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Minireview

Asymmetry of cilia and of mice and men

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ABSTRACT Evidence is given for the opinion that cilia in the early embryo, by their work, determine the laterality of the body; without ciliary work body laterality would be randomized. More exactly, monocilia in the primitive node are responsible for this determination. They have been described as being of the 9+0 type, but with dynein arms and with a gyrating movement. The orientation of the monocilia on the epithelium is of no importance but the direction of their gyration is, as may also be the shape of the node. The chirality of the cilia is thus reflected directly in the asymmetry of the body. The dynein arms go clockwise as seen from the base to tip and the ciliary rotation is in the same direction. The resulting water flow is towards the left and so is the movement of the forming heart. In most subgroups of the immotile-cilia syndrome this mechanism does not work and equally many individuals will be born with situs inversus as with situs solitus. An exception is the immotile-cilia subgroup, named 'microtubule transposition', which is characterized by all cilia having a 9+0 structure throughout most of their length.

KEY WORDS: *situs inversus, immotile-cilia syndrome, primitive node, body asymmetry, nodal cilia*

The problem: what is the left-right axis and how is it determined?

Two of the body axes are determined at a very early embryological stage, in some vertebrates even in the zygote: the anterio-posterior axis and the dorsal-ventral one. In mammals, these axes can be discerned in the embryonic disc of the blastocyst. From a geometric point of view, the third axis, left-right, is thereby specified, but the two body sides will differ anatomically. The left body half in mammals normally will contain a.o. most of the heart, stomach, spleen, whereas the right half will contain caecum and most of the liver. The question is: which is the mechanism that specifies the correct body asymmetry?

This problem has been treated by several authors during the last decades (see Brown and Wolpert, 1990; Horwich and Brueckner, 1993; Lander *et al.*, 1998; Wright, 1998 for reviews). The first asymmetry to be seen in an embryo is a shift of the early heart anlage to the left side. Before that stage various factors have been recognized that are expressed in one body side only (Harvey, 1998). These factors are demonstrable by histochemical methods.

The mechanisms that specify the proper body asymmetry are preferably studied in situations where the mechanism is defective, i.e. where there is a random determination of body asymmetry. In such cases there will be equally many individuals with situs solitus (= the normal position of inner organs) and situs inversus totalis (= body organs are mirrored). The immotile-cilia syndrome in man is a striking example hereof.

The immotile-cilia syndrome

The first cases of immotile-cilia syndrome to be identified were recognized by their spermatozoa being immotile and by an ultrastructural feature, namely the lack of dynein arms in the sperm tail. (Dynein is the force-generating component of the ciliary motor). Soon the cilia were also found to be immotile and deficient in dynein arms (Afzelius, 1976). As it could be expected, the patients have various problems from the organs with ciliated epithelia such as those of respiratory tracts, frontal sinuses, and middle ear.

A further feature was completely unexpected: three of the four first cases to be identified had situs inversus; the fourth case was the brother to one of the other three cases. What could be the cause-effect relationship? There are, à priori, four possibilities:

(1) The presence in a patient of both features is merely coincidental; there is no cause-effect relationship.
(2) Situs inversus will cause the cilia and the spermatozoa to be defective.
(3) Ciliary immotility in the early embryo entails a random determination of body asymmetry.
(4) There is a factor which will influence both ciliary structure/function and laterality of body asymmetry.

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The first alternative could immediately be ruled out. A chance association clearly is highly improbable. Situs inversus occurs with a prevalence of about one in 10,000 persons in the normal population of Scandinavia (Torgersen, 1947). Now, after over two decades, several hundreds of patients with the immotile-cilia syndrome have been identified out of which about 50% have situs inversus. The syndrome has been found to be a heterogeneous one with at least a dozen subgroups. In nearly all subgroups about 50% situs inversus is found, whereas in a few subgroups there are no cases with situs inversus. The probable reason for the difference in this respect will be treated below.

An early hypothesis of the frequent co-existence of ciliary immotility and situs inversus was proposed, namely that cilia on the epithelia of the normal embryos, "have a certain position and a fixed beat direction and that their beating somehow is instrumental in determining the visceral situs" (Afzelius, 1976). When the cilia are immotile, on the other hand, a random determination of body asymmetry was assumed, resulting in equally many cases of situs inversus and situs solitus.

A possibility to test this hypothesis presented itself when W.M. Layton (1976) described a mouse mutant, named iv/iv, in which 50% of the homozygotes have situs inversus; they shared with the immotile-cilia syndrome the trait 'random determination of situs laterality.' (The designation iv stands for inversed viscera). The fact that these iv/iv mice have healthy respiratory tracts and thus presumably have functioning cilia was not considered incompatible with the idea that their embryonic cilia could be defective or missing. It is conceivable that embryonic cilia differ from those in the ciliated epithelia, just as there are men who have motile spermatozoa but immotile cilia and other characteristics of the immotile-cilia syndrome (Jonsson *et al.*, 1982) or have motile cilia but immotile spermatozoa (Walt *et al.*, 1983). Not all ciliary axonemes have an identical composition.

As the first visible asymmetry in the murine wildtype embryo is a leftward turn of the heart we examined by scanning and transmission electron microscopy the forming heart of embryos in the 8th embryonic day, searching for cilia. Short cilia were indeed found, and were so called monocilia, e.g. one cilium per cell but they were devoid of dynein arms and hence unlikely to be able of any motility. No difference could be detected between these embryonic cilia from homozygous iv/iv mice and those from normal mice (Layton and Afzelius, unpublished data).

More recently, a number of histochemically discernible factors have been found, which are expressed asymmetrically: their genes have been named *nodal, lefty-2, Pitx2*. They are demonstrable in the left body side and at a stage before the heart shifts leftwards (Harvey, 1998). This seemed to give a blow to the hypothesis of embryonic cilia being responsible for determining the first visible asymmetry, that of the forming heart.

There is another weakness of the original hypothesis. If it is true that the embryonic cilia have a certain position and a fixed beat direction, there must be some factor that informs the cilia about which is the left body half and which the right one.

Some other hypotheses

Several other hypothesis have since been suggested. In an anonymous editorial note in Scientific American (1976), it was proposed that the gene for situs determination could be located close to, but separate from, the gene or genes responsible for the dynein arms of the cilia. The two defects were presumed to 'be closely linked genetically.' Supp *et al.* (1998) are of the same opinion; they thus conceive two adjacent genes, one responsible for ciliary movements, the other for left-right axis formation. This hypothesis appears unlikely in view of the pronounced heterogeneity of the immotile-cilia syndrome as seen by electron microscopy. More than 200 genes are coding for the various ciliary proteins (Chapelin *et al.*, 1997), and it seems likely that mutations in the different genes give rise to different subgroups of the disease. There are also proteins in the cytoplasm that are responsible for assembly and transport of dynein arms or spokes to the cilium (Porter *et al.*, 1999; Rosenbaum *et al.*, 1999). Likewise, the proper human situs is assumed to be determined by several genes on different chromosomes (Carmi *et al.*, 1992; Koiffmann *et al.*, 1993).

Three further hypotheses have later been proposed and are discussed in another paper (Afzelius, 1995). A common feature of these hypotheses is that the primary defect is assumed to reside in the cytoskeleton, thus in the interior of the cell and that both ciliary motility and situs laterality are influenced by this cytoplasmic defect. They thus exemplify the fourth possibility listed above. Later hypotheses are among similar lines. Recently Supp *et al.* (1997) have isolated a gene that is a component of a dynein motor complex and participates in the determination of the body laterality. The suggested mechanism to provide an initial left-right bias in the embryo is an "asymmetric movement along a microtubule scaffolding which is oriented relative to the anterior-posterior and dorsal-ventral axes" (Supp *et al.*, 1998).

Motility of nodal cilia

A major step in the exploration of the cause of left-right asymmetry came with the work by Nonaka *et al.* (1998), who examined the early embryo of a mouse mutant that is characterized by a defect in a kinesin-like motor-protein named murine KIF3B. The homozygotes in this strain will not develop beyond mid-gestation but it was noted that, as in the iv/iv-homozygotes, about 50% of the embryos have situs inversus. The early homozygous embryos differ from heterozygotes or controls in that certain monocilia are missing, namely monocilia that in the normal embryo would be located in the primitive node, thus at the anterior end of what will develop into the primitive streak.

Nonaka *et al.* (1998) further found that nodal cilia in normal mice are motile and rotate counter-clockwise, when observed from tip to base. This creates a leftward transport of substance that comes with the extra-embryonal fluid. This transport is the earliest asymmetric trait that has been found; it occurs before the factors nodal, lefty-2, and Pitx2 are recognized.

The nodal cilia lack the two central microtubules and are hence of the 9+0 type, which is of interest in that the cilia then will have a three-dimensional movement. A gyrating movement of the nodal cilia was actually also recorded. Whereas normal 9+2 cilia have a rather flat downstroke (effective stroke) and a recovery stroke in which the cilium returns, bending to the left (Parducz, 1967), the 9+0 cilia have a rotating movement, which however like that of the 9+2 cilia is counter-clockwise, as seen from above. The trajectory for a motile 9+0 cilium thus is a cone, that for a 9+2 cilium a half-cone. For the difference in movement between 9+2 and 9+0 cilia

(or flagella), see Ishijima *et al.* (1988). (Different kinds of cilia may beat with the recovery stroke bending to the left or to the right; the latter situation occurs in some tracheal cilia described by Sanderson and Sleigh, 1981).

The gyrating movement of cilia has no directional component but cilia have a counter-clockwise gyration; the orientation of monocilia relative to right or left and anterior or posterior is hence immaterial. The shape of the primitive node is likely to be of importance. In the early mouse embryo it is somewhat triangular with an acute angle anteriorly (Nonaka *et al.*, 1998; Vogan and Tabin, 1999). As a consequence hereof the flow at the tip of the triangle is more cramped and impeded than is the flow at the base of the triangle; the leftward flow at the posterior side of each monocilium will dominate over the rightward flow at the anterior side of a monocilium. A leftward flow was demonstrated by vital microscopy.

It hence appears that 9+0 monocilia by their gyration may be responsible for the creation of the first asymmetric feature of the body. The dynein arms have a counter-clockwise orientation when seen from tip to base of the cilia; the direction of the gyration of the monocilia is the same. As a consequence, the extraembryonal fluid will be transported leftwards and so will the forming heart. It is concluded that the asymmetry of the cilia is responsible for the asymmetry of the mammalian body. When cilia and their dynein arms are missing (or else non-functional) body asymmetry apparently is randomly determined.

Cases of random determination of left-right asymmetry

Three recessive murine mutations are presently known in which body asymmetry is randomly determined:

(a) The mice studied by Nonaka *et al.* (1998) in which the defective gene is that coding for a kinesin that is assumed to participate in the assembly of cilia. The human equivalent would be the subgroup of immotile-cilia syndrome in which there are no cilia, the ciliary aplasia described by De Santi *et al.* (1988).
(b) Another genetic mouse mutant with random left-right asymmetry determination and with absent cilia (Chen *et al.*, 1998).
(c) The mice mutant studied by Layton (1976). The *iv* gene is located in mouse chromosome 12 with a distance of 5 Mbases from Igh-C (Hanzlik *et al.*, 1990). This would be equivalent to human chromosome 14q3 (Brueckner *et al.*, 1989).

Two of these three mouse mutants with random determination of body asymmetry have defective or rather absent cilia; it is an open question whether nodal cilia are normal, defective or absent in iv/iv mice.

There are also other models for the human immotile-cilia syndrome. Male rats of the mutant named WIC-Hyd have a random determination of body asymmetry and have cilia that lack dynein arms and are immotile (Torikata *et al.*, 1991). It has been described as the first useful model for human immotile-cilia syndrome.

The human immotile-cilia syndrome in itself is an assembly of different subgroups: disorders affecting outer dynein arms, inner dynein arms, outer and inner dynein arms, spokes, ciliary assembly, ciliary orientation, etc. It appears likely that the cause of the disease in each case is a defective gene coding for a ciliary component. Further, it appears likely that the randomization of left-right asymmetry is a consequence the cilia being defective or lacking. The locus of the defective gene, in each case, is unknown. Pan *et al.* (1998) have studied a child with paternal isodisomi of chromosome 7 associated with immotile cilia and situs inversus. Richer *et al.* (1977) have described a man with trisomi 12 mosaicism with immotile cilia and situs inversus. For other subgroups of the immotile-cilia syndrome we have no clue which chromosome may contain the defective gene. Among the many possible candidate genes of the syndrome are those of the several (probably 15) dynein heavy chains that have been mapped to various chromosomes by Chapelin *et al.* (1997).

On the heterogeneity of the immotile-cilia syndrome

As mentioned above there are many subgroups of the immotile-cilia syndrome; their number can be estimated to be between 12 and 20. It is of interest that half of the affected persons in nearly each subgroup have situs inversus – a random determination of body asymmetry. One or two groups are exceptions to this rule:

One subgroup is the so called 'microtubular transposition effect' (Sturgess *et al.*, 1980, 1986; Escalier *et al.*, 1982; Levison *et al.*, 1983; Jorissen *et al.*, 1997). Cilia from persons in this subgroup have dynein arms on their nine microtubular doublets and are hence capable of some motility. However, the two central microtubules are almost entirely lacking, which gives them a 9+0 structure. As a result, the cilia are unable to beat with a normal, effective stroke; presumably they beat like monocilia. Symptoms from the airways, middle ear, frontal sinuses and the other ciliated organs are the same as in the other subgroups of immotile-cilia syndrome. However, a lack of central microtubules will obviously not influence the movements of the nodal cilia, which in normal mouse embryos, presumably also in human embryos, are of the 9+0 type.

A second subgroup, if such it is, is characterized by overlong cilia, with a length that is about twice the normal one (Afzelius *et al.*, 1985; Niggeman *et al.*, 1991). The symptoms from the airways are the same as in other subgroups, except that no patients with situs inversus have been seen in this numerically still rather small subgroup. Whether their monocilia also are long and their gyrating movement thereby influenced is not known.

Recent reviews of the determination of the left-right axis emphasize the existence of various factors (nodal, lefty, etc.) that have an asymmetric distribution in the early embryo (Brown and Wolpert, 1990; Horwich and Brueckner, 1993; Harvey, 1998). This asymmetric expression is regarded as a means whereby laterality information is propagated. The hypothesis of ciliary asymmetry being responsible for body asymmetry is not discussed in these papers. The observations by Nonaka *et al.* (1998) have, however, provided strong evidence for the cilia being the primary mover in the long chain of events that will lead to a body with a non-random asymmetry. The co-occurrence of ciliary dysfunction with random determination of body asymmetry in at least two mouse mutants, in a rat mutant, and in the human or canine immotile-cilia syndrome are other indications that cilia may be responsible for determining situs position.

Predictions

The strength of the hypothesis of 'the nodal cilia as first mover' can be tested in various ways, for example by finding out whether

the following predictions can be verified: the primitive node (Hensen's node) has monociliated cells in all vertebrates and their cilia move as described by Nonaka *et al.* (1998). The nodal cilia in iv/iv mice or WIC-Hyd rats will not display this kind of movement. A high percentage of situs inversus would result from immersing a group of amphibian or fish eggs in a highly viscous methyl cellulose solution during the embryonic stage when the primitive node is present. The same outcome can be predicted for embryos, which during primitive streak stage are transiently exposed to deciliating substances such as dibucain or nickel ions.

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