryonic disc develops within a cup-shape "egg cylinder" and consists of ectoderm and endoderm that are inverted compared with most other chordates. Thus, the embryo first develops in a dorsally flexed position and then undergoes rotation to a ventrally flexed position. This process is commonly called "axial rotation" or "turning", and this is assumed to be the second gross morphological sign of body asymmetry in mouse and rat following cardiac looping. Snell (1941) has suggested that one useful consequence of the inversion of the germ layers is that the embryo is very compact, and that much of the space normally occupied by the yolk sac cavity in other mammals is eliminated. Such inversion is seen also in rabbit, guinea pig and other closely related mammals (Snell, 1941).

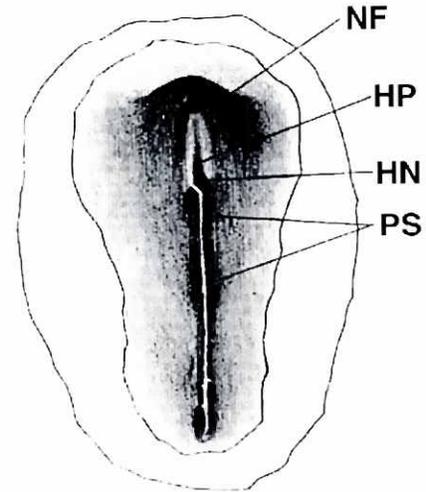
According to Long and Burlingame (1938), axial rotation was first observed in mouse by Raven (1894) and in rat by Widakowich (1909). Since then, many investigators have described this process in mouse (e.g., Snell, 1941; Theiler, 1972; Poelmann *et al.*, 1987; Kaufman, 1990, 1992) and in rat (Deuchar, 1969, 1971, 1975a,b; Deuchar and Park, 1975) based on the assumption that only one asymmetric movement is involved in this process. However, Fujinaga, *et al.* (1995a) reported that axial rotation of the rat embryo actually consists of three movements which start at different stages. From the top (dorsal) view, they are: 1) clockwise twisting of the upper body at stage 12/s7-8; 2) anti-clockwise twisting of the middle body at stage 13/s11-12; and 3) anti-clockwise twisting of the lower body ("tail") at stage 14/s15-16. Axial rotation is almost completed by stage 14/s17-18 resulting in several asymmetric structures, e.g., chorioallantoic placenta, "tail" and umbilical vessels normally on the right side of the embryo, and vitelline vessels on the left side of the embryo (Fig. 6 and Table 1). The vitelline vessels are always located on the opposite side of the chorioallantoic placenta, as explained by Fujinaga *et al.* (1995a). In some books the vitelline vessels are illustrated as if they merge with the umbilical vessels as the axial rotation progresses, e.g., Kaufman (1992) and Hogan *et al.* (1994); however, these vessels never merge during development.

Although movements of the upper and lower bodies during the axial rotation appear to be anatomically separate, it is possible that the mechanisms for these movements are linked because twisting always seems to be in the same direction; otherwise an abnormal "squirrel shape" would result. In the early 70's, Deuchar and colleagues performed a series of microsurgical experiments addressing this issue of linked movements, and concluded that the two movements are independent (Deuchar, 1969, 1971, 1975a,b; Deuchar and Parker, 1975). The investigators transected embryos at either the cervical or mid-trunk levels on GDs 9 or 10 to examine the effects on axial rotation, making the assumption that the transection would not affect rotation of the upper and middle bodies if different mechanisms controlled the movement of each part. However, their results are difficult to interpret because unsatisfactory culture methods of the time led to a high incidence of abnormal control embryos. For example, only 67.6% (25/37, Deuchar, 1971) and 48.0% (24/50, Deuchar, 1975a) of control embryos completed normal axial rotation in their experiments, whereas almost 100% would be expected to do so with current culture methods. Furthermore, the transection surgery itself, especially on GD9, caused too many confounding traumatic effects.

To date, the underlying mechanisms for asymmetric movement involved in axial rotation are not well understood. Poelmann *et al.*

**Fig. 3. Chick embryo at stage 5 showing asymmetric Hensen's node.**

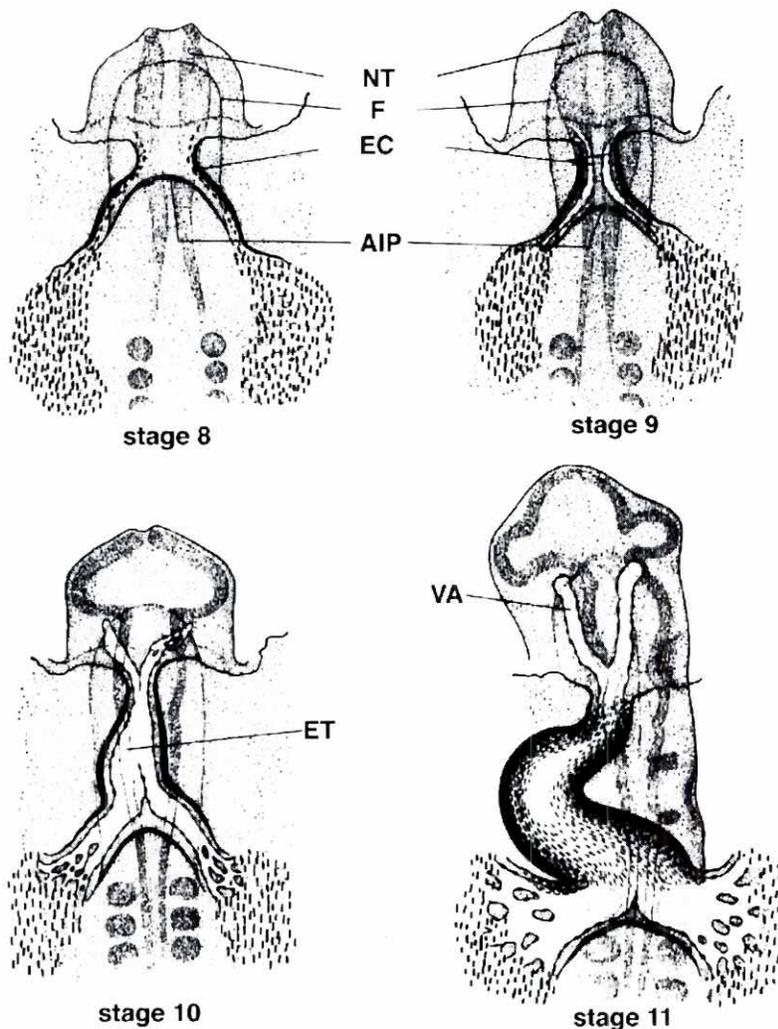
Modified from Wetzel (1929, p. 220). Abbreviations: HN, Hensen's node (Knoten); HP, head process (Chordaanlage); NF, neural fold (Querer Hirnwulst); PS, primitive streak (Vorderer Teil des Streifens). The words in parenthesis are the labels used in Wetzel's illustration.



(1987) reported asymmetric mitotic activity of cells near the rotating part of the neuroectoderm, and suggested that asymmetric growth and activity of the neural tube play important roles. Also, asymmetric mitotic activity was demonstrated in the somatopleure by Miller and Runner (1978). Deuchar (1975a) suggested that coordinated activity of the somites may play a role. In addition, Poelmann *et al.* (1987) suggested that looping of the heart is unlikely to be involved in determining sidedness of axial rotation because the first signs of asymmetric activity within the neuroectoderm are seen before cardiac looping becomes evident. Furthermore, rat embryos subjected to  $\alpha 1$  adrenergic stimulation show various combinations of sidedness of the heart, chorioallantoic placenta and "tail" (Fig. 8 and Table 2). Thus, sidedness of cardiac looping probably occurs by mechanisms independent from those of axial rotation. If this is the case, are they totally independent? Data from  $\alpha 1$  adrenoceptor stimulation-induced situs inversus suggest that the truth may lie somewhere in between. For example, total situs inversus occurred in approximately one third of affected embryos (Table 2; Fujinaga and Baden, 1991c), an incidence virtually identical to that found in another set of similar experiments (Fujinaga and Baden, 1991b) and in an *in vivo* study of nitrous oxide (Fujinaga *et al.*, 1991). If the "tail" and chorioallantoic placenta are always on the same side as the heart, all affected embryos would have total situs inversus. Whereas, if the sidedness of each structure is determined independently, only one seventh of the affected embryos would have total situs inversus. Certainly, correlation of the sidedness of different structures remains to be elucidated. In addition, this emphasizes the need to examine at least three asymmetric structures, i.e., the heart, "tail" and chorioallantoic placenta/vitelline vessels, to obtain a full picture of sidedness at this developmental stage.

### Axial rotation in bird

Although the term "axial rotation" may not be appropriate, a body rotation similar to the axial rotation in mouse and rat is observed in the bird. In chick, it starts at stage 11/s13 and is completed at stage 20 (cited from Poelmann *et al.*, 1987). While the rotation in mouse and rat is 180 degrees, it is only 90 degrees



**Fig. 4. Chick embryos showing the process of cardiac looping from ventral view.** Modified from DeHaan (1965, p. 391). Abbreviations: AIP, anterior intestinal portal; EC, endocardium; ET, endocardial tube; F, foregut; NT, neural tube; VA, ventral aorta.

in chick. According to Romanoff (1960), the first observations on the orientation of the embryo was made by Pander (1817), who reported that the AP axis of the embryo lays along the short diameter of the egg. Baer (1828) confirmed this report and found that the chick embryo is normally seen with its head directed to the left when the egg is held so that its blunt end is nearest the observer and blastoderm uppermost. While some investigators have suggested the direction of cardiac looping determines the direction of axial rotation (e.g., Waddington, 1937; Itasaki *et al.*, 1991; Hoyle *et al.*, 1992), others have suggested that these two processes are independent (e.g., Icardo and Ojeda, 1984).

#### Limb buds

Limb buds are mirror image symmetric structures. For years, they have been models studied for development of symmetric structures in amphibia (e.g., Harrison, 1921) and in chick (e.g., Hamburger, 1938; Chaube, 1959; cited from Oppenheimer (1974).

In mouse and rat, limbs are usually considered to be symmetric structures. Nevertheless, forelimb buds at the beginning of development are known to be asymmetric; the left forelimb bud is normally larger than the right. According to observations in mouse by Milaire (1985), active proliferation of somatopleural mesoderm in the region of the forelimb buds starts at stage 13/s10, and the forelimb buds themselves first become visible at stage 14/s16-17. Even by stage 14/s17, the volume of left forelimb bud slightly exceeds that of right one.

#### Asymmetric gene/protein expression during development

##### Genes in bird

In 1995, Levin *et al.* reported for the first time that several patterning genes, e.g., chicken *activin receptor 1la* (*cAct-R1la*), *Sonic hedgehog* (*Shh*), *nodal-related 1* (*cNR-1*), and *HNF3 $\beta$* , which have been implicated in cell signaling and in the regulation of the DV and AP axes are expressed asymmetrically in chick embryo during development before the first sign of body asymmetry becomes apparent. (Most figures and illustrations shown in their manuscript are dorsal views of the chick embryo, although some are ventral views. Thus, indications of "left" or "right" in the figures and illustrations directly correspond to actual left and right sidedness. One should keep in mind, however, that when ventral view is used in other reports, for example to show cardiac looping, left and right sidedness in the figure or illustration becomes inverted. In addition, no statement was made about the number of embryos examined for normal expression of these genes).

*Act-R1la* is a receptor for activin, a member of TGF- $\beta$  family, which is known to play a role in tissue differentiation. *cAct-R1la* is initially expressed more strongly on the right side of the primitive streak at stage 4, and then exclusively in the right half of Hensen's node; this expression is only in the ectoderm. At stage 5, it is expressed symmetrically in areas lateral to Hensen's node that roughly coincide with the areas that have heart-forming potency. In contrast, the gene for another receptor subtype for activin, *cAct-R1lb*, is expressed symmetrically first in the primitive streak and then within Hensen's node at stage 4.

*Sonic hedgehog* (*Shh*) is a vertebrate homolog of *Drosophila melanogaster hedgehog* which has been shown to be an important signaling gene during development. *Shh* is initially expressed symmetrically throughout the node. With the onset of the expression of *cAct-R1la* in the right side of the node, *Shh* expression becomes restricted to the left side lasting until stage 7. This expression at stage 4<sup>+</sup> is in the ectoderm, whereas midline expression of *Shh* anterior to the node at stage 5 is exclusively in mesodermal cells. A similar asymmetric expression pattern of *Shh* was also reported by Chen *et al.* (1996) in quail embryos.

*cNR-1* is a chick homolog of the mouse gene *nodal* which is also a member of the TGF- $\beta$  gene family. *cNR-1* is expressed symmetrically in and lateral to the middle two thirds of the primitive streak,

but not in Hensen's node, until stage 4<sup>+</sup> and disappears by stage 4<sup>-</sup>. *cNR-1* expression reappears during stage 7, only on the left side and just lateral and anterior to Hensen's node and is followed by a much larger patch of expression in the lateral plate mesoderm. This large patch remains asymmetric only on the left side until at least stage 11, while a smaller medial region of expression eventually appears on the right side as well at stage 9. These expressions are in mesodermal cells.

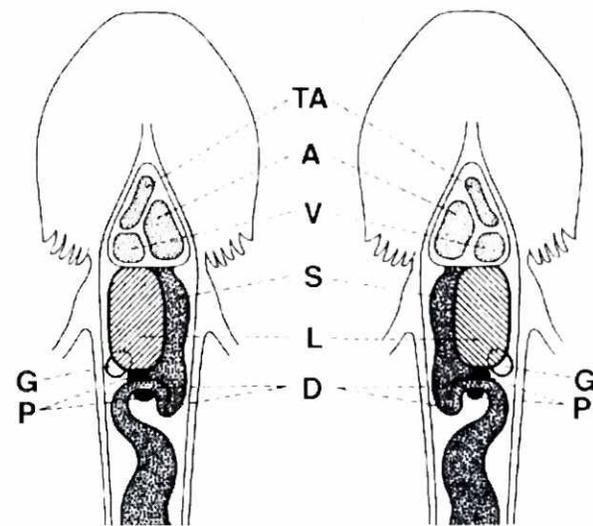
In addition, the investigators found that *HNF3 $\beta$*  is symmetrically expressed in Hensen's node by stage 5, but exhibits a brief and transient period of asymmetric expression during stage 4. *HNF3 $\beta$*  is a winged-helix transcription factor that may regulate *Shh*. The following genes were found to be expressed only symmetrically: *cNot*, *FGF4*, *goosecoid*, *Msx1*, *Hoxb-8*, and *engrailed* (Levin *et al.*, 1995).

From these observations, the investigators hypothesized that "an activin-like molecule may be responsible for inducing asymmetric *cAct-RIIa* expression and setting up the LR asymmetry in the expression of *Shh*." (Levin *et al.*, 1995). To test this hypothesis, the investigators performed the following experiments. First, they placed heparin acrylic beads pre-soaked in activin on the left side of Hensen's node and caused the appearance of *cAct-RIIa* expression and disappearance of *Shh* expression on the same side. Second, the investigators implanted the cell pellets prepared from chick embryo fibroblast cells expressing *Shh*, and examined the expression of *Shh* and *cNR-1*. When implantation was made on the right side of Hensen's node, the side where *Shh* is not normally expressed, ectopic expression of *cNR-1* was observed on the same side. But when implantation was made on the left side, the side where *Shh* and later *cNR-1* are normally expressed, the endogenous *cNR-1* expression domain was unaffected and no ectopic *cNR-1* expression was observed. Third, they implanted *Shh*-expressing cell pellets on the right side of Hensen's node at stage 4, and caused a 50.0% (11/22) incidence of inverted cardiac looping examined at stage 12; New's culture method (1955) was used, which showed a 13.0% (3/23) incidence of inverted cardiac looping in the control group. Fourth, beads pre-soaked in activin were placed on the left side of Hensen's node at stages 3<sup>+</sup> and 4 caused a 50.0% (8/16) incidence of inverted cardiac looping; the control group showed no inversion (n = 13) in this experiment by modifying the culture method.

Based on these results Levin *et al.* (1995) has proposed a model of the molecular pathway that determines the LR axis (Fig. 7). Commenting on Levin *et al.*'s work, Wolpert and Brown (1995) have speculated that *Vg1*, which belongs to the same family as activin, becomes localized at the vegetable pole in amphibia and is involved in axis specification, might be also expressed asymmetrically.

### Genes in mouse

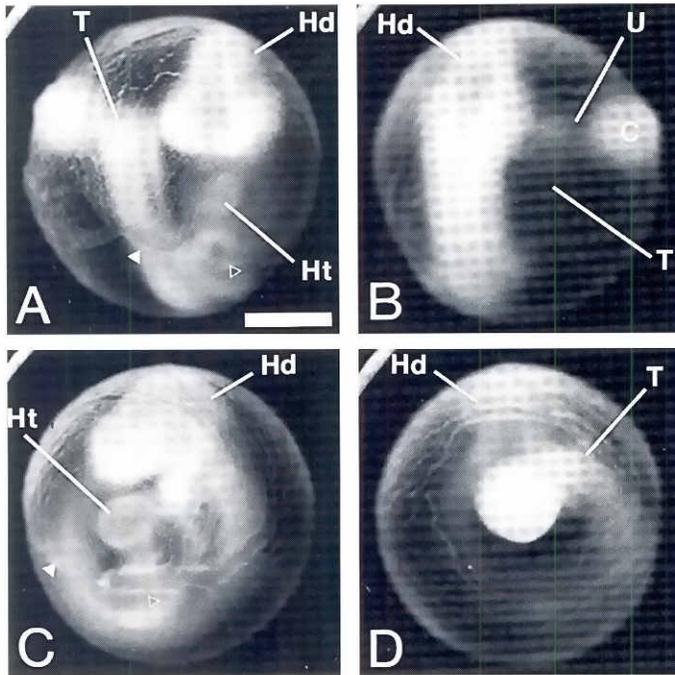
In 1996, three groups of investigators simultaneously reported asymmetric expression of similar genes in the mouse as in the bird. Collignon *et al.* (1996) and Lowe *et al.* (1996) reported that mouse *nodal* is expressed asymmetrically around the node and in the lateral plate, i.e., bilateral but stronger expression on the left side of the node and only left-sided expression in the lateral plate. According to the data presented by Lowe *et al.* (1996), expression of *nodal* was symmetric around the node at the "0-1 somite stage" (n = 5). As the development advanced, the incidence of embryos showing asymmetric *nodal* expression around the node increased



**Fig. 5. Illustration of newt embryos showing asymmetric body structures. (Left) Situs solitus. (Right) Total situs inversus.** Modified from Woellwarth (1950, p. 182). Abbreviations: A, atrium; D, duodenum; L, liver; S, stomach; TA, truncus arteriosus; V, ventricle.

while those with symmetric expression decreased (n = 6-13 for each number of somite stage). Interestingly, the incidence never reached 100% (except for the "9-10 somite stage" when *nodal* expression was no longer observed in 5 out of 7 embryos). In contrast to the expression around the node, *nodal* expression in the lateral plate was either left-sided or not expressed in the embryos examined. Expression began at the "1-2 somite stage" (2 out of 9 embryos), the incidence of expression increased as the development advanced, and it reached 100% at the "5-6 somite stage" (n = 10). The incidence then gradually declined, and no expression was observed at the "9-10 somite stage" (n = 7). In addition, Lowe *et al.* (1996) observed that *nodal* expression in the lateral plate expanded from a small area lateral to the node to include the entire area of the lateral plate, and then became restricted to the anterior area of the lateral plate; *nodal* expression did not extend into the heart. Although somewhat similar but not totally consistent findings were reported by Collignon *et al.* (1996), it is difficult to interpret this group's report because the data were not fully presented.

Another group, Meno *et al.* (1996), found a new member of TGF- $\beta$  family gene among a number of undifferentiated cell-specific complementary DNA clones that they obtained by applying a systematic subtraction procedure to the P19 embryonal carcinoma cells. The investigators named it "*lefty*", probably because they subsequently found it to be expressed exclusively on the left side of embryo during a certain stage of development. The investigators also reported locating this gene on chromosome 1. The expression of *lefty* was first detectable at stage 9 (so-called early primitive streak stage) in the anterior half of the primitive streak. At stage 10a (so-called mid primitive streak stage) the expression extended along the primitive streak except at "its caudal and rostral extremities". However, at both stages, the expression was symmetric. At the "presomite headfold stage" (probably stage 11c, the stage commonly called as "neural fold stage" by embryologists) *lefty* expression became transiently undetectable. At the "3-4 somite stage", *lefty* was again expressed highly in the lateral plate and in



**Fig. 6.** Four different views of rat embryo within the intact visceral yolk sac at stage 15 on GD 11 after the completion of axial rotation. (A) Frontal view. (B) Back view. (C) Left lateral view. (D) Right lateral view. Derived from Fujinaga *et al.* (1995, p. 95). Abbreviations: C, chorioallantoic placenta; Hd, head; Ht, heart; T, tail; U, umbilical cord. Symbols: open arrowhead, vitelline vein; closed arrowhead, vitelline artery. Bar, 1 mm.

the prospective floorplate (but not in the notochord/notochordal plate); the former expression was only on the left side, and the latter was asymmetric only in the anterior region ("at the level of midbrain and hindbrain"). After the "6-8 somite stage", *lefty* expression became undetectable. Unfortunately, however, full data including the numbers of embryos examined were not reported in this series of experiments, thus the relationship between *lefty* and *nodal* expression remains unclear.

These investigators subsequently examined *nodal* and *lefty* expression in *iv* and *inv* mutation mice. Homozygotes of the former show approximately a 50% incidence of situs inversus and homozygotes of the latter almost 100% incidence of situs inversus (discussed later). Lowe *et al.* (1996) found that *nodal* expression in the lateral plate showed various patterns in approximately 30 *iv* homozygotes that they examined at various stages, i.e., left or right one-sided expression, no expression, and expression in both sides. In embryos obtained by mating *inv* heterozygous male and female animals, the investigators found that all 4 embryos confirmed as homozygotes by genotyping showed right sided (inverted) *nodal* expression, whereas, all of approximately 20 embryos confirmed as heterozygotes or wild type showed left sided expression. Collignon *et al.* (1996) also conducted similar experiments on the *inv* mutation. Their results are mostly, but not completely consistent, with those of Lowe *et al.* However, interpretation of this group's experiments is difficult because full data were not presented. Meno *et al.* (1996) found that among 7 *iv* homozygotes that they examined, *lefty* expression was inverted in 3 and was normal in 4 embryos. In addition, the investigators found ectopic expression of *lefty* in the somatopleure of all embryos. In embryos

obtained by mating *inv* heterozygous male and female animals, the investigators found that approximately 25% (5/17, 29.4%) showed inverted *lefty* expression, and the remaining normal expression. Although genotyping was not done for this experiment, the investigators did not find any inverted *lefty* expression in 16 embryos obtained by mating *inv* heterozygous and wild type animals. Thus, they speculated that *lefty* expression was inverted in all *inv* homozygous embryos. From these results, Beddington (1996) suggested that both *nodal* and *lefty* "may be involved in the execution of left-right asymmetry, possibly by effecting differential growth such that both the heart tube and the embryo turn away from where their molecules are being produced".

Collignon *et al.* (1996) also examined the embryos obtained by mating "double-heterozygotes for mutations in *nodal* and *HNF3 $\beta$* " (term cited from Beddington, 1996). They found that all of the *HNF3 $\beta$*  heterozygous embryos showed bilateral *nodal* expression in the lateral plate and the direction of axial rotation was randomized (the number of examined embryos was not reported). Furthermore, 7 out of ten double-heterozygotes showed "defects in the positioning of the abdominal viscera and the heart" when examined on GD 15. However, the investigators did not find asymmetric expression of *HNF3 $\beta$*  itself during normal development unlike in chick (Levin *et al.*, 1995). Although their report suggests the interaction between *HNF3 $\beta$*  and *nodal*, the involvement of *HNF3 $\beta$*  in the development of the LR axis remains unclear in mouse.

### Proteins

In 1991, Brown *et al.* reported that they analyzed the profiles of proteins obtained from left and right halves of rat embryo by two-dimensional gel electrophoresis between stage 11 and stage 16/s30. Among approximately 600 proteins that were "resolvable", no protein showed a difference between left and right.

In 1995, Britz-Cunningham *et al.* described in their manuscript a personal communication from C.W. Lo that transgenic mice containing a connexin 43 promoter-driven:lacZ reporter gene showed an symmetric pattern in various tissues in incipient connexin 43 expression. In contrast, no asymmetric expression of connexin 43 protein (Yancey *et al.*, 1992) and gene (Ruangvoravat and Lo, 1992) was reported in wild type mouse, although these investigators may have overlooked it.

In 1996, Tsuda *et al.* reported asymmetric staining by F-22 in chick during development. F-22 is a monoclonal antibody to an extracellular matrix protein that is extracted from the interphotoreceptor matrix of mouse eyes (Miezewska *et al.*, 1994). The investigators named this protein "flectin" from the Latin word "flectere" that means "to bend" or "to loop". F-22 staining was first detected at stage 7/s3 in the extracellular matrix of the precardiac mesoderm in the left side, and shortly later in the right side. After the completion of cardiac looping at stage 22, F-22 staining was decreased in both sides. The investigators have suggested that the delay in synthesis of this extracellular matrix protein on the right side may lead to asymmetric cardiac looping. The investigators also suggested that flectin expression may be modulated by retinoids, although the data were very preliminary.

### Genes in Amphibia

In *Xenopus laevis*, Lowe *et al.* (1996) examined three *nodal*-related genes, and found only *Xnr-1* (*Xenopus* related *nodal* gene-1) showed similar stage- and spatial-specific asymmetric expression in the lateral plate as seen in the mouse.

## Incidence of situs inversus in normal population

### Mammal-human

Many investigators cite the thesis by Karashima (1912) for an earlier, thorough historical summary of situs inversus; however, it is not readily accessible today. According to the literature citing Karashima's work, e.g., Lineback (1920), Sherk (1922), and Wood and Blalock (1940), "transposition of visceral organs" was already reported in 17th century by Fabricius (Italy), Servicus (Italy) and Riolan (France). In 1824, Küchenmeister reported "transposition of visceral organs" in a living person soon after the development of percussion and auscultation as diagnostic tools (no citation). In 1897, Vehsemeyer first demonstrated dextrocardia by X-ray.

Studies on total situs inversus in adults have reported incidences ranging between 1:4000 and 1:500,000 (reviewed by, e.g., Johnson, 1949; Varano and Merklin, 1960; Gray *et al.*, 1994). This wide range probably reflects different methods used for detection, e.g., X-ray survey or hospital records. Today, most authors assume the incidence to be between 1:10,000 and 1:20,000 in adults. However, the incidence of situs inversus among newborns is expected to be much higher, because situs inversus is accompanied frequently by other malformations, particularly cardiac, which lower survival (Cockayne, 1938; Warkany, 1971).

### Mammal-mouse/rat

Similar to the human, situs inversus is rarely observed in adult mice (discussed by Hummel and Chapman, 1959). Situs inversus is also seldom observed among fetuses at late gestational periods. For example, according to the control data reported by Charles River Laboratories (Wilmington, Massachusetts, USA), only 11 out

of 30,134 rat fetuses (0.037%) examined on GD 20 or 21 had situs inversus (Anonymous, 1995). In contrast, relatively high incidences of inverted "tail" (lower body which normally flexes to the right side of the embryo) were reported during the embryonic period in mouse, 3.2% (4/127) on GD 10 (Layton, 1976), 5.2% (16/308) on GD 10 and 6.2% (11/177) on GD 11 (Endo and Sakai, 1987). These investigators did not examine sidedness of other structures including the heart. In rat, Fujinaga *et al.* (1990b) examined 549 embryos on GD 11 and found 1 embryo with an inverted "tail" but a normal heart, 1 embryo with an inverted heart but a normal "tail" and 1 with an inverted "tail" and heart, i.e., 2 (0.37%) with an inverted "tail" or heart and 3 (0.55%) with both inverted. These data may suggest that embryos and fetuses with situs inversus may be lost during the fetal period and/or sidedness of the "tail" (lower body) may not always reflect the sidedness of visceral organs.

### Bird

Numerous reports of inverted embryonic head direction of varying incidence, usually attributed to inverted axial rotation, are found in the literature. However, the AP axis of the embryo is not always perpendicular to the axis of the egg, e.g., it may be completely inverted, and many investigators have reported such variations; reviewed by, e.g., Bartelmez (1918) and Romanoff (1960, pp. 141-142). This author has found it extremely difficult to review the large volume of reports on inversion of the head as they are mostly old, poorly accessible, and usually do not distinguish between inversion of the axis itself and head direction. For example, although Romanoff (1960, pp. 141-142) stated that Dalton (1881) reported a 12% (12/100) incidence of inverted head direction in fowl embryos, none of them actually showed inverted head direction but the AP axis was inverted in those embryos in relation

TABLE 1

### SUMMARY OF DEVELOPMENTAL EVENTS AND STAGES IN RAT EMBRYO WHEN EARLY SIGNS OF LR ASYMMETRY APPEAR

Approx. GD	Modified Theiler's stage <sup>2</sup>	General developmental events	Events related to the LR axis, including the heart, axial rotation and limb buds
7	9	Primitive streak appears	
8	10a	Amniotic folds become visible	
9	10b	Amnion formation finishes	
	10b1	Notochordal process is absent	
	10b2	Notochordal process is present	
	11a	Neural groove becomes visible	Asymmetry in neural plates* <sup>1</sup>
		Allantoic bud begins to develop	
	11b	Neural folds become visible	
	11c	Foregut pocket becomes visible	Cardiac primordial cells appear
	12 /s1-2	First pair of somites become visible	
	12 /s3-4		Contraction of cardiac cells begins
10	12 /s5-6		Bilateral heart tubes meet in the midline / Primitive heart tube begins to beat
			Heart looping begins*
	12 /s7-8	Cephalic neural tube begins to close	Twisting of upper body begins*
		Hematopoiesis begins in the yolk sac	
	13 /s9-10	Allantois makes connection with chorion	
		Yolk sac circulation becomes visible	
	13 /s11-12		Twisting of middle body begins*
	14 /s13-14		
	14 /s15-16	Cephalic neural tube is almost closed / Embryo detaches from the yolk sac	Twisting of lower body ("tail") begins*
11	14 /s17-18		Axial rotation finishes* / Forelimb buds become visible*

Modified from Fujinaga (1995, p. 594). \*Asymmetric events. <sup>1</sup>Long and Burlingame (1939). <sup>2</sup>Fujinaga *et al.* (1992b)

to the axis of the egg. A review by Bartelmez (1918) is also not clear in this respect.

The incidence of inverted cardiac looping *in ovo* is probably very low, because most investigators do not report this finding. (Development of inverted cardiac looping under culture conditions is frequently observed, and is discussed later). Salazar del Río (1974) has reported that none of 82 embryos examined *in ovo* at stage 12 showed inverted cardiac looping.

### Amphibia

In the newt, *Triturus alpestris*, Wehrmaker (1969) found a 0.9% (33/3801) incidence of larvae showing at least one of the following asymmetric structures: alimentary canal, heart or nuclei habenu-lae. Of the larvae with situs inversus, 27.3% (9/33) had total situs inversus and 72.7% (24/33) had partial situs inversus. According to Oppenheimer (1974, p. 870), Mangold (1921) reported the incidence of situs inversus as 1.8% (4/228) in *Triturus Taeniatus* and 2.1% (13/610) in *Triturus alpestris*. According to Wehrmaker (1969), several other investigators also reported situs inversus in normal populations, e.g., 0.8% by Woellwarth (1947, 1948; no citations) and 4.4% by Kraft (1968b) in *Triturus alpestris*, and 2.1% (1/48) by Zwanzig (1938) in *Siredon mexicanum*. In frogs, Wehrmarker (1964, 1969) reported that no situs inversus was reported in a sample of 870 of *Bufo bufo* and 3,948 in *Rana temporaria*, respectively. The individual etiology of spontaneously occurring situs inversus in amphibia was discussed by Wehrmaker (1969, p. 11-13).

### Fish

In zebrafish, Danos and Yost (1996) reported the incidence of inverted heart to be 1% (n=1227) and 2% (n=321) in wild type animals obtained from crossing fish heterozygous for *ntl* and *flt* mutations (discussed below), respectively. (The genotype of the phenotypically wild type animals could be either wild type or heterozygous for these mutations).

## Mutations associated with situs inversus

### Mammal-human

*Mutation of heterotaxy locus (HTX) on X chromosome, "X-linked laterality sequence (syndrome)"*

In 1987, Mathias *et al.* reported the association of situs inversus, complex cardiac defects and splenic anomalies, and demonstrated their X-linked recessive inheritance. Since then, similar cases have been reported by other investigators (e.g., Mikkilä *et al.*, 1994). In 1993, Casey *et al.* studied a family reported by Mathias *et al.* (1987), and identified the mutation by mapping assignment to Xq24-q27.1. The investigators named this locus the heterotaxy locus (*HTX*), and it is now recognized that this is the first mapping assignment of a gene related to the LR axis development in human.

### Case reports of situs inversus with identified chromosomal abnormalities

In 1991, Wilson *et al.* reported a case of an electively terminated fetus at 22 weeks of gestation with partial situs inversus, complex heart defects and other abnormalities similar to "polyasplenia sequence". The investigators found balanced translocations having the break points 12q13.1 and 13p13 both in the fetus and

mother, although the mother had no morphological abnormalities including situs inversus. The karyotype was determined as 46,XX,t(12;13)(q13.1;p13). The investigators pointed out that the human counterparts of 2 loci, *int-1* and *HOX 3*, involved in *Drosophila* early pattern formation are located near the translocation breakpoint 12q13.1. The investigators have suggested these genes may be involved in causing situs inversus.

In 1992, Carmi *et al.* reported two cases of full term newborns with partial situs inversus identified at birth. The karyotypes were determined for a male with interstitial deletion of proximal 10q:46,XX,del(10)(pter→q21::q23→qter), and for a female with derivative chromosome 13 formed by a terminal deletion of the region 13q31→qter:46,XX,-13+der(13)(q31→qter).

In 1993, Koiffmann *et al.* reported a case of a male newborn with partial situs inversus, polysplenia, as well as limb, craniofacial and cardiac defects. The karyotype was determined to be 46,XY,ins(7;8)(q22;q12q24); the parents' chromosomes were normal.

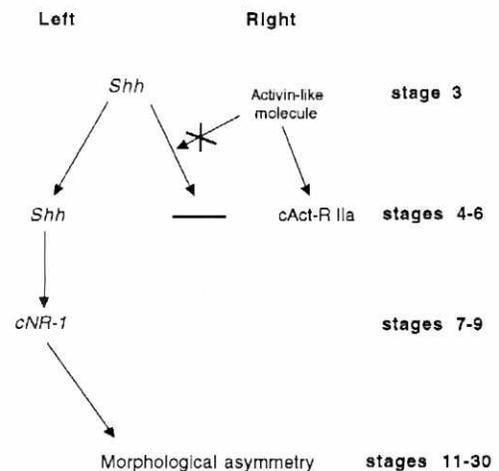
In 1993, Genuardi *et al.* reported a case of a male newborn with various abnormalities including partial situs inversus, asplenia and multiple limb split hand/split foot anomalies. Although chromosomal analysis was not performed for this individual, the investigators found a balanced translocation on chromosome 7 in many family members with similar congenital abnormalities.

Many other reported cases of situs inversus with identified chromosomal abnormalities are found in the literature, e.g., trisomy 13 (Drut *et al.*, 1992), a case of monosomy 22 with mesocardia, midline liver, accessory spleen and other anomalies (DeCicco *et al.*, 1973), and a case of 18p- with cranial meningocele (Meinecke *et al.*, 1990; cited from Carmi *et al.*, 1992).

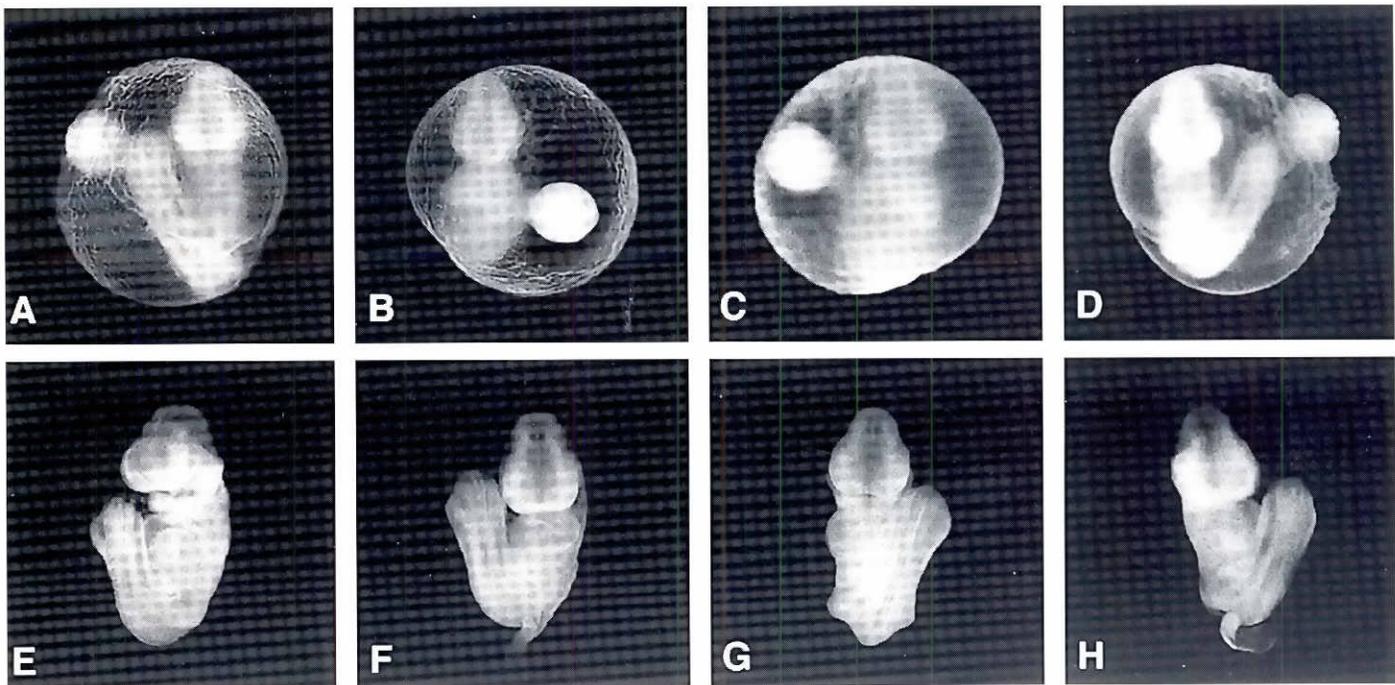
### Mammal-mouse/rat

#### *iv* mutation mouse

In 1948, Tihen *et al.* reported a mutation in the mouse with autosomal recessive inheritance (assigned symbol *v*) in which homozygous animals showed an approximately 50% incidence of



**Fig. 7. A model of the molecular pathway of the LR axis determination proposed by Levin *et al.*** Modified from Levin *et al.* (1995, p. 812). Interactions of several genes have been proposed to lead to morphological asymmetry. See the text for details.



**Fig. 8. Rat embryos at stage 15 on GD 11 showing abnormal sidedness of the asymmetric structures, the chorioallantoic placenta/vitelline vessels, tail (lower body) and heart, in different combinations. (A-D) Frontal view of the embryo within the intact yolk sac. (E-H) Frontal view of the embryo after the yolk sac and amnion have been removed. (A) Normal tail and chorioallantoic placenta. (B) Normal tail but inverted chorioallantoic placenta. (C) Normal chorioallantoic placenta but inverted tail. (D) Inverted tail and chorioallantoic placenta. (E) Normal tail and heart. (F) Normal tail but inverted heart. (G) Normal heart but inverted tail. (H) Inverted tail and heart. Derived from Fujinaga *et al.* (1995a, p. 104).**

total situs inversus; 12.8% (29/227) of offspring obtained by breeding heterozygous animals were found to show total situs inversus. According to Layton (1976), most homozygous animals died at a few weeks of age, and the mutation died out. In 1959, Hummel and Chapman recovered a similar mutation from a non-inbred stock of mouse, and assigned the gene symbol *iv*. Homozygous animals showed approximately a 50% incidence of total situs inversus (50.3%, 255/507). In 1976, Layton confirmed that the incidence of total situs inversus was approximately 50% (51.0%, 234/459) in homozygous animals. In addition, the investigator examined 173 homozygous embryos on GD 10 and found 91 (52.6%) with an inverted left-sided "tail" (lower body). Based on these findings, Layton proposed a hypothesis that, "The normal allele at the *iv* locus exhibits complete dominance and controls normal visceral asymmetry, and absence of this control allows the situs of visceral asymmetry to be determined in a random fashion". In 1989, Brueckner *et al.* first mapped *iv* locus to chromosome 12, a finding later confirmed by the same and other investigators (Hanzlik *et al.*, 1990; Brueckner *et al.*, 1991; Singh *et al.*, 1991; de Meeus *et al.*, 1992, 1993, 1995; McGrath *et al.*, 1992). Brueckner *et al.* (1996) have suggested that "a gene bearing homology to a human cytoskeleton-associated phosphoprotein is an excellent candidate for the mouse *iv* gene".

Not only situs inversus but also many other malformations including venous heterotaxia, isomerism ("partial situs inversus"), spleen abnormalities and cardiac defects are known to occur in homozygous *iv* animals (Layton, 1976, 1978, 1985; Layton and Manasek, 1980; Icardo, 1990; Biddle *et al.*, 1991a; Icardo and Sanchez de Vega, 1991; Seo *et al.*, 1992; Layton *et al.*, 1993; Ho

*et al.*, 1995; Icardo and Colvee, 1995; Icardo *et al.*, 1995; Seo and Kim, 1995). Approximately 40% of homozygous animals show various types of venous heterotaxia, which occurs independent of total situs inversus (Layton *et al.*, 1993).

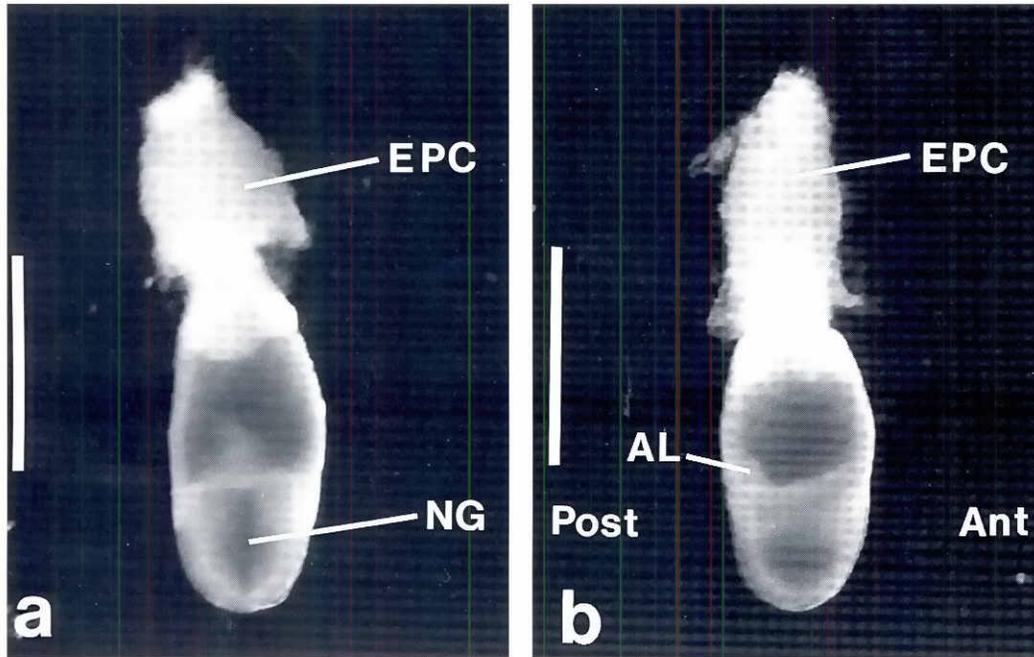
In 1984, Handel and Kennedy examined tracheal cilia and sperm tails of six male homozygous animals (SI/Col strain), of which three had inverted internal organs (no description of which organs), and found no abnormalities in ciliary structure by electron

TABLE 2

**FREQUENCY TABLE OF INVERTED ASYMMETRIC STRUCTURES IN RAT EMBRYOS TREATED WITH PHENYLEPHRINE**

Inverted CAP	Inverted tail	Inverted heart	No. of embryos	% affected embryos	% of total
+	+	+	24	31.6	15.8
-	-	+	29	38.2	19.1
+	-	+	0	0.0	0.0
+	+	-	10	13.2	6.6
-	+	-	0	0.0	0.0
+	-	-	8	10.5	5.3
-	+	+	5	6.6	3.3
-	-	-	76	-	50.0
Total			152	100.0	100.0

+, presence; -, absence. Embryos were cultured from stage 11a with 4 h or more of 50 μM phenylephrine treatment. Derived from Fujinaga and Baden (1991c).



**Fig. 9. Rat embryo at stage 11a (early neural plate stage) on GD 9 when  $\alpha$ 1 adrenergic stimulation can cause situs inversus. (a) Frontal view. The neural groove is visible in the midline. (b) Lateral view. The neural fold is yet not prominent. Abbreviations: AL, allantois; Ant, anterior; EPC, ectoplacental cone; NG, neural groove; Post, posterior. Bar, 1 mm. Derived from Fujinaga *et al.* (1994a, p. 559).**

microscopy and normal motility by phase contrast microscopy. Thus, the investigators doubted that any link between the *iv* mutation and ciliary dysfunction is likely.

In 1989, Brown *et al.* examined the effects of chemicals that are known to cause asymmetric limb defects, e.g., acetazolamide and misonidazole, and found that sidedness was reversed in *iv* homozygous embryos/fetuses. The investigators suggested that "the mechanisms of induction of asymmetric defects is not related to any intrinsic difference between the development of left and right limbs, but is connected to visceral asymmetry." In 1991, Kocher-Becker *et al.* produced double homozygous mouse for *iv* and *polydactyly (py)* (a recessive mutation producing asymmetric limb defects, i.e., polydactylism), and found a strong correlation between the sidedness of visceral organs and limb defects.

In 1990, Brown *et al.* reported a chimera study aiming to answer the question whether the presence of normal cells in *iv* homozygous embryos could prevent situs inversus. However, the results were inconclusive. In the same report, the investigators also conducted "reciprocal embryo transfers" at stage 5 (blastocyst stage) between wild type and *iv* homozygous animals. Among 147 wild type blastocysts transferred to *iv* homozygous mothers ( $n=14$ ), 26 fetuses (17.7%) were viable on GD 18, and all of them showed situs solitus. Among 228 *iv* homozygous blastocysts transferred to wild type mothers ( $n=16$ ), 46 embryos (20.2%) were viable on GD 10. Of these, 20 (43.5%) showed total situs inversus, 4 (8.7%) showed partial situs inversus, and 22 (47.8%) showed situs solitus. (No explanation was made for examining the embryos/fetuses at totally different stages, i.e., the former on GD 10, and the latter on GD 18.) Based on these results, the investigators have suggested that maternal environment plays no role in development of the LR axis.

In 1991, Van Keuren *et al.* reported the comparison of profiles of proteins obtained from wild type and *iv* mutant mice at stages 11, 12 and 13 by two-dimensional gel electrophoresis. Approximately

1000 proteins were detected at each stage, and the investigators found several proteins expressed differently in two groups including those expressed only in wild type or *iv* mutant mouse. Among them, one protein (Spot 7112 in their study) was expressed only in wild type but not in *iv* mutant mouse at stage 12, thus the investigators have suggested this protein to be a candidate for determination of sidedness of body asymmetry.

In addition, there is a series of studies by Collins using *iv* mutation mouse to study inheritance of "handedness" (1968, 1969, 1975, 1988).

#### *legless* insertional mutation mouse

In 1988, McNeish *et al.* reported a transgenic insertional mutation mouse that exhibited a characteristic pattern of malformations including limb and craniofacial abnormalities. The investigators named this mutation "*legless*", because the hind limbs of homozygous animals were uniformly truncated at the distal end of the femur showing a "legless" appearance. Subsequently, the investigators found approximately a 50% incidence of situs inversus in homozygous animals examined during the perinatal period, GD 18 or birth (McNeish *et al.*, 1990). In 26 homozygous animals examined, 12 (46.2%) showed total situs inversus and 2 (7.8%) showed partial situs inversus, whereas none of 18 wild type animals showed situs inversus. Results of heterozygous animals ( $n=38$ ) were not reported. Furthermore, Schreiner *et al.* (1993) found in homozygous animals that forelimb defects were more severe and predominantly on the left side in those with situs inversus (78%,  $n=23$ ), but they occurred on the right side with situs solitus (75%,  $n=16$ ). Subsequently, sequences flanking the transgene insertions were cloned and mapped to chromosome 12, near the *iv* locus (Singh *et al.*, 1991).

#### *inv* insertional mutation mouse

In 1993, Yokoyama *et al.* created a mutant strain of mouse that resulted in "a reversal of left-right polarity (situs inversus) in 100

TABLE 3

**EXAMPLES OF SYNDROMES ACCOMPANIED BY SITUS INVERSUS**

<i>Autosomal dominant</i>	
	Goldblatt-Viljoen-radial ray hypoplasia
<i>Autosomal recessive</i>	
	Crawford-renal-hepatic-pancreatic dysplasia
	Ellis-van Creveld syndrome
	Fullana-caudal deficiency
	Johanson-Blizzard syndrome
	Kartagener syndrome
	Knobloch-Layer-detached retina; encephalocele
	Marden-Walker syndrome
	Renal-hepatic-pancreatic dysplasia
	Short rib-polydactyly syndrome
<i>X-linked recessive</i>	
	Mathias-X-linked laterality sequence
<i>Uncertain inheritance</i>	
	Agnathia malformation complex
	Axial mesodermal dysplasia
	Catell-Manzke-cleft palate
	DK-phocomelia
	Ivemark syndrome
	Renal dysplasia-total situs inversus-multisystem fibrosis complex
	Stratton-Parker-growth hormone deficiency

Derived from Winter and Baraitser (1991).

percent of the homozygous transgenic mice tested". The investigators were trying to create a stock of pigmented mice by injecting into the one-cell stage albino mouse the gene that carries the genetic code for tyrosinase, the first enzyme in the pathway of melanin synthesis. Incidentally the investigators found that some newborns had inverted internal organs including the stomach, spleen, heart, lung and liver. The mutant animals had severe jaundice and growth retardation, and died within 7 days after birth. In subsequent histological examinations, they found dilated tubules and abnormal glomeruli in the kidney. To further study the situs inversus, they examined a total of 74 fetuses between on GD 16 and 19 obtained by breeding heterozygous animals, and found 17 (23.0%) fetuses with situs inversus. The investigators examined 15 of those with situs inversus, and found one with polysplenia and another with "normal cardiovascular orientation". In addition, the investigators examined another 60 embryos on GD 9, and found all 16 homozygous embryos to have inverted vitelline vessels. They also examined 28 embryos among the 60 for sidedness of cardiac looping, and found 6 out of 8 homozygous embryos had inverted cardiac looping and another two with uncertain sidedness. Twenty-one other wild type or heterozygous embryos that were examined had normal cardiac looping. The investigators eventually proved this mutation to have recessive inheritance, cloned the transgenic integration site on chromosome 4, and named the locus defined by the transgenic insertion "inversion of embryonic turning" (symbol *inv*). Although the data were not presented, the investigators reported also that they did not find defects in the dynein arms of the cilia.

Although many investigators have referred to this mutation as a model of complete inversion of the LR axis, and some have even described it as a "Genetic 'Master switch' for left-right symmetry" (Ewing, 1993), this is obviously a hasty conclusion. As the original

investigators discussed, the mechanism ("pathway") that involves *inv* certainly does not determine sidedness of all asymmetric body structures as some homozygous animals had normal or ambiguous sidedness of some structures (Yokoyama *et al.*, 1993, 1995; Morishima *et al.*, 1995). In addition, although there is no doubt that homozygous animals show an incidence much higher than 50% of inverted vitelline vessels, this author has concerns about their observations on sidedness of the vitelline vessels. On GD 9, the stage when the investigators examined the embryo, there are two types of vessels coming out of the embryo toward the yolk sac (more precisely, visceral yolk sac). The vitelline vessels normally come out from the left side of the middle part of the embryo and attach to the yolk sac just beneath the embryo, thus they are relatively "short". The umbilical vessels normally come out from the lower part of the embryo and attach to the chorioallantoic placenta on the yolk sac that is normally located on the right side of the embryo; they are relatively "long". The vessel illustrated (Fig. 4B in their report) does not correspond well to either of these two vessel types. Furthermore, the shaded area in the same illustration indicated as "yolk cavity" is actually the exocoelom. Thus, it is possible that the unfamiliarity of these investigators with embryology may have led to a higher than actual incidence of inverted vitelline vessels. In addition, as discussed earlier, "tail" sidedness must also be examined to obtain a complete view of sidedness of axial rotation.

In a subsequent study, Overbeek and Yokoyama (1996) reported that mice homozygous for both *inv* and *iv* showed "random specification of left-right polarity", i.e., approximately 50% of animals showed situs inversus. In addition, the investigators reported that when *inv* homozygous of 8-cell stage were aggregated with wild-type, the resultant chimeric animals showed normal sidedness, implying that "*inv/inv* cells can respond appropriately to normal polarity information provided by wild-type cells."

TABLE 4

**FREQUENCY TABLE OF SITUS INVERSUS IN MOUSE EMBRYOS CULTURED FROM DIFFERENT STAGES WITH OR WITHOUT PHENYLEPHRINE**

Start of culture	Control	Phenylephrine (50 $\mu$ M)
stage 10b	52.2% (12/23)	—
stage 11a	30.0% (12/40)	43.8% (14/32)
stage 11b	27.6% (8/29)	57.1% (16/28)*
stage 11c	5.9% (1/17)	25.0% (5/20)
stage 12/s1-2	4.8% (1/21)	7.1% (1/14)

\* $p < 0.05$  vs Control by Chi-squared test. Numbers in the parenthesis indicate "No. of embryos with situs inversus assessed at stage 15/No. of embryos examined." Experiments were performed for the purpose of this manuscript using the same methods as described in Fujinaga *et al.* (1994a). However, the ectoplacental cone was left intact for mouse embryos, because the chorion protruded when the ectoplacental cone was removed, unlike in rat embryos. In addition, phenylephrine was added to the culture medium for the whole culture period in these experiments. Timed-pregnant CD-1 mice were obtained from Charles-River Laboratory Animals (Hollister, California).

*Ft insertional mutation mouse*

In 1994, Van der Hoeven *et al.* reported an insertional mutation mouse characterized by fused toes (Ft) on the forelimbs and thymic hyperplasia in heterozygous animals. The preference in sidedness of these lesions was not reported. Transgene insertion was mapped to the D region of chromosome 8, and the investigators have suggested that programmed cell death is affected by the mutation. Interestingly, homozygous embryos showed head malformations as well as inverted "tail" (lower body). The investigators examined 85 embryos on GD 9, and found 18 homozygous embryos. Eight of these embryos were examined for sidedness of the "tail", and five (62.5%) were found to be inverted. No cardiac abnormality including sidedness was reported. These homozygous embryos were speculated to die on GD 10.

*nt mutation mouse*

In 1996, Melloy *et al.* reported a mutation recovered from a mouse stock of mixed backgrounds. The mutation shows recessive inheritance, and putative homozygous embryos show failure in axial rotation, thus the investigators named it as "no turning (*nt*)". Approximately half of "mutant embryos" show inverted cardiac looping, suggesting sidedness is determined randomly. Regardless of the direction of cardiac looping, development of the heart does not progress further than the looping stage. In addition, the heart of "mutant embryos" shows only periodic contraction suggesting abnormal cardiac function. Because the phenotypes of this mutation are different from those of *iv* or *inv* mutation mice, the investigators have suggested the existence of another gene involved in the LR axis development.

*Connexin 43 gene mutation mouse*

Connexin 43 is the major protein of gap junctions in the mammalian heart. Gap junctions are the transmembrane channels that allow ions and small molecules to pass between adjacent cells. It is known that connexin 43 unites ventricular cardiomyocytes in an electrical syncytium and participates in heart beat synchronization. Also, connexin 43 may be involved in cardiac development. In 1995, Ewart *et al.* produced 6 independently derived lines of transgenic mice that express connexin 43 under regulation of the pan-active cytomegalovirus promoter, and found cranial neural tube defects and failure of the brain ventricles to fully expand, accompanied by exencephaly. Four lines also exhibited "defects in left/right patterning as indicated by inversion of embryonic turning and malrotation of the heart". The same group (Sullivan *et al.*, 1995) also produced another two lines of transgenic mice in which expression of connexin 43 is inhibited. They reported cranial neural tube defects in these mice; defects in laterality were described as under investigation. In contrast, Reaume *et al.* (1995) produced mice with "knock-out" of the connexin 43 gene, but did not find situs inversus or any abnormal "topological arrangement of all the major vessels of the heart". In human, mutations of connexin 43 gene are perhaps associated with asplenia/polysplenia syndromes (Britz-Cunningham *et al.*, 1995; discussed later).

*Mgat-1 knock-out mouse*

In 1994, two groups of investigators generated mutation mice that lacked a functional *Mgat-1* gene on chromosome 11 that encodes N-acetylglucosaminyltransferase I, a key enzyme in biosynthesis of complex N-linked oligosaccharide structures on cell surfaces and extracellular proteins. Metzler *et al.* (1994) reported that homozygous

embryos on GD 9 showed neural tube defects ("highly convoluted neural tube with an irregularly shaped suture line") and impaired vascularization ("accumulations of red blood cells in unexpected compartments" and "vascular hypertrophy") accompanied by severe growth retardation, which led to lethality by GD 10. The investigators also reported that homozygous embryos showed "abnormalities in the completion of turning, often with the tail oriented towards the left side" and that "heart loop structure was found to be inverted in a majority of such embryos". However, the number of embryos examined as well as the incidence of abnormal embryos were not reported. Furthermore, the picture of the "embryo with left sided tail" (Fig. 4A in their manuscript) actually shows normal tail sidedness. Ioffe and Stanley (1994) reported similar phenotypes as those reported by Metzler *et al.* except that they did not report any abnormalities in heart and "tail" sidedness. Therefore, further studies are needed to confirm that the *Mgat-1* knock-out mouse is actually accompanied by situs inversus.

*Dh mutation mouse*

In 1954, Carter reported a mutant strain of mouse that the investigator first thought to be heterozygous for luxate (symbol *lx*). Members of the luxoid strain of mice are characterized by a twisting of fore- or hind-limbs associated with the reduction or loss of certain long-bones and oligo- or polydactyly (Searle, 1964). The mutation reported by Carter (1954) subsequently turned out to be a new luxoid mutation, and was named dominant hemimelia (*Dh*). Fetuses that are heterozygous or homozygous for *Dh* mutation have a high incidence of venous heterotaxia, accompanied by inversion of other major organs (Searle, 1959, 1964; Green, 1967). Homozygous animals usually die within four days of birth. Heterozygous animals may show a lack of spleen, small stomach, hydropic kidneys, and asymmetric limb defects.

*Mouse trisomy 16*

Trisomy 16 in the mouse is regarded as an animal model of Down's syndrome in humans. In 1991, Buselmaier *et al.* examined 109 fetuses and found a 20% incidence of "situs inversus of the aortic arch". Interestingly, asymmetric hypoplasia of the thymus was also frequently observed, although a preference in sidedness was not reported.

**Fish***No tail (ntl) mutation and Floating head (flh) mutation*

In 1996, Danos and Yost reported that *ntl* and *flh* mutants in zebrafish have randomized left-right orientation and defective notochords; 50% (n= 248) and 44% (n= 54) incidence of inverted heart, respectively. Because these genes encode for the transcriptional factors, and are expressed in the organizer and notochord, structures which also regulate the DV and AP axis development, the investigators have suggested that "the notochord may coordinate the development of all three axes in the vertebrate."

**Syndromes associated with situs inversus****Mammal-human***Kartagener syndrome/Immotile-cilia syndrome*

According to the literature (e.g., Miller and Divertie, 1972; Rott, 1979; Afzelius and Mossberg, 1989), Siewer reported the first

definite case of situs inversus associated with bronchiectasis in 1904. In 1933, Kartagener reported a detailed study of similar cases recognizing their common etiology. Since then, similar cases have been reported by many other investigators (reviewed by, e.g., Kartagener and Stucki, 1962), and today, the triad of situs inversus, paranasal sinusitis, and bronchiectasis is called the Kartagener syndrome or triad. In 1976, Afzelius reported 4 males with immotile sperm who were identified by the absence of dynein arms of the cilia. Three of them had chronic sinusitis/bronchitis and total situs inversus; thus, the investigator suggested that the underlying mechanism of these symptoms is immotile cilia. In subsequent studies, Afzelius and colleagues demonstrated that Kartagener syndrome represents a part of more generalized disorder which is characterized by dysmotility or complete immotility of the cilia, and proposed the name "immotile-cilia syndrome" (Eliasson *et al.*, 1977; Afzelius, 1979). It is now also called "primary ciliary dyskinesia". Today, immotile-cilia syndrome is recognized as a genetically heterogeneous disease, because many genes are known to be involved in construction or function of the cilia and an error in any of them may result in ciliary malfunction (Afzelius and Mossberg, 1989). It is suggested that various genes responsible for different subgroups of the immotile-cilia syndrome are located on most chromosomes in humans, and this explains why attempts to localize the gene for Kartagener syndrome or immotile-cilia syndrome have been unsuccessful (Afzelius and Mossberg, 1989).

Afzelius suggested in the original report (1976) that "cilia on the embryonic epithelia have a certain position and a fixed direction (in normal embryos), and that their beating somehow is instrumental in determining the visceral situs". Afzelius also suggested that "in the normal course of development, dextral rotation of the viscera takes place, so that bilateral symmetry gives rise to spiral symmetry", "a malrotation may occur when the ciliary movements causing rotation are lacking", and "chance alone will determine whether the viscera will take place for the normal or the reversed position during embryogenesis, when normal dynein arms are missing". While reviewing the literature, this author noticed that many investigators describe the incidence of situs inversus among the patients with immotile-cilia syndrome to be 50% citing this speculation by Afzelius (1976). However, there is no report to date that directly demonstrates a 50% incidence of situs inversus among the patients with immotile-cilia syndrome. Several studies have estimated that only 1/8 of the progeny from carrier parents of immotile-cilia syndrome show situs inversus (Rott, 1979; Moreno and Murphy, 1981; Sturgess *et al.*, 1986). Based on the assumptions that immotile-cilia syndrome shows autosomal recessive inheritance, these studies support the suggestion made by Afzelius that only half of symptomatic patients will have situs inversus by chance. However, as mentioned earlier, immotile-cilia syndrome is a genetically heterogeneous disease, thus these assumptions may only apply to certain subgroups of this syndrome.

Several animal models of immotile-cilia syndrome have been reported in different species (reviewed by Afzelius, 1995). In dog models, situs inversus was reported in high incidences; reviewed by, e.g., Morrison *et al.* (1987) and Edwards *et al.* (1992). The WIC-Hyd rat, a rat model of immotile-cilia syndrome, has a high incidence of situs inversus (discussed later). However, there are other animal models of immotile-cilia syndrome without situs inversus. In mouse, the *hydrocephalic-polydactyly* (*hpy*) mutation on chromosome 6 has defects in cilia and flagella and is associated

with post-natal hydrocephalus, male sterility and reduced reproductive performance in females. Bryan (1983) examined more than 60 homozygous animals but found none with situs inversus. Although it is unclear whether the investigators specifically examined animals for the its existence, situs inversus has not been reported in several other animal models of immotile-cilia syndrome, e.g., cat (Roperto *et al.*, 1994) and pig (Roperto *et al.*, 1991, 1993). These reports support the above assumption that not all subgroups of immotile-cilia syndrome show situs inversus.

In 1994, Sulik *et al.* reported in the mouse that a "population of motile cilia extending into the yolk sac cavity from the notochordal plate" begins to form a prominent feature in the ventral midline of the embryo at stage 11, the critical stage when  $\alpha 1$  adrenergic stimulation induces situs inversus (discussed later). Thus, this suggests a functional significance for the cilia on the LR axis development.

#### *Asplenia/polysplenia/isomerism/heterotaxia syndrome*

Asplenia (absence of spleen) syndrome, more commonly called the "Ivemark syndrome" (Ivemark, 1955), is characterized by bilateral right sidedness (so-called "dextroisomerism"), e.g., symmetrical liver and three-lobes in each lung (Van Mierop and Wiglesworth, 1962). Polysplenia (multiple spleen) syndrome is characterized by bilateral left-sidedness (so-called "levoisomerism"), e.g., symmetrical liver with absence of the gallbladder, absence of the hepatic segment of the inferior vena cava, and each lung having two-lobes (Moller *et al.*, 1967). Although asplenia and polysplenia had been described as separate abnormalities of different origins, their etiologies are now assumed to be closely related because both are associated with similar abnormalities and are sometimes seen within a family (Polhemus and Schafer, 1952; Zlotogora and Elian, 1981; Arnold *et al.*, 1983; Niikawa *et al.*, 1983; reviewed by, e.g., Aylsworth, 1993). There are also reports of polysplenia with dynein arm defects (Teichberg *et al.*, 1982) and Kartagener syndrome (Schidlow *et al.*, 1982). Some investigators use the term "polyasplenia" which is a composite term of asplenia and polysplenia. Other terms, such as, "(visceroatrial) heterotaxia (or heterotaxy) syndrome", "situs ambiguus" and "laterality sequences", have been used to describe similar cases. Certainly, this is a group of genetically heterogeneous diseases. One should remember, however, that isolated cases of asplenia ("absence of spleen") or polysplenia ("accessory spleen") without isomerism also occur; while the former group is rare, the latter cases are fairly common (Aylsworth, 1993).

Although most reported cases are sporadic, many investigators have suggested autosomal recessive inheritance, e.g., Simpson and Zellweger (1973), Rose *et al.* (1975), Chen and Monteleone (1977), Hurwitz and Caskey (1982), Arnold *et al.* (1983), and McChane *et al.* (1989). In contrast, Alonso *et al.* (1995) reported cases suggesting autosomal dominant inheritance. In 1995, Britz-Cunningham *et al.* suggested that defects of connexin 43 may be the underlying mechanism of asplenia and polysplenia. The investigators examined 30 children with a variety of congenital heart disease and found mutations of connexin 43 gene in 6 children. Of these 6, three had asplenia syndrome, two had polysplenia syndrome, and one had a mixed type. As discussed earlier, there is some supportive evidence in mouse for their assertion. However, there are some arguments that these connexin 43 defects may only represent a subtype of these syndromes (Casey and Ballabio, 1995; Fletcher *et al.*, 1995; Splitt *et al.*, 1995).

**Short rib-polydactyly syndrome**

Short rib-polydactyly syndrome is a group of lethal skeletal dysplasias inherited in an autosomal recessive fashion whose characteristic phenotype is short horizontal ribs and long tubular bone changes. The syndrome has been classified into at least four types, and total situs inversus has been reported in type I (Richardson *et al.*, 1977), type III (Bernstein *et al.*, 1985; de Sierra *et al.*, 1992), and in a yet unidentified type (Tsai *et al.*, 1992). Both type I and type III are thought to be allelic disorders representing variability in expression of the same mutation or different mutations at the same gene locus (Sillence, 1980; Bernstein *et al.*, 1985). In 1994, Urioste *et al.* reported that balanced pericentric inversion of chromosome 4 was found in a newborn with clinical and radiological manifestations of this syndrome.

**Agnathia malformation complex**

Agnathia (virtual absence of the mandible) is a rare developmental abnormality associated with secondary abnormalities in structures related to the mandible. According to Persutte *et al.* (1990), Ahfeld first described agnathia in 1882, although Hennekam (1990) found a picture of such a case in a book by Vrolik published in 1849. Cases with agnathia are basically classified into two groups, those accompanied by holoprosencephaly and those without. (The term holoprosencephaly indicates a spectrum of facial and cerebral malformations of graded severity, ranging from cyclopia, i.e., single median eye in a single median orbit, to an almost normal but short face and brain; O'Rahilly and Müller, 1992). Several investigators have reported cases of agnathia accompanied by situs inversus in both groups suggesting a common etiology (Pauli *et al.*, 1981; Santana *et al.*, 1987; Leech *et al.*, 1988; Krassikoff and Sekhon, 1989; Robinson and Lenke, 1989; Meinecke *et al.*, 1990; Persutte *et al.*, 1990). Thus, the term "agnathia malformation complex" has been suggested (Persutte *et al.*, 1990). Leech *et al.* (1988) proposed the concept that this spectrum of abnormalities represents "a developmental field defect affecting mainly midline embryonic structures" (Opitz and Gilbert, 1982).

Although most reported cases to date are sporadic, an unbalanced translocation resulting in duplication 6p and monosomy 18p was reported in one case (Krassikoff and Sekhon, 1989), thus Meinecke *et al.* (1990) suggested that monosomy 18p is the cause of situs inversus in agnathia malformation complex.

**Renal dysplasia-total situs inversus-multisystem fibrosis complex**

In 1992, Pinar and Rogers reported a case of a stillborn female fetus showing total situs inversus associated with renal dysplasia and multisystem fibrosis. The investigators reviewed several other similar cases (Yoshikawa *et al.*, 1981; Bernstein *et al.*, 1987; Hiraoka *et al.*, 1988), and claimed these cases to be a new syndrome.

**Other syndromes/cases accompanied by situs inversus**

Many more syndromes and cases have been reported to be accompanied with either total or partial situs inversus. It is beyond the scope of this manuscript to review them all, but some are shown in Table 3.

**Mammal-mouse/rat****NOD mouse**

The NOD (nonobese diabetic) mouse is an animal model for insulin-dependent diabetes mellitus (Makino *et al.*, 1980). Morishima *et al.* (1991) found a high incidence of situs inversus ("visceroatrial heterotaxy syndrome") in fetuses obtained from dams after the onset of diabetes mellitus but not from those before the onset of diabetes mellitus. The investigators examined 158 fetuses of the former, and found 57 (36.1%) had situs inversus as well as many other abnormalities including various types of cardiac defects. Of these, 52.6% (30/57) showed dextroisomerism, whereas 1.5% (1/57) showed levoisomerism. Based on these results, the investigators concluded that maternal diabetes mellitus had an influence on development of sidedness of asymmetric body structures and have suggested that the NOD mouse might be a useful model for human asplenia syndrome.

**WIC-Hyd rat**

In 1987, Koto *et al.* (1987a,b) reported a mutant strain of rat with a X-linked recessive gene (assigned symbol *hyd*) in which homozygous animals showed "primary ciliary dyskinesia" accompanied by total situs inversus and hydrocephalus. The investigators examined 234 fetuses obtained by breeding heterozygous females and wild type males. In 135 males, total situs inversus was observed in 59.0% of those with hydrocephalus (36/61) but in none of those without hydrocephalus (n= 74). In contrast, among 99 females, no situs inversus was observed in those with or without hydrocephalus. In subsequent studies ultrastructural and functional defects of the cilia were demonstrated (Torikata *et al.*, 1991; Shimizu and Koto, 1992). Igarashi *et al.* (1994) studied the effects of acetazolamide and dichlorophenamide, carbonic anhydrase inhibitors known to cause right sided forelimb defects in wild type animals, and found that forelimb defects were observed only on the left side in both *in vivo* and *in vitro* experiments in homozygous animals.

**Incidence of situs inversus in embryos grown *in vitro*****Mammal-mouse/rat**

The whole embryo culture system developed by New and colleagues in late 60's and early 70's (reviewed by New, 1978) allows investigators to culture mouse and rat embryos during the so-called early postimplantation period. In 1991, Fujinaga and Baden (1991a) reported that rat embryos cultured from stage 9 showed high frequencies of situs inversus, i.e., 55.3% (21/41) when the ectoplacental cone was left intact and 61.0% (25/41) when the ectoplacental cone was dissected away at the time of explantation. In subsequent studies, the investigators also found that the incidence of situs inversus decreased when the culture was started from a later stage reaching almost no situs inversus by approximately stage 11a/11b (Fujinaga and Baden, 1991c, 1992). These findings were later confirmed by others (Brown *et al.*, 1992; Flynn *et al.*, 1993a), although the incidence of situs inversus varied among investigators and among different experiments even by the same investigator. The cause of situs inversus under these circumstances is as yet unknown. Fujinaga *et al.* (1991) have speculated that abnormal flattening of the embryonic disc that occurs *in vitro* may be the cause, whereas others have speculated that it may be

culture conditions such as serum factors in the culture medium (McCarthy and Brown, 1992; Flynn *et al.*, 1993a,b). Furthermore, Lander and Brown (1994a) have suggested that formation of free radical superoxide anion resulting from the relative hyperoxia that occurs during the explantation procedure and immature mitochondrial development in the embryo may be the cause.

Although this author could not find any published data, situs inversus is also known to occur in mouse embryos cultured with the whole embryo culture system. This author conducted an experiment for this manuscript, and confirmed that mouse embryos develop a higher frequency of situs inversus than rat embryos (Table 4).

### Bird

Salazar del Río (1974) reported various incidences of inverted cardiac looping when chick embryos were cultured by different methods, e.g., 30.0% (30/100) by Paper ring technique, 18.0% (18/100) by Spratt's technique (1947), and 6.0% (3/50) by New's technique (1955). A similar incidence of inverted cardiac looping was also reported by Hoyle *et al.* (1992) using New's technique, i.e., 8.5% vs 0.2% in *in ovo*; but the number of examined embryos was not presented. Cooke (1995) also reported that in chick embryos, cultured from the primitive streak stage (stages 2-4) by New's technique, 8.0% (6/75) showed inverted cardiac looping and 9.3% (7/75) showed unclear looping. Interestingly, all of them showed normal sidedness of Hensen's node when examined during the culture (Cooke, 1995).

### Amphibia

In *Triturus alpestris*, Wehrmaker (1969) found a 2.6% (50/1947) incidence of larvae showing at least one of the following asymmetric structures, i.e., alimentary canal, heart or nuclei habenulae, when collected eggs were reared on the glass blades. In larvae with situs inversus, 42.0% (21/50) showed total situs inversus, and 58.0% (29/50) showed partial situs inversus. In *Xenopus laevis*, Danos and Yost (1995) reported a 2.6% (n= 151) incidence of inverted heart when cultured from the four-cell stage.

## Situs inversus caused by experimental manipulations

### Mammal-human

#### Separate twins

Although not truly an experimental manipulation, the "twins condition" is a type of experimental setting that nature creates. According to Gray *et al.* (1994), "the oldest and most persistent theory of visceral inversion is that twins are often (or always) mirror images of each other and that a single individual with situs inversus represents a survivor whose normal twin has died early *in utero*." Several early studies with small numbers of cases reported a relationship between twins and situs inversus (reviewed by Torgersen, 1950). However, many later studies with larger numbers do not indicate such a relationship (e.g., Torgersen, 1950; Hay and Wehrung, 1970; Schinzel *et al.*, 1979; Layde *et al.*, 1980; Källén, 1986).

#### Conjoined twins

Various types of conjoined twins (double monsters) and parasitic twins (unequal conjoined twins) may occur as an accident in the development of monozygotic twins (reviewed by, e.g., Patten, 1953, and Phelan, 1993). The term "Siamese twins" is used when the partners of monozygotic twins are connected to each other only by a common skin or liver bridge (Sadler, 1990, p. 112). Conjoined twins are commonly classified according to the regions of fusion, e.g., thoracopagus (chest to chest), omphalopagus (abdomen to abdomen), pygopagus (rump to rump) and craniopagus (head to head). The term "dicephalus twins" indicates a single trunk and two heads. Inversion in sidedness of organs was noticed in old literature (discussed by Morrill, 1919). According to Oppenheimer (1974), Förster (1861) first reported that visceral inversion is frequently observed in one member of conjoined twins. Although numbers of similar reports have been made since then, Oppenheimer (1974) described that, "The literature is vast, extremely confusing, and virtually impossible to evaluate, since hard data are rare, especially in the older articles, and the interpretations

TABLE 5

### SOME LANDMARKS IN THE HISTORY OF LR AXIS INVESTIGATION IN VERTEBRATES

Human	Experimental animals
17th C. Reports of situs inversus on autopsy	3rd C. BC Aristotle's description of situs inversus*
1824 Report of situs inversus in living human	
1897 Reports of situs inversus diagnosed by X-ray	
1933 Kartagener syndrome	1900's Spemann's experiments in amphibia
1955 Ivemark syndrome	1948 <i>v</i> mutation mouse
1976 Immotile-cilia syndrome	1958 <i>iv</i> mutation mouse
1987 X-linked laterality sequence	
1993 Mapping of <i>HTX</i> locus in X chromosome	1989 Mapping of <i>iv</i> locus (chromosome 12)
	1993 <i>inv</i> mutation mouse (chromosome 4)
	1995 Asymmetric gene expression in bird
	1996 Asymmetric gene expression in mouse
	1996 <i>nt</i> mutation mouse

\*Cited from Gray *et al.* (1994, p. 1055)

of the data that are available are often discordant." Recent studies have confirmed a high incidence of situs inversus in dicephalus twins, particularly when fusion of the hearts occur at the atrial level (Seo *et al.*, 1985; Cunniff *et al.*, 1988; Gerlis *et al.*, 1993).

### **Mammal-rat**

#### *Removal of the allantois*

During the process of axial rotation, the "tail" (lower body) is connected to the chorion by the allantois which originates from the caudal end of the embryonic disc and grows across the extraembryonic coelomic cavity towards the chorionic plate/ectoplacental cone. Because the allantois establishes anatomical connection with the chorionic plate around the time when axial rotation takes place, Fujinaga and Baden (1993a) hypothesized that the allantois itself and possibly its connection between the chorion and embryo are important in determining "tail" sidedness. To test this hypothesis, the investigators surgically removed either the allantois or chorion from rat embryos at stage 11b/11c and examined development of "tail" sidedness. Removal of the allantois resulted in approximately a 50% (49.1%, 27/55) incidence of inverted "tail", whereas, removal of the chorion resulted in a 20.2% (23/114) incidence of inverted "tail". (In any groups, sidedness of the heart and chorioallantoic placenta were not affected.) In subsequent studies, the investigators found that an allantois transplanted from another embryo could prevent this effect, i.e., from 51.1% (45/88) to 14.3% (3/21) (Fujinaga *et al.*, 1995a). Furthermore, transecting the allantois from the embryo after establishing the connection with the chorionic plate resulted in only a small increased incidence of inverted "tail", e.g., between 8.0% at stage 12/s7-8 and 17.2% at stage 14/s15-16 (n= 25-50). Based on these results, the investigators concluded that the presence of the allantois is necessary for normal sidedness of the "tail" since its removal resulted in approximately a 50% incidence of inverted "tail" probably as a result of random determination, whereas anatomical connection between the "tail" and chorion plays only a partial role, if any. Furthermore, since the results indicated that removal of the allantois affected only one of three asymmetric structures, i.e., the "tail", the investigators hypothesized that the allantois produces a chemical factor which determines normal sidedness of the "tail".

### **Bird**

#### *Removal of cardiogenic area*

Orts-Llorca (1964) removed a wide portion of the left or right ectoderm of the cardiogenic area in chick embryos ranging from stage 4 to stage 8 and examined sidedness of cardiac looping (cited from Lepori, 1967). The investigator found that removal of ectoderm consistently caused cardiac looping to occur toward the opposite side. Nadal-Ginard and García (1972) reported that when the caudal part of the left cardiac primordium was surgically removed from chick embryo at stage 9, 91.4% (32/35) of embryos developed inverted cardiac looping.

#### *Interference of splanchnopleure movement*

Lepori (1967) examined the development of cardiac looping when a small glass rod was placed on the side of the chick embryo at stage 7 to interfere with "lateromedial movements" of the splanchnopleure necessary for closing of the foregut pocket. When

the rod was placed on the left side, 44.0% (33/75) showed inverted looping; another 33.3% (25/75) showed other types of abnormal looping, e.g., double hearts, median heart and S-shaped heart. In contrast, only 7.7% (3/39) showed inverted cardiac looping when the rod was placed on the right side. The investigator also performed a lateral cut in the splanchnopleure or in the splanchnopleure and somatopleure together at stage 8 or 9 to prompt lateromedial movements of the splanchnopleure (n= 10 or 12 in each group). When a cut was made on the right side, 100% showed inverted looping, compared with 0% on the left side. From these experiments, the investigator concluded that the movements of bilateral cardiac primordia play an important role in cardiac looping.

#### *Transplantation experiments*

Salazar del Río (1974) surgically transplanted cardiogenic areas of chick embryo at stage 5, and found the following incidences of inverted heart: embryos with two left cardiogenic areas (0%, 0/39), embryos with two right cardiogenic areas (19.6%, 9/46), embryos in which two areas were exchanged (4.3%, 1/23), embryos without left cardiogenic area (81.3%, 13/16), embryos without right cardiogenic area (0%, 0/10), and embryos without surgery (6%, 3/50). In the 23 embryos in which two areas were exchanged, 43.5% (10/23) showed cardiac bifida, and only 52.2% (12/23) developed normal heart. Based on these results, the investigator concluded, "when there is integration of the areas to the hemiblastoderm in which they are placed, in most of the cases a bulboventricular loop is formed to the right. This fact suggests that in stage 5 of the chick embryo, the areas can be integrated into the contralateral hemiblastoderm and that their intrinsic properties for forming one type of loop are not determined in this stage".

Hoyle *et al.* (1992) performed similar experiments as those of Salazar del Río but at various stages, i.e., stages 4, 5 and 6. In addition, transplantation was performed not only between chicks at the same stage but also at different stages. After a complex and probably retrospective analysis of only those animals which had normal cardiac looping except for its sidedness (40%, 87/218), the investigators concluded that embryos with two right cardiogenic areas showed more inverted cardiac looping than those with two left cardiogenic areas. Among those with two right cardiogenic areas, 46.2% (6/13) showed inverted cardiac looping when transplantation was performed between chicks at stage 6, but only 13% (1/7) showed inverted looping when transplantation was performed between chicks at stage 4 or 5. Although the difference was not statistically significant, the investigators suggested that there is "an intrinsic change" in the precardiac mesoderm between stages 5 and 6 that later influenced the direction of cardiac looping.

Easton *et al.* (1992) performed an experiment in which pieces of mesoderm were dissected either from the anterior or posterior regions of the precardiac areas of the quail embryo at stages 5-7 and grafted into either the anterior or the posterior level of the precardiac region of the chick at the same stage, and the effects on cardiac looping were examined. The investigators produced a total of 178 various chimeras (4 different types), and found a 5-14% incidence of inverted cardiac looping. Although all control embryos (n= 15) showed normal cardiac looping and the results appear to be statistically significant, the investigators did not discuss these results. Rather, the investigators paid attention to the significantly higher incidence of failure in cardiac looping when the posterior region was grafted to the anterior region, and suggested that "a morphogen is secreted by the posterior end of the precardiac mesoderm and this

plays a role in controlling the cessation of looping". However, no description was made of the sidedness of the host or donor regions, which make their results difficult to interpret.

## Amphibia

### Early works

According to Oppenheimer (1974), Spemann reported as early as 1901 and 1903 that "situs inversus viscerum" could occur in one member of a pair of twins produced by constriction of the newt egg. According to Wehrmaker (1969) who reviewed old literature including that written in German and French, various surgical manipulations were reported to cause situs inversus when they were performed before the midneurula stage. Wehrmaker (1969) summarized those experiments that produce situs inversus as follows: 1) "Replantation of a middle piece of the medullary plate with or without the underlying mesoderm and entoderm" (Spemann, 1906, 1907; Pressler, 1911; Meyer, 1913; Woellwarth, 1950). 2) "Median constriction of the egg during cleavage or at the blastula stage" (Spemann and Falkenberg, 1919). 3) "Extirpation, at the gastrula or neurula stage of parts of the embryo, particularly from the left side" (Wilhelmi, 1921; Zwanzig, 1938; Woellwarth, 1950). 4) "Various modifications of these basic experiments" (Dalcq, 1942, 1947; Dalcq and Halter, 1943; Takaya, 1951a,b, 1952, 1953a,b, 1955; Wilens, 1957; Yoshida, 1958; Kraft, 1968a,b). See also Oppenheimer (1974) for a review of these studies.

### Perturbation of extracellular matrix

In 1992, Yost reported experiments in *Xenopus laevis* embryo in which "localized perturbation of a small patch of extracellular matrix by microsurgery was correlated with localized randomization of left-right asymmetries" and "global perturbation of the extracellular matrix by microinjection of arginine-glycine-aspartic acid peptides or heparinase into the blastocoel resulted in global randomization of left-right asymmetries." In the former experiment, the investigator transplanted a small ectoderm patch at stage 10, and examined the effects on sidedness of the heart, upper gut (liver, gall bladder and pancreas) and lower gut (placement and handedness of the intestinal coil) of the host embryo at stage 45; location of the transplanted ectoderm was assessed at stage 28 (the tailbud stage). When a transplanted ectodermal patch did not "appose" the location of the underlying organs of the host embryo, the sidedness of the asymmetric structure in the host embryo was normal ( $n = 12-25$ ). However, when a transplanted ectoderm "directly apposed" an organ the sidedness was randomized ( $n = 12-40$ ). From these results, the investigator concluded that "local perturbation of animal pole ectoderm early in gastrulation induces localized randomization of the left-right asymmetry in underlying organs without altering the orientation of nearby organs." In the latter experiment, the investigator microinjected arginine-glycine-aspartic acid peptides or heparinase, which inhibited deposition of extracellular matrix measured by fibronectin fluorescence and did not affect normal gastrulation and formation of the AP and DV asymmetries, into the blastocoels at stage 10. While none of the embryos in uninjected control group ( $n = 381$ ), buffer injected control group ( $n = 272$ ), or heat-inactivated heparinase injected group ( $n = 87$ ) showed situs inversus, the embryos in arginine-glycine-aspartic acid peptides injected group ( $n = 37$ ) and heparinase injected group ( $n = 20$ ) showed a 46% and a 40% incidence of situs inversus, respectively. In addition, these treatments did not cause

situs inversus when the injection occurred at stage 12 when the fibronectin-rich extracellular matrix had already been deposited on the basal surface of animal pole ectoderm. From these results, the investigator suggested that "global perturbation of fibronectin-rich extracellular matrix deposition leads to global randomization of left-right asymmetry".

### Perturbation of dorsal-anterior development

To examine whether alteration in dorsal-anterior development interferes with normal cardiac looping, Danos and Yost (1995) either subjected the first cell cycle of *Xenopus laevis* to UV irradiation or injected *Xwnt-8* DNA into dorsal blastomeres. Cardiac looping was inverted corresponding to the severity of dorsal-anterior perturbation and to the extent of anterior notochord regression as measured by the DAI scale (Kao and Elinson, 1988). For example, embryos with a DAI score 5 (normal in all external respects) showed a 3% and 1% incidence of cardiac inversion in each experimental group, respectively. Whereas, embryos with a DAI score 4 (reduced forehead and small and sometimes joined eyes) and a DAI score 3 (fused or cycloic eyes, and at least some visible retinal pigment) showed approximately a 22% and a 50% incidence of cardiac inversion in the respective experimental groups. (Embryos with DAI score 2 or lower were excluded from the experiment because of severe heart abnormalities). In addition, the investigators examined the effects of injection of *Xwnt-8* DNA into cardiac progenitor blastomeres, and found it did not affect either sidedness of cardiac looping or dorsal-anterior development and notochord formation. From these experiments, the investigators concluded that disrupting development of dorsal-anterior cells, including cells that give rise to the "Organizer region" and the notochord, results in the randomization of cardiac looping.

## Chemicals/conditions causing situs inversus

### Chemicals/conditions causing situs inversus alone

#### $\alpha 1$ Adrenoceptor agonist

In 1987, Fujinaga *et al.* reported that nitrous oxide, the most commonly used inhalational anesthetic in clinical practice, caused a high incidence of right-sided (inverted) aortic arch in rats in addition to other abnormalities that had been reported previously, e.g., rib and vertebral abnormalities (Fink *et al.*, 1967; Shepard and Fink, 1968). Furthermore, the investigators found that the most susceptible period for causing a right-sided aortic arch is when nitrous oxide exposure is begun in the morning of GD 8 and lasting for 24 h until GD 9 (Fujinaga *et al.*, 1989). In a subsequent study, the investigators demonstrated that nitrous oxide causes situs inversus, and a right-sided aortic arch is one of the phenotypes (Fujinaga *et al.*, 1990a). In addition, situs inversus was produced in an *in vitro* whole embryo culture system, although the incidence of situs inversus varied in each experiment (Fujinaga and Baden, 1989, 1994; Baden and Fujinaga, 1991). Because nitrous oxide is clinically known to have sympathomimetic properties, the investigators hypothesized that adrenoceptor stimulation is the cause for situs inversus, and demonstrated using the whole embryo culture system that phenylephrine, an  $\alpha 1$  adrenoceptor agonist, causes situs inversus without other accompanying abnormalities (Fujinaga and Baden, 1990, 1991b). These findings were later confirmed by other investigators (McCarthy *et al.*, 1990; Brown *et al.*, 1991; Flynn *et al.*, 1993a).

In subsequent studies, the investigators demonstrated that stimulation of  $\alpha 1$  but not of  $\alpha 2$  or  $\beta$  adrenoceptor (other classes of adrenoceptors) causes situs inversus, the maximum incidence of  $\alpha 1$  adrenoceptor stimulation-induced situs inversus is approximately 50%, and the critical period is at stage 11a (Fig. 9) and at least 4 h of treatment is needed (Fujinaga and Baden, 1991b,c; Fujinaga *et al.*, 1992c). The investigators also showed that prazosin, an  $\alpha 1$  adrenoceptor antagonist, alone does not cause situs inversus suggesting that catecholamines (endogenous ligands of adrenoceptors) themselves are not involved in normal development of the LR axis (Fujinaga *et al.*, 1992c), and that  $\alpha 1$  adrenoceptor stimulation results in interference with whatever signal transduction pathway is actually involved in normal development of the LR axis leading to a random determination of the sidedness. Using various compounds known to stimulate or inhibit signal transduction pathways related with  $\alpha 1$  adrenoceptors at different sites, the investigators demonstrated further that the effect is mediated by an  $\alpha 1A$  but not an  $\alpha 1B$  receptor subtype, and by  $Ca^{2+}$ /calmodulin dependent protein kinase II but not protein kinase C (Fujinaga *et al.*, 1994a,b, 1995b). Interestingly, KN-62, a  $Ca^{2+}$ /calmodulin dependent protein kinase II antagonist alone also caused situs inversus at higher concentrations. In the most recent study, the investigators examined the expression of early immediate genes that are known to be induced by adrenoceptor stimulation in other experimental models, and have found that expression of *c-myc* but not *c-fos*, *c-jun*, *jun-b* or *egr-1* is induced in the stage 11a embryo by phenylephrine suggesting that *c-myc* may be involved (Fujinaga *et al.* 1995c).

In mouse,  $\alpha 1$  adrenoceptor stimulation also causes situs inversus, although the significance of the effect is less than in the rat because of the high incidence of situs inversus that occurs under *in vitro* conditions in control embryos as described earlier (Table 4). In addition, mouse embryos show susceptibility to  $\alpha 1$  adrenoceptor stimulation until at stage 11b, whereas rat embryo is much less susceptible at the same stage.

In chick, Bezold and McQuinn (1995) examined the effects of various concentrations of phenylephrine ranging from 50  $\mu$ M to 2 mM (LD50). However, the investigators did not find a higher incidence of situs inversus, but they found malformations in heart, brain, neural tube and somites in a dose-dependent fashion.

#### Cytochalasins

Cytochalasins are known to cause disorganization of actin bundles, thus they have been used to examine the roles of actin bundles during development. In chick, cytochalasins added in the culture medium were originally reported to inhibit cardiac looping leading to a symmetric heart by Manasek (1976; cytochalasin B) and by Ghaskadbi and Mulherkar (1984; cytochalasin H); other abnormalities were reported including neural tube defects, microcephaly and shortening of body axis (Ghaskadbi and Mulherkar, 1984). In 1991, Itasaki *et al.* demonstrated that when a crystal of cytochalasin B was applied to the right side of the caudal part of the heart tube at stage 9/s8 and stage 9/s9, cardiac looping was inverted in 71.4% (15/21) and 66.7% (6/9) of the embryos, respectively; no other abnormalities were reported. In addition, the investigators found that the same treatment at stage 10/s10 did not cause an inversion. Cytochalasin B application on the left side did not show any effects, which led the investigators to conclude that actin bundles on the right side of the caudal part of the heart generate tension and cause normal cardiac looping.

#### Lidocaine

Lidocaine is one of the most commonly used local anesthetics in clinical practice. Using the rat whole embryo culture system, Fujinaga and Baden (1993b) reported that 250  $\mu$ M lidocaine administered from stage 11a caused a 60.0% (15/25) incidence of situs inversus accompanied with no other abnormalities, although higher concentrations of lidocaine caused growth retardation and other non-specific abnormalities as well.

#### Chemicals/conditions causing situs inversus accompanied by other abnormalities

##### Ionizing (X-rays) radiation

Ionizing radiation is well known to cause various abnormalities in human and animals, and these effects have been reviewed elsewhere, e.g., Shepard (1995). It can also cause situs inversus associated with many other abnormalities, including cardiac defects. For example, in the rat, Wilson *et al.* (1953) reported that an exposure greater than 100 rad resulted in a high incidence of situs inversus of both total and partial types, especially when the exposure occurred on GD 8 ("9th day of gestation"): 13% (n= 178) from a 100 rad exposure and 55% (n= 43) from 200 rad (Wilson, 1954). Similar findings have been reported by other investigators, e.g., Kirrmann and Wolff (1964). Ionizing radiation causes similar effects in amphibia but with lower incidences of situs inversus, e.g., 6.8% (4/59) by Woellwarth (1950) and 3.0% (83/2,748) by Wehrmaker (1969).

##### Ultraviolet (UV) radiation

As described earlier, Yost and colleague reported that UV radiation during the first cell cycle of the *Xenopus laevis* egg caused a high incidence of inverted cardiac looping corresponding to the severity of dorsal-anterior perturbation and to the extent of anterior notochord regression as measured by the DAI scale (Yost, 1991; Danos and Yost, 1995).

Clarke *et al.* (1991) also applied UV irradiation to the uncleaved fertilized *Xenopus laevis* egg and produced embryos without notochords. In these embryos, extracellular electrophysiological recordings from motor axons showed that normal alternation of locomotor activity on the left and right side of the embryo was lost. The investigators have suggested that the notochord and/or normal floor plate structure are important for the development of sidedness of spinal cord connections. However, the investigators did not report any effects on cardiac looping or other asymmetric structures.

##### Lithium

Lithium is known to inhibit phosphoinositide metabolism as reviewed by, e.g., Berridge *et al.* (1989) and Birch (1991). Lithium is now recognized as a human teratogen and is suspected to increase the risk of congenital heart defects, especially Ebstein's anomaly; reviewed elsewhere, e.g., Shepard (1995). Nevertheless, to this authors knowledge, situs inversus that is clearly identified as lithium-induced, has not been reported in human. Lithium is also a well known teratogen in experimental mammals and has been studied by investigators in various species (reviewed by, e.g., Klug *et al.*, 1992). Among numerous studies in mammals, there is only one study reporting lithium-induced situs inversus in rat (Brown *et al.*, 1991). Using the rat whole embryo culture system, the investigators treated the embryos with 16 or 32 mM lithium

chloride for 4 hours from different stages, i.e., stages 10b, 11a, 11b, and 11c, and observed the following frequency of situs inversus; 58.3% (7/12), 54.5% (18/33), 32.0% (8/25), and 15.0% (3/20), respectively. Further, they reported that the embryos showed other defects, although the incidences or phenotypes of the defects were not reported. In amphibia, some investigators reported that lithium caused situs inversus in frog, e.g., Takaya (1951b) and in newt, e.g., Woelwarth (1950) and Wehrmaker (1969), while others did not in frog, e.g., Wehrmaker (1969), Kao and Elinson (1988) and Drysdale *et al.* (1994).

#### Retinoic acid

Retinoic acids, e.g., 13-*cis*-retinoic acid, are analogs of vitamin A, and are commonly used for treatment of acne. Exogenously administered retinoic acids are metabolized to active all-*trans*-retinoic acid and 9-*cis* retinoic acid. Although these active retinoic acids are now well known to play important roles in development (reviewed by, e.g., Tabin, 1991), they are also known to cause a wide range of malformations in human and experimental animals and are recognized as human teratogens (Anonymous, 1991). Only a few studies have reported retinoic acid-induced situs inversus which is usually accompanied by many other abnormalities. In hamster, Shenefelt (1972) reported that single administration of retinoic acid at different stages of development caused various types of abnormalities including situs inversus. The highest incidence of situs inversus was 20% when administration occurred on GD 6. In rat, Miura *et al.* (1990) reported that 40, 60, and 80 mg/kg of 13-*cis*-retinoic acid administered intraperitoneally on GD 8 caused "heterotaxy syndrome" in 2.6% (5/192), 7.0% (12/172) and 16.7% (24/144) of fetuses examined on GD 18, respectively. In mouse, Pexieder *et al.* (1995) reported that 20 mg/kg of all-*trans*-retinoic acid administered intraperitoneally on GD 7 caused a 22.5% incidence of transposition of great arteries accompanied with many other cardiac defects. In chick, Chen and Solursh (1992) reported that when anion exchange beads soaked with 50 ng/ml retinoic acid were placed on anterior or left side of the embryonic axis at stage 4, 56.3% (9/16) showed inverted cardiac looping. However, embryos had many other abnormalities including neural tube defects and head abnormalities. Although the number of embryos examined was small ( $n=5-11$ ), the effect was somehow dose-dependent as well as stage-dependent, i.e., treatment at stages 3 and 5 caused only a 25.0% (2/8) and 16.7% (1/6) incidence of inverted cardiac looping, respectively.

To the contrary, many studies do not report situs inversus caused by retinoic acid in chick (e.g., Osmond *et al.*, 1991), fish (e.g., Stainier and Fishman, 1992) and *Xenopus laevis* (e.g., Sater and Jacobson, 1989; Sive *et al.*, 1990; Ruiz i Altaba and Jessell, 1991; Sive and Cheng, 1991; Drysdale *et al.*, 1994). Studies in mammals are reviewed elsewhere, e.g., Shepard, 1995. In addition, studies of the effects of all-*trans*-retinoic acid on *legless* insertional mutation mice, which show predominant left-sided forelimb defects, did not report the effects on its sidedness (Singh *et al.*, 1991; Scott *et al.*, 1994).

#### Trypan blue

Trypan is an azo dye, and its teratogenic effects have been studied by many investigators since the report by Gillman *et al.* in 1948 in rat. Trypan blue is cited by many investigators as causing situs inversus based on the study by Fox and Goss (1957, 1958).

These investigators injected 1% trypan blue subcutaneously in timed-pregnant rats, and produced frequent abnormalities in the heart and great vessels, including transpositions of auriculae, atria, left superior vena cavae and arterial trunks. However, abnormal sidedness of other asymmetric structures was not reported. Christie (1961) confirmed the reports by Fox and Goss, and suggested that cardiac defects are due to inverted cardiac looping. Monie *et al.* (1966) also reported that trypan blue causes a high incidence of inverted great vessels and hearts in rat. However, *in vitro* studies using the rat whole embryo culture system did not report such effects of trypan blue, e.g., Turbow (1965, 1966) and Gulamhusein *et al.* (1982), although many other abnormalities were reported.

#### Colchicine

Colchicine is a plant alkaloid isolated from *Colchicum autumnale* used as an antiinflammatory agent against gouty arthritis (Insel, 1990). Colchicine is known to inhibit DNA polymerase, prevent microtubule assembly and arrest cell division; thus, it has been widely used as an experimental tool. The teratogenic effects of colchicine and its derivatives have been reported in experimental animals by many investigators, e.g., in hamster by Ferm (1963) and in mouse by Sieber *et al.* (1978). Commonly reported defects are microphthalmia and exencephaly, but no situs inversus has been reported. In 1991, Brown *et al.* reported colcemid causes situs inversus in rat whole embryo culture system. The investigators studied the effects of 75 minutes exposure of 0.4  $\mu\text{g/ml}$  colcemid to the embryos at stages 10b, 11a, 11b and 11c, and found 83.3% (5/6), 31.0% (9/29), 43.9% (18/41) and 0% (0/12) incidence of situs inversus, respectively. Although the data were not presented, the investigators also reported that colcemid caused other defects. In chick, Icardo and Ojeda (1984) reported that colchicine prevented the completion of cardiac looping, i.e., "counterclockwise movement of the heart loop", but did not report an inversion of cardiac looping.

#### Atracurium

Atracurium is a skeletal muscle relaxant commonly used for clinical anesthesia. Using rat whole embryo culture system, Fujinaga *et al.* (1992a) reported that embryos cultured from stage 11b in medium containing atracurium showed an approximately 40% incidence of situs inversus at concentrations that also caused growth retardation and other abnormalities, i.e., more than 75  $\mu\text{g/ml}$  ( $n=29-35$ ). In contrast, no other muscle relaxants, i.e., d-tubocurarine, pancuronium and vecuronium, caused situs inversus.

#### Angiotensin II

Angiotensin II is a key hormone in the renin-angiotensin system that is involved in the regulation of blood pressure and electrolyte metabolism. Using rat whole embryo culture system, Price *et al.* (1996a) reported that "10 ng to 10  $\mu\text{g/ml}$ " angiotensin II caused a 25% incidence of situs inversus accompanied by increased growth of the embryo measured by crown-rump length. Angiotensin II receptors and  $\alpha_1$  adrenoceptors (described above) consist of seven transmembrane domains and link to similar intracellular signal transduction pathways. Thus, the underlying mechanism of angiotensin II-induced situs inversus may be similar to that of  $\alpha_1$  adrenoceptor stimulation-induced situs inversus.

**Hyperthermia/heat shock**

Although hyperthermia or heat shock are sometimes cited as causes of situs inversus, numerous studies in various animals do not report such effects *in vivo*, as reviewed by, e.g., Edwards (1986) and *in vitro*, e.g., Cockroft and New (1978) and Mirkes (1985). To this author's knowledge, only Nakashima *et al.* (1991) reported that mouse embryos explanted at stage 12/s3-5 and exposed to 43°C caused a 5.6% (1/18) and 12.5% (2/18) incidence of inverted "tail" (lower body) when the exposure lasted for 10 or 15 min, respectively. Furthermore, these embryos had growth retardation and other abnormalities including neural tube defects.

**Electric current**

According to Wehrmaker (1969), Kraft (1968a) produced situs inversus by exposing the newt, *Triturus alpestris*, at the neurula stage to the electric current of an electrophoresis apparatus.

**Chemicals/conditions inhibiting development of cardiac asymmetry (looping)****Inhibition of proteoglycan synthesis**

Proteoglycans are proteins containing covalently linked glycosaminoglycans and are found in the extracellular matrix of various tissues. They are known to have important functions in determining the structural organization of the matrix. In *Xenopus laevis*, Yost (1990) reported that *p*-nitrophenyl- $\beta$ -D-xylopyranoside ( $\beta$ -xyloside), an inhibitor of proteoglycan synthesis, interfered with cardiac looping in a dose- (n= 24-48) and stage- (n= 30-50) dependent fashion. The maximum incidence of perturbation was 100%, although  $\beta$ -xyloside did not cause inversion of cardiac looping. In addition, Yost found that *p*-nitrophenyl- $\alpha$ -D-xylopyranoside, an analog which does not inhibit proteoglycan synthesis, showed no effect, and concluded that proteoglycan synthesis during stage 12 (late gastrula) and stage 15 (early neurula) is necessary for cardiac looping.

On the other hand, studies in rats (Morriss-Kay *et al.*, 1986; Baldwin and Solursh, 1989) showed that *Streptomyces* hyaluronidase, an enzyme that specifically degrades hyaluronic acid (a glycosaminoglycan), did not inhibit cardiac looping. In the chick, Schoenwolf and Fisher (1983) reported that *Streptomyces* hyaluronidase administered at stages 8 and 9 *in ovo* caused neural tube closure defects, but did not report any effects on cardiac looping.

**Inhibition of basic helix-loop-helix (bHLH) transcription factors expression**

Members of bHLH family are known to regulate various developmental events including myogenesis, neurogenesis and hematopoiesis. Srivastava *et al.* (1995) reported that chick embryos cultured from stage 8 with a combination of antisense oligonucleotides specific to two bHLH genes, *dHAND* and *eHAND*, showed no cardiac looping (14 of 18 embryos, and 11 of 15 embryos with two different sets of antisense oligonucleotides). Neither single antisense oligonucleotides nor random oligonucleotides used as controls inhibited cardiac looping. In addition, neither gene showed asymmetric expression in the embryo. The investigators have suggested that *dHAND* and *eHAND* regulate one or more genes required for cardiac looping, and cardiac looping is arrested in the absence of the encoded proteins.

**Asymmetric abnormalities****Syndromes associate with asymmetric abnormalities in human****Hemihyperplasia (hemihypertrophy)**

Hemihyperplasia is defined as asymmetric overgrowth of one or more external body parts. The overgrowth may involve a part or entire half of the body, and is often associated with various malformations (reviewed by, e.g., Hoyme, 1993). Georgmáneau *et al.* (1983) collected nine cases of hemihypertrophy, and found that the left side was more affected (7:2). Syndromes that are known to be associated with hemihyperplasia are, e.g., Beckwith-Wiedemann syndrome, Klippel-Trenaunay-Weber syndrome, Neurofibromatosis, Langer-Giedion syndrome, Enchondromatosis, Maffucci syndrome, McCune-Albright syndrome, Proteus, and Epidermal nevus (cited from Hoyme, 1993).

**Hemihypoplasia/hemiatrophy**

Hemihypoplasia is defined as body asymmetry accompanying undergrowth or atrophy of one or more external body parts. The growth deficiency or atrophy may involve an entire half of the body, or part. Syndromes associated with hemihypoplasia are, e.g., Russell-Silver syndrome, Poland syndrome, Facio-auriculo-vertebral spectrum, CHILD syndrome, Goltz syndrome, and the Oromandibular-limb hypogenesis spectrum (cited from Hoyme, 1993). Syndromes associated with hemiatrophy are, e.g., Parry-Romberg syndrome, Dyke-Davidoff-Masson syndrome, Sturge-Weber syndrome, and Incontinentia pigmenti (cited from Hoyme, 1993).

**Congenital abnormalities with preference in sidedness**

Slight asymmetry in bilateral structures, e.g., long bones, ribs and vertebrae, are often seen in normal healthy humans (reviewed by Schnall and Smith, 1974). Interestingly, unilateral congenital abnormalities often show a significant preference in sidedness (Schnall and Smith, 1974). Examples that show left preference are postaxial polydactyly (77%, n= 242) and cleft lip (68%, n= 2,403), while those that show right preference are Poland's anomaly (68%, n= 71), inguinal hernia (67%, n= 1,310) and hemifacial microsomia (62%, n= 200).

**Mutations causing asymmetric abnormalities in mouse**

Several mutations in the mouse are known to cause asymmetric limb defects (reviewed by Scott, 1985). They are: Dominant hemimelia (symbol *Dh*) (Searle, 1964; Green, 1967), Luxate (symbol *lx*) (Carter, 1954), Polydactyly (symbol *py*) (Kocher and Kocher-Becker, 1980), Postaxial hemimelia (symbol *px*) (Searle, 1964), Postaxial polydactyly (symbol *Tu*) (Center, 1955), Tail short (symbol *Ts*) (Deol, 1961).

**Chemicals causing asymmetric limb defects****Acetazolamide**

Acetazolamide is a potent inhibitor of carbonic anhydrase, an enzyme that catalyzes the formation of bicarbonate. In 1965, Layton and Hallesy reported that acetazolamide supplied in a diet during pregnancy caused forelimb defects in rat which were predominantly on the right side. Their findings have been confirmed by many investigators (reviewed by, e.g., Hirsch and Scott,

1983), and their underlying mechanisms have been actively investigated since then, e.g., Biddle *et al.* (1991b, 1993).

#### *Other chemicals*

Many other chemicals are also known to cause asymmetric limb defects in different animals (reviewed by Scott, 1985) including cadmium (Barr, 1973; Layton and Layton, 1979) and aspirin (Gulamhusein *et al.*, 1980; Klein *et al.*, 1981).

#### **Chemicals/conditions reported to cause other asymmetric defects**

##### *Chemicals*

In a series of studies, Fantel and colleagues have reported that nitroheterocyclic compounds, e.g., niridazol, cause right sided asymmetric necrosis in rat using whole embryo culture system (Fantel *et al.*, 1986; Greenaway *et al.*, 1986). These findings have been confirmed by other investigators (e.g., Coakley and Brown, 1986; Narburgh and Brown, 1994).

In 1995, Wilby reported that 0.5 ml/kg/day glycerol formal administered subcutaneously from GD 6 through GD 15 in rat caused a high incidence of asymmetric thyroid accompanied by various types of cardiac defects. Although there was no preference for sidedness of asymmetric thyroid, a third of affected fetuses with only one lobe of thyroid were on the right side.

##### *Hypoxia/hyperoxia*

Using the rat whole embryo culture system, Fantel *et al.* reported that hypoxic conditions cause necrotic lesions on the right side of the embryo, whereas hyperoxic conditions cause necrotic lesions on the left side (Fantel *et al.*, 1990, 1991). The investigators have suggested that asymmetric lesions may relate to precocious functional maturation of mitochondria on the left side of the embryo. Under hypoxic conditions mitochondrial immaturity on the right side of embryo may result in inadequate energy generation, either directly or as a result of redox cycling. However, under hyperoxic conditions functionally mature mitochondria on the left side may induce "leakage" of superoxide leading to necrotic lesions on the left side.

#### **Model/hypothesis for LR axis development**

Many investigators have proposed models or hypotheses explaining how sidedness of the LR axis is determined or how situs inversus occurs. However, it is beyond the capability of this author to summarize them in a consistent manner, mainly because the concept of the LR axis itself varies among investigators, since each model or hypothesis was built based on the existing knowledge of the time. Furthermore, inconsistency and vagueness of terminology make a parallel comparison difficult. Thus, these models and hypotheses are cited here using the proponents' own words as much as possible to avoid any possibility of misinterpretation by this author.

##### **Spemann (1906), "Microstructure hypothesis"**

Based on the experiments in amphibia, Spemann (1906) suggested that there is an asymmetric submicroscopic structure that determines sidedness, and that "this structure surrounds the main axis of the egg in a manner that would cause a primary difference

between the right and left sides of the ensuing embryo" (cited from Wehrmaker, 1969). Spemann also suggested that, "The microstructure is probably formed before or during fertilization, for external factors capable of orienting a monostrophic asymmetry obviously do not exist".

##### **Spemann (1906, 1907), "Hypothesis of microstructure co-rotation"**

According to Wehrmaker (1969), "The idea that a displacement, relative to the main axis of the body, in the position of the microstructure may result *in situs inversus*" was proposed by Spemann based on the classical experiments in which the investigator "... excised a mid portion of the medullary plate, namely the region behind the eye anlagen, together with the underlying roof of archenteron, rotated this portion by 180° to exchange right and left, and replanted it." "The rotation thus obtained of the microstructure - a passive 'co-rotation' - seemed to have been the cause of the ensuing situs inversus." "However, when Von Woellwarth (1950) demonstrated that replantation of unrotated midportions is equally effective in provoking reversal", this hypothesis was refuted.

##### **Spemann and Falkenberg (1919), "Hypothesis of microstructure reversal"**

According to Wehrmaker (1969, p. 24), this hypothesis was founded "on the peculiarity that twins produced by median constriction often differ in sidedness: the twin formed by the left half of the egg is nearly always regular, whereas the other is frequently reverse, i.e., the mirror image of the left twin." Wehrmaker explained, "There is an inorganic analogy to this curious reaction: the products of experimental fission of the calcite crystal are also mirror images of each other. Therefore, the behavior of this crystal might serve as a model to explain the occurrence of situs inversus, at least in twins: since it is beyond doubt that the submicroscopic structure of the reversed secondary crystal is reversed, one might assume that the asymmetric carrier structure of the reversed *Triturus* twin becomes reversed when the egg is 'split'."

##### **Wilhelmi (1921), "Maternal anisotropic distribution of protein or mRNA"**

Levin *et al.* (1995) discussed that Wilhelmi (1921) has proposed the ideal that "maternal anisotropic distribution of protein or mRNA" is involved in generation of LR asymmetry.

##### **Cockayne (1938), First demonstration of genetic inheritance of situs inversus**

Many investigators have given credit to Cockayne (1938) as the first investigator to demonstrate that "total situs inversus" in human is determined by a recessive autosomal gene.

##### **Harrison (1945), "Polarized protein molecules"**

According to Oppenheimer (1974), Harrison has suggested that "... the asymmetry of the limb rudiment ... may be brought about by the change in constitution of the structural elements in a manner similar to the building up of the asymmetric molecules in carbon compounds" (1921, p. 113), and "changes in the ultramicroscopic elements" (1936, p. 246) as the possible basis for changes in asymmetrical relationships, and named this element "polarized protein molecules" (1945).

**Wehrmaker (1969), "Defect hypotheses" or "Hypotheses of left-hand defects"**

Wehrmaker (1969) summarized the existing hypotheses that consider "a defect on the left side of the embryo essential for reversal", the so called "Defect hypotheses" or "Hypotheses of left-hand defects". These hypotheses imply that "... normally, the left side is preponderant; but when material is removed from or any other injury inflicted on this side, the right side may become dominant, the shift resulting *in situs inversus*. Conversely, damage to the right side of the embryo would only enhance the original advantage of the left and therefore not lead to reversal."

Wehrmaker (1969) also suggested that the mechanism of action of the defect has been interpreted differently, and proposed the following six possibilities each based on a different concept of normal development of the LR axis (cited without amendment). 1) "Hypothesis of reversal by misbending" based on the "Bending hypothesis" by Spemann and Falkenberg (1919). If the left side becomes too weak, the developing gut will bend in the reverse direction. 2) "Hypothesis of removal of left-hand factor" based on the "Left-factor hypothesis" by Wilhelmi (1921). The defect deprives the left side of a peculiar factor necessary for the development of the regular situs. 3) "Hypothesis of elimination of left antagonist" based on "Hypothesis of dominance of left antagonist" by Ludwig (1932) and Zwanzig (1938). From two antagonistic factors, one for regular growth concentrated on the left and one for reversed accumulated on the right, the defect "hits" only the left. Consequently, the right antagonist becomes dominant. 4) "Hypothesis of reversed asymmetric induction" based on "Induction hypothesis (Hypothesis of asymmetric induction)" by Woellwarth (1950). The defect interferes with the normal asymmetry of an induction process taking place in the region of the organizer. If the normal preponderance of induction to the left is lost, situs inversus will occur. 5) "Hypothesis of suppression of left rudiment" based on "Hypothesis of dominance of left rudiment" by Zwirner and Kuhlo (1964). The defect weakens the left cardiac rudiment to an extent that the right rudiment becomes capable of determining the situs of the definitive heart, which is the product of fusion of the two. Since the right anlage, in contrast to the left, is disposed to develop into the reversed form, the definitive heart will become reversed if the right side is dominant. 6) "Hypothesis of reversal by a determinative stimulus" based on "Hypothesis of physiological (not merely mechanical) determination of sidedness" by Kraft (1968b). The defect provides a stimulus that interferes at the "physiological" level with the events determining sidedness in normal development, no matter whether they consist in asymmetric induction or asymmetric self-differentiation. Reversal is thus not a mere reaction to a change in the relative mechanical strength of the left and right sides of the embryo. This hypothesis includes, as one of several conceivable possibilities, that of reversal by reversed asymmetric induction.

**Wehrmaker (1969), "Regulation hypothesis" or "Hypothesis of reversal by regulatory movements"**

Based on experiments in amphibia, Wehrmaker (1969) suggested that "asymmetry in *Triturus* is oriented in two steps: during oogenesis, sidedness is only determined preliminarily, thus remaining reversible, and the definitive fixation (final determination) takes place during later embryogenesis." The investigator proposed three possible relations between microstructure and situs inversus: 1) The molecules of the asymmetric carrier structure might be reversible

("crystallographic" reversal). 2) The arrangement of the carrier structure relative to the main axis of the body might be alterable ("rotational" or "rearrangement" reversal). 3) The asymmetric developmental process initiated or controlled by the carrier structure might be susceptible to interference resulting in non-regular situs (reversal due to disturbance or "interferential" reversal).

The investigator summarized, "Presumably, a microstructure that is asymmetric in itself and capable of determining the orientation of the asymmetric organs is present in the egg and transmitted to all rudiments so that each of them possesses an intrinsic orienting tendency. Reversal is probably an incidental effect of the disturbance of normal development. Surgical methods, such as replantation, constriction, or extirpation, and non-surgical methods, such as X-irradiation or lithium treatment, provoke regulatory movements, in the course of which the anlagen are rearranged. The rudiments may become rotated with respect to their original orientations, and their intrinsic microstructures may therefore assume abnormal relative positions to the geometrical axes of the embryo. This change in the relative positions is the direct cause of situs inversus, the degree of reversal depending on the angle of rotation. In the small egg, the three organs are often affected similarly, though the looseness of the connections between the rearranging cell complexes permits local differences in the amount of rotation and hence in the degree of reversal. This interpretation is termed the *regulation hypothesis* of situs inversus."

**Afzelius (1976)**

Based on the studies on immotile-cilia syndrome, Afzelius (1976) has suggested that "... cilia on the embryonic epithelia have a certain position and a fixed direction (in normal embryos), and that their beating somehow is instrumental in determining the visceral situs", "in the normal course of development, dextral rotation of the viscera takes place, so that bilateral symmetry gives rise to spiral symmetry", "a malrotation may occur when the ciliary movements causing rotation are lacking", and "chance alone will determine whether the viscera will take place for the normal or the reversed position during embryogenesis, when normal dynein arms are missing".

**Layton (1976) and Kurnit et al. (1987), "Stochastic single-gene model"**

Based on the studies on *iv* mutation mouse that approximately 50% of homozygous animals show total situs inversus, Layton (1976) has suggested that "the normal allele at the *iv* locus specifies situs solitus, i.e., normal laterality of visceral situs. Absence of this control allows situs to be determined in a totally random fashion." In 1987, Kurnit *et al.* (1987) have provided mathematical support for this theory.

**Corballis and Morgan (1978)**

Corballis and Morgan (1978) suggested that "... many asymmetries appear to be under the influence of a left-right maturational gradient, which often seems to favor earlier or more rapid development on the left than on the right. If the leading side is damaged or restricted, this gradient may be reversed so that growth occurs with the opposite polarity." "However we must also suppose that the leading side normally exerts an inhibitory influence on the lagging side ..." An extensive discussion on this hypothesis by many investigators is presented in the manuscript by Morgan and Corballis (1978).

**Almirantis and Nicolis (1987)**

The basis of these investigators' hypothesis is that "chirality at the molecular level is the primordial source for macroscopic asymmetry" "... two-step mechanism for the explanation of the systematic asymmetric visceral arrangement in vertebrates is proposed. A two-variable reaction-diffusion system displaying a symmetry-breaking bifurcation is considered, and it is demonstrated that a slight asymmetry of the boundary conditions can give rise to a marked asymmetry in the resulting dissipative structure in both one- and three-dimensional systems. A criterion is formulated allowing classification of reaction-diffusion systems operating in a three-dimensional space with regard to their ability to incorporate slight asymmetries at the boundaries in the form of a chiral dissipative structure."

The difference between their model and that of Brown and Wolpert (1990) is explained in a subsequent manuscript (Almirantis, 1995) as, "... Brown and Wolpert consider the combination of the F molecules alignment with a supposed polarization of cells with respect to the plane of bilateral symmetry as the final carrier of left-right asymmetry, before the involvement of the reaction-diffusion system; whereas in our model this role is played by the primary shallow left-right gradient triggered by the alignment of the microstructures. ... The experimental finding in higher organisms of a polarization of cells with respect to the midline would be of interest, as it is a necessary (but not sufficient) condition of the Brown and Wolpert model."

**Brown and Wolpert (1990)**

Brown and Wolpert (1990) have proposed a model, "... comprising three components. (i) A process termed conversion, in which a molecular handedness is converted into handedness at the cellular level. A specific model for this process is put forward, based on cell polarity and transport of cellular constituents by a handed molecule. (ii) A mechanism for random generation of asymmetry, which could involve a reaction-diffusion process, so that the concentration of a molecule is higher on one side than the other. The handedness generated by conversion could consistently bias this mechanism to one side. (iii) A tissue-specific interpretation process which responds to the difference between the two sides, and results in the development of different structures on the left and right."

**Jefferies (1991), "Dexiothetism"**

Jefferies (1991) has suggested that, "a common ancestor of the chordates and echinoderms took to crawling right-side downwards on the mud of the sea-floor, pulling itself along by a posterior tail, and the asymmetries of these creatures can be simply explained by an original loss of structures on the right-hand side, arising from a process called 'dexiothetism' whereby an originally bilaterally symmetrical ancestor lay down on its right-handed side."

**Horwich and Brueckner (1993)**

Horwich and Brueckner (1993) have suggested that "... genes whose defects lead to situs inversus, as distinct from heterotaxia, act early, at the one or few-cell stage of development, to set up left/right positional information. By contrast, genes whose defects lead to heterotaxia are proposed to act later in development, during translation of initial positional information, provided by the early genes, into cues for later asymmetric movement of the visceral

organs." (The investigators use the term "heterotaxia" as partial situs inversus distinguished from total situs inversus.) The investigators also have speculated that "... it is dynein in the cytoplasm, associated with the mitotic spindle apparatus of the early embryo, that plays a very early role in left-right determination, acting to produce directional information."

**Klar (1994), Asymmetric imprinting and segregation of DNA**

Klar (1994) has proposed a model that "DNA replication produces different chromatids, and that these specific chromatids of both homologs are non-randomly segregated to daughter cells to specify the left-right axis of the embryo". The investigator has described that, "In its simplest form, in a specific cell division during early development of the embryo, two developmentally different sister cells are produced. Their developmental difference confers left-right asymmetry to the animal later in development. The novel feature of the model is that the developmental difference of the two sister cells is dictated by the non-random inheritance of Watson and Crick strands of a specific parental chromosome such that one daughter cell inherits a chromosome of each homolog with a developmentally important gene in an active state, while the other cell inherits an inactive, or weakly expressed, gene." This model subsequently became an issue of debate among several investigators (Brown and Lander, 1995; Beddington and Solter, 1995; Klar, 1995).

**Levin et al. (1995), "A model of molecular pathway of the LR axis determination"**

As mentioned earlier, Levin et al. (1995) have proposed a model of molecular pathway of the LR axis determination based on their studies in chick embryo (Fig. 7).

**Additional notes**

Many more human syndromes and cases involving situs inversus have been reported that could not be covered in this review. Undoubtedly, the author has overlooked many more chemicals or mutations that cause or are associated with situs inversus in experimental animals. In addition, it is possible that some investigators overlook the effects of certain chemicals. For example, the timing of chemical administration is important for the causation of situs inversus. Timing of embryonic/fetal examination is equally important, because embryos/fetuses with situs inversus often have other abnormalities leading to early death. Furthermore, unless examined carefully, situs inversus is often overlooked. These comments also apply to studies of mutations in animals.

While reviewing the literature, this author has found many interesting discussions that could not be incorporated in the text but might be useful for investigators working on LR axis development, e.g., Morrill (1919), Sumner and Huestis (1921), Boycott et al. (1930), Cockayne (1938), Torgersen (1949), Wehrmaker (1969), Oppenheimer (1974), Morgan (1977), Corballis and Morgan (1978), Morgan and Corballis (1978), Freeman and Lundelius (1982), and series of discussions in the book by Bock and Marsh (1991). In addition, this author was unable to review numerous studies reported in languages other than English but cited in other investigators' manuscripts, e.g., Wehrmaker (1969) and Oppenheimer (1974).

TABLE 6

**MOUSE AND HUMAN CHROMOSOMES AND THEIR MUTATIONS KNOWN OR SUSPECTED TO AFFECT THE LR AXIS DEVELOPMENT**

Mouse	Human	
<i>lefty</i>	<b>1</b>	
	4	short rib-polydactyly syndrome
	7	balanced translocation
<i>inv</i>	<b>4</b>	(9)
<i>Ft</i>	8	
<i>Mgat-1</i>	11	(5)
	10	monosomy 10q
	12	translocation 12/13
	13	monosomy 13q, trisomy 13
<i>iv, legless</i>	<b>12</b>	14 14q
	18	monosomy 18p, Agnathia malf. complex
trisomy 16	16	
	22	monosomy 22
	<b>X</b>	<i>HTX</i> , X-linked laterality sequence
nt	?	
Dh	?	

Confirmed chromosomes are bold-faced. Human chromosomes known to be homologous to mouse chromosomes are shown in parenthesis.

This author also found the book "The New Ambidextrous Universe. Revised Edition." by Gardner (1990) very useful. Also, Oppenheimer (1974) suggested the following books: "Symmetry and Asymmetry" by Bacher (1973), "Symmetry" by Weyl (1952), and "Das Rechts-Links-Problem im Tierreich und beim Menschen" by Ludwig (1932, reprint in 1970; German). In addition, the following references were not incorporated in this text but might be interesting to read as they review or comment on the LR axis or related issues: Galloway (1990), Brown (1991), Wilkins (1991), Gurdon (1992), Morgan (1992), Wilson (1992), Brown and Lander (1993), Hori (1993), Yokoyama (1993), Fishman and Lavine (1994), Jefferies and Brown (1995), Norberg (1995), Roush (1995), Speakman and Thomson (1995), Yost (1995a,b), Beddington (1996) and Dickman (1996).

Finally, this author summarized some landmarks in the history of LR axis investigation in vertebrates (Table 5), and mouse and human chromosomes and their mutations currently known or suspected to affect the LR axis development (Table 6).

## Summary

Development of the LR axis is not only of fundamental importance in developmental biology but also of clinical importance since failure to establish proper sidedness of organs such as the heart can lead to considerable pathology. The evidence to date indicates that multiple mechanisms are involved in controlling the normal development of the LR axis, including several genes. Various experimental manipulations, genetic mutations, use of chemicals or altered environmental conditions can interfere with these control mechanisms leading to partial or total abnormal sidedness of various asymmetric body structures. Although various models and hypotheses have been proposed to date, much more work is needed to fully understand the normal and the pathological development of the LR axis.

**KEY WORDS:** *left-right body axis, asymmetry, situs inversus, vertebrate*

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