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The Organization of the Oocyte and Embryogenesis in Twinning and Fusion Malformations

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Abstract. Certain congenital malformations occur in excess among twins and also among first-degree relatives of twins. In the general population, these anomalies are familially associated with each other, and, like twinning, familially associated with unusual brain function asymmetry. They affect structures built by fusion of bilateral embryonic halves and remodeled under major influence of neural crest mesenchyme. This conjunction of associations suggests that twinning, symmetry development, and this group of malformations might share causal elements at least some of which are heritable. The problem here is the absence of zygosity differences in these relationships, because of which they cannot be explained within the biology of twinning as it has been understood. A potential resolution is offered by way of a mechanism common to MZ and DZ twinning, involving a relationship between oocyte organization and the determination of body symmetries.

Key words: Twinning, Left handedness, Malformations, Epidemiology

INTRODUCTION

Development ... "is a scientific territory the majority of biologists of our day shall see only as Moses saw the promised land, from afar and without power to enter" [29].

It may serve us well here to consider the rate of change of our thoughts on the oocyte and embryogenesis over the 100 years since Giard [29] wrote on the same subject. In especially the recent part of that interval, at once everything, and nothing much, has changed. The primary motivation for this paper is a concern for the quality of conceptual tools at hand for further exploration of that still compellingly mysterious ground. We will argue here that the twin biology component of the toolkit is of that same vintage and

overdue for replacement. It is our intention to be constructive; of we attack one or more of your favorite ideas, please know that we do so only in the hope of making room for what might be a better one.

"The etiology of MZ twinning is unclear. There is an excess if congenital malformations in twins entirely due to their increased incidence in MZ twins. There may be a common factor in the causation of both".

This statement, a direct quote from a very recent paper which shall remain anonymous, can be found in more or less dogmatic form in any number of other sources. It represents prevailing opinion on its subject according to the literature of the past few decades. Certain facts as we find them force us to argue here that it represents little that is very real or useful; the bit of truth in each sentence has for too long sufficed to veil great ignorance.

The biology of twinning events has implications for understanding the cellular mechanisms of embryogenesis. When differences between twin and singleton embryogenesis can be documented and understood, and their observable consequences can be assigned to specific differences, we should be able to undertake productive new approaches to human developmental biology, and particularly to the biology of anomalous development. Twins are of interest to such questions because their embryos pass through those early stages differently. That much has long seemed obvious for MZ twins. We will argue here that our most basic assumptions have kept us from seeing that something very similar must also be true for DZ twins, which changes all of the rules.

The belief that sex-pairing differences represent zygosity differences in the association of twinning and malformation is so deeply ingrained that few of us give it a thought. In several papers given at this Congress, anomalies were attributed to MZ twinning on the basis of no better evidence than same sex, and that attribution was defended when challenged, in a manner suggesting mental deficiency in anyone who might suppose otherwise.

What we really see in such data is a difference in malformation frequency between the members of same-sex and opposite-sex pairs. It is only by application of a certain logic, with certain apparently plausible assumptions (for which assumptions [1] is the earliest *cited* source we have found), that we have translated observed sex-pairing differences into a belief in zygosity differences. In another paper in these proceedings [5], we have analyzed that logic and its assumptions as they have been applied to mortality, and found it false. It is precisely the same logic, with the same assumptions, which has given us our belief in zygosity differences in frequency of malformations:

Because of the very low joint frequency of twinning and any individual malformation, samples of twins large enough for epidemiologic study have been found only in public records from large populations. Such records are blind to zygosity beyond sex-pairing. (Even now that we are able to genotype a pair with neither twin alive [18], who will pay for genotyping that many twin pairs?). Because we know only that opposite-sex pairs are DZ, and that same-sex pairs include all of the MZs and about half of the DZs, we must estimate overall zygosity fractions by the Weinberg difference method.

For that purpose, we are required to assume that sex-pairing among DZs has made no difference in any prenatal anomaly, in order that binomial distribution of DZ sex pairing at conception might still be the case at birth.

We then apply that same logic, with the same assumptions, to any and all anomalies,

where any departure from those assumptions will be concentrated. The standard assumption that the frequency of any anomaly among SS-DZs is the same as that observed among the OS-DZs is just another way of saying that any difference associated with sex-pairing is due to zygosity. If that is true, it deserves demonstration more substantial than this tautology. If it is not true, it changes everything. For mortality, it cannot be true [5].

Most of what follows will have something to do with symmetry. That will mean not only the relative size or functionality of paired structures on opposite sides of midline, but the underlying developmental plan by which consistent side-to-side differences come to be. For reasons which should become clear, we will mostly be concerned with brain, face, and heart. Because the left-right dimension has different meaning at every different point in the sagittal plane, the phrase "body symmetry" will imply the entire body plan.

We find very little in the literature concerning the biology of OS twinship. The first 110 years of Galton-style, "genetic" twin studies has systematically avoided OS pairs. The over-riding concern has been for within-pair differences, and gender is clearly a source of variance considered extraneous from that perspective. It does not really seem safe to assume that cogestation with a co-twin of the other gender could make no difference in human development. The members of OS twin pairs, perhaps especially the females, experience what might well be a unique gestational environment. We can provide an example.

Rats and mice have gender-dependent behavioral asymmetries, like handedness in humans. Individual gender phenotype with respect to those behavioral asymmetries varies as a function of the prenatal hormonal environment. That environment can be changed experimentally, by hormone injections to the pregnant dam, but it also changes naturally as a function of the genders of adjacent fetuses. The female is rather more sensitive. When a female rat pup is gestated with a high number or proportion of males, between two males, or downstream in uterine circulation from one or more males, her behavioral asymmetry phenotype tends to be changed in a masculine direction [17,30].

We have been able to make a direct test of the possibility that OS twinship affects human sex-symmetry relationships. The experiment concerns gender difference in asymmetries of dental diameters. Asymmetry of dental diameters differs enuogh between sexes that gender can be identified with over 95% accuracy by classification functions based on dental diameter asymmetries. A discriminant function sex-classification rule which correctly identifies the genders of 96% of SS twins of both zygosities misclassifies 80% of the OS-DZs ($\chi^2_1 = 76.1$), 70% of the males and 90% of the females. Among DZs only, a discriminant function sex-classification rule which correctly identifies genders of 95% of the SS-DZs misclassifies 60% of the OS-DZs, 40% of the males, 80% of the females ($\chi^2_1 = 21.6$).

The sex-pairing difference in mortality affects the sexes differently, the OS vs SS reduction of mortality approaches 60% in males, but is just 1/3 for females.

We are forced to conclude that sex-pairing is indeed strongly correlated with developmental differences among DZ twins, with respect to minor aspects of symmetry development within normal limits and with respect to mortality, and that the differences interact with individual gender. We believe that the malformations in question here lie on a continuum between those points, and will be found to be subject to the same influences when the distributions of these malformations can be similarly analyzed.

With specific reference to malformations, we have been unable to find a dataset with

a sufficient number of zygosity-diagnosed cases to perform the same tests we applied to the results for mortality. In one study of 1,424 genotyped pairs [20] and another of 546 genotyped pairs [15], there was no significant zygosity difference in total malformations. SS and OS DZs were pooled, and no sex-pairing effect could be assessed from the published values. Even a sample of that size, accumulated at great expense of time and effort, is of little use to demonstrate differences in frequencies as low as those of individual malformations.

To apply the tests we have used on mortality to individual malformations, or even to *appropriately* grouped malformations, seems likely to require the collection of many thousands of twin pairs, well diagnosed – in uniform terminology – with respect to their anomalies and zygosities. It will require the inclusion of stillborn and aborted twins, ideally including autopsies. And it will be far more efficient if certain information about both parents is included. While we are waiting for an army and a treasury not yet assembled to carry out those tasks, let us make what progress we may with what is at hand.

Quite reasonably, no one really seems to believe that all malformations have the same causes. The malformations most strongly associated with twinning seem to be neural tube defects, congenital heart defects, and facial clefting. These may well represent either more or less than the true full set of twinning-associated malformations, but they have been repeatedly implicated [10,15,35,36,38,45,46,48,51,52].

All of these anomalies, in various groupings, have also been reported to be more frequent in siblings and/or offspring of twins [14,41,51]. With rare exception, those relatives of twins are not themselves twins, and their births are not readily supposed to have been subject to any extra scrutiny that might lead to relative overreporting.

The excess malformations among siblings and offspring of twins do not differ significantly or even consistently as a function of the zygosity of the twins in the affected families.

This particular cluster of observations has two fundamental implications. First, the association of these malformations with twinning is not caused by twinship itself, but by one or more familial causal elements common to twinning and malformation. Second: given that the association does not differ with zygosity, then MZ and DZ twinning processes must share some part of their causes.

Children afflicted with these anomalies have an excess of nonrighthanded (NRH) parents [6,23-25,50]. Twins and their sibs and offspring have excess frequencies of these anomalies, and twins also have an excess of NRH parents. The excess NRH among parents of twins does not differ as a function of zygosity [2], or of sex-pairing among the DZs (this last point is new information; it was not tested in [2]).

The malformations we have listed all affect structures built from bilateral halves, fused near embryonic midline and remodeled under the influence of neural crest mesenchime. We find it convenient to call them "fusion malformations". Because they share this dependency on developmental symmetry operations, and because they share with each other, and with twinning, a familial relationship with unusual brain function asymmetry, any unifying mechanism must probably involve control of embryogenic symmetry determination.

As we use it here, "embryogenic body symmetry determination" does not mean simply the elaboration of left-vs-right specializations. Related mechanisms must be involved whenever the progress of any stage of development requires tissues to develop

differently at different distance from midline, or requires that developmental behavior be integrated across midline. Although perhaps most obviously crucial in the closures of the brain, face, heart, gut and groin, bilateral asymmetry of varying degree is the rule rather than the exception in all animal morphogenesis. These processes and their underlying mechanisms are ubiquitous and fundamental.

Some further examples, not all malformations in the usual sense of the word, but concerning the association of twinning with unusual symmetry development, may be useful. Dental diameters of twins are substantially more symmetrical than those of singletons; zygosity differences are very few and small compared to the twin-singleton differences [4]. Sharma reports in these proceedings a large twin excess of doubled occipital hair whorl; there is no zygosity difference. No zygosity difference is apparent in the large twin excess of reading disability reported in [34] (reading is probably the brain's most symmetry-dependent function). Burn and Corney show an association between twinning, congenital heart defects, and the sidedness (directional asymmetry) of the occipital hair whorl [10].

Because prevailing theory allows no relationship between the biologies of MZ and DZ twinning processes, an excess of some anomaly in twins, without a clear zygosity difference, could be explained only as a consequence of twinship itself. But when the anomaly is also excessive among relatives who share with the twins some of *Everything but* the fact of being twins, and there is still no zygosity difference, then a new interpretation is required. The idea of unrelated origins for MZ and DZ twinning is unable to accomodate these observations.

A quote above includes the statement "The etiology of MZ twinning is unclear". Our only problem with that statement is the implication by omission that the etiology of DZ twinning is clear. We confess a long history of increasing doubt, arising from results like those above. In reference to the physiology of ovulation as now understood, a heritable tendency to double ovulation is far from being a simple idea, and remains unproven in spite of many efforts. (It might be simpler if it could involve reduced asymmetry by way of weakness in monthly alternation between ovaries. That alternation implies asymmetric innervation of the ovaries, which has been demonstrated by Gerendai [28] and Burden et al [9]).

If MZ and DZ twinning share part of their causes, then indeed neither mechanism is at all clear.

We have all supposed that multiple births resulting from the induction of ovulation would represent only events of the DZ type. In an elegant test of the independence of MZ and DZ events from a quite different approach, Derom and her colleagues have shown [19] that induced multiple pregnancies include a significant excess of MZ events.

PROPOSITION OF A POTENTIAL UNIFYING HYPOTHESIS

What seems necessary at this point is a testable mechanism capable of taking effect before or after fertilization to produce either DZ or MZ twinning. The mechanism should be related to the determination of body symmetry, in order to explain relationships between body symmetry development and twinning of both zygosity types. It must be both (at least partially) heritable and significantly subject to non-Mendelian variation. Its heritable

component(s) are probably not Mendelian; at least some of them are not defined by the individual's own genotype. It must be such that either parent may contribute. We think there may be a way.

The mechanism we propose has the following elements:

1) The exquisite asymmetry of meiotic cell divisions, and the establishment of three-dimensional body symmetry in early embryogenesis, demonstrate a high degree of spatial organization which must be highly reliable and species-specific. We propose that all developmental processes requiring asymmetric or cross-midline spatial organization depend at least in part on a system of internal spatial referents.

That organization cannot be created in its entirety *de novo* by the individual genotype, but must at least in part be carried across generations in the germ line as a legacy of spatial organization brought to the zygote by the gametes. Major determinants of this organization are the cytoskeleton and stable effector molecules. Those effector molecules must include, among others, messenger RNAs made and stored in organized spatial arrangements within the cytoskeleton of the oocyte during oogenesis. This should be demonstrable in mammalian oocytes and zygotes by techniques similar to those which have made it plain in other vertebrates [16,26,47].

For purposes of our present thoughts, the organizing structure itself – the cytoskeleton and the system of mechanisms by which its components are built and put in place – is probably more at issue than the specific substances it serves to organize. Demonstration of the organization will most probably prove the existence of organizing mechanisms well before those mechanisms themselves can be demonstrated.

2) Spatial organization of the gametes and of the zygote is vulnerable to disturbances of both genomic and environmental origin. Any process which changes the concentration, placement, or activity of one or more of these effectors may be expected to disturb development. The range of temporo-spatial specificity of developmental disturbance should be commensurate with the action of the effector(s) in question, and may range from catastrophic failure of early embryogenesis to a subtle trace that will become apparent only when organization of a specific tissue becomes critical much later in life.

3) Disturbance of oocyte organization prior to the second meiotic division may cause or allow that division to occur prior to fertilization, and symmetrically. Such division has been seen with substantial frequency when mature rodent oocytes are allowed to age before fertilization [20,21,27,44,49]. Both daughter cells from such division are haploid potential gametes [20,49]. As these cells are not normal representatives of either secondary oocytes or polar bodies, we will call them *"tertiary oocyte"*.

The occurrence of twinning, aneuploidy, fusion malformations and excess mortality as a result of such gamete aging manipulations has been repeatedly demonstrated [7,11, 12,21,22,52]. The twinning observed under those conditions has usually been called MZ. However, the obviously MZ incomplete twinning events seen in amphibians, reptiles, and birds are much less common in mammals, and no test of zygosity has ever been made. The best evidence in its favor, double embryos in single zonae [7] demonstrates uniovular origin, but does not distinguish MZ twinning from DZ development of terziary oocytes.

This prospect provides the necessary alternative (perhaps we should instead say "additional") mechanism of DZ twinning. It involves absence or reduction of the normal asymmetry of meiotic cytokinesis. Twin-singleton differences in handedness, dental diameters and hair whorls reviewed above all involve reduction, in twins, of asymmetries

found in normal singletons.

That human DZ twinning may be induced by gamete aging is clearly indicated by the results of Harlap and her colleagues [33]. It remains only to demonstrate and quantify the occurrence of this type of DZ twin in the general twin population. That analysis is under way here.

You will almost certainly rebel at first against the notion that some sizable fraction of DZ twin pairs must also be derived from single oocytes. This is not a new idea. It has been known as "polar body" or "dispermatic monovular" twinning. You probably think it has been proven that it happens very rarely, if at all. In fact, none of the several tests of its existence had any realistic prospect of discovering it at any frequency. Of all the intended tests, reviewed in [8,42], those which indicated awareness of the effects of recombination were unable to accommodate it in their analyses due to the lack of mapping. As it happens, the blood antigens tested are nearly all coded by loci well away from centromeres. By neglecting to genotype *both* parents, most of what sensitivity might have remained was discarded. Due to dramatic advances in mapping, this prospect is now subject to critical testing. Preliminary evidence from this more critical approach suggests that the "two-egg" form of DZ twinning may just as easily be the exception as the rule. We are not trying to say that "two-egg" DZ twins never occur, but only that there must be another mechanism operating at a substantial frequency.

4) Disturbances of the same or a similar substance or process taking effect after fertilization may cause MZ twinning.

None of us dares claim to know exactly how the basic plan of the body is defined in early embryogenesis, but MZ twinning requires that the cells which define one body symmetry in 99.7% of viable human embryos must somehow define two of them. A clear majority of such events occur within the confines of a single intact trophoblast, demonstrated by monochorionicity [3]. We reject any supposed analogy between human MZ twinning and the effects of separating early starfish blastomeres or tying newt embryos almost in half, and believe that "splitting" is a consequence and not a cause of the MZ twinning event. The following thoughts represent for us the mechanical boundaries of plausibile speculation.

Trophoblast differentiation visibly indicates the dorsoventral axis. We have argued from the distribution of MZ placentation that definition of the other two axes (anterior-posterior and left-right) moves to completion during the rearrangement of the inner cell mass to form the bilaminar disc embryo. This process normally terminates in the eighth day of gestation [3].

Consider the result projected onto the bilaminar disc embryo. (This is only a geometric convenience; you may find it useful to imagine flattening a dough ball *from within*). The simplest mechanism we can propose for enforcement of a plan upon a disc would require the definition of only one, distinctly eccentric focus (cell). The other end of the second axis could be defined by the cell most distant from that focus. The third axis is defined by default, perpendicular to the other two.

Each axis could acquire its original definition from a cell division which asymmetrically partitions the distribution of one or more effectors. MZ twinning requires duplication of one axis, which could arise from absence or reduction of the normal asymmetry of such a cell division.

We suggest that the second axis determined is the anterior-posterior, the ends of which

are represented by the prochordal plate and primitive streak, and that duplication of this axis is the more likely focus of MZ twinning. This suggestion is primarily intuitive, its best concrete basis being the order in which the differentiated axes become visible; the prochordal plate and primitive streak visibly define the anterior-posterior dimension before (left-right) lateral margins of the embryo are visibly defined by the onset of neurulation. Bear in mind that our concern here is for the conditions of commitment of these developmental processes, much earlier than the time of their first microscopically visible appearance.

It may be that dichorionic MZ twinning represents duplication of the first (dorsoventral) axis, monochorionic-diamnionic twinning duplication of the second (anterior-posterior), and monoamnionic twinning the duplication of the third (left-right).

It seems reasonable to suppose that the determinant(s) of cytokinetic asymmetries in meiosis and embryogenesis are few in number, and depend at least in part on the same or closely related effectors in all manifestations. Thus we envision a single system involved in both MZ and DZ twinning and multiple manifestations of developmental (a)symmetry. If both MZ and DZ twinning are related to either transmitted weaknesses or imposed disturbances in this system, then the many observed relationships between twinning of both types and anomalies of symmetry-dependent development may find a unifying theme.

Under that theme, DZ as well as MZ twinning would represent anomalous embryogenesis with excessive risks of malformation and mortality. Our results on zygosity distribution of mortality indicate that prenatal mortality is at least no worse for MZs than for DZs [5]. The DZ version of this mechanism, involving earlier and possibly broader changes in partitioning of structural determinants, might even be expected to be more closely correlated with developmental disturbance. Among SS twins, prenatal mortality actually seems to be worse for DZs than for MZs, especially in blacks whose DZ twinning frequency is supposed to be higher.

Among DZs, the apparent protective effects of OS gestation require another level of explanation. We have shown a clear effect on a developmental sex-asymmetry relationship. Sex difference is closer to rule than to exception among the many asymmetries of mammalian form and function, and also among anomalies of symmetry-dependent development such as the fusion malformations. Hormonal modulation of tissue-specific gender-dependent developmental rates most readily come to mind [17,30], but understanding of that whole area of human biology is primitive at best. There are associations of sex ratio deviations with time-in-cycle of insemination [31,32,37], and with elevations of DZ twinning rate on return of soldiers from war [43], both situations associated with effects of gamete aging. Any deviation from equal sex fractions would increase the fraction of SS among DZ conceptions, suggesting in turn that DZ twins developing from tertiary oocytes may be enriched in SSDZs. If we can demonstrate the existence of tertiary oocyte twins, we will in the same experiments demonstrate their sex-pairing distribution.

The most difficult part is to relate both parents equally to DZ twinning. Our handedness results require it [2], and so do the Mormon pedigree results of Carmelli et al [13]. Excess malformations in the offspring of twins are not significantly or consistently associated with the gender of the parent twin. Independence of MZ and DZ twinning processes, especially with DZ twinning supposed to occur only by way of double ovulation, cannot explain a paternal contribution to the probability of DZ twinning. By whatever

mechanism, DZ twinning must by definition occur before fertilization, while the sperm may contribute only afterward. Two types of paternal contribution prior to fertilization come to mind, potentially subject to both transmissible and nontransmissible variation and both affecting probability of fertilizing a pair of tertiary oocytes: a behavioral contribution to the timing of insemination, or some characteristic of the sperm interacting with some limitation of fertilizability of tertiary oocytes. Absent clear human precedent for either, we postpone further speculation on that aspect, but it will require further consideration.

We are well aware that this proposal departs very substantially from prevailing theory, but we and others have clearly nonrandom observations which might be explained this way, but cannot be explained within prevailing theory. Most of what is new here is a matter of perspective; the meanings previously assigned to certain observations have depended intimately upon certain assumptions. Those assumptions form a highly integrated system and it proves extremely difficult to challenge just one. The one we end up challenging is the most basic. At least most of the elements of our proposed mechanism have reasonable precedent [16,21,26,39,40], and are subject to tests within reach of existing or rapidly-developing methods.

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