Human Reproduction, Vol.24, No.6 pp. 1255–1266, 2009
Advanced Access publication on February 27, 2009 doi:10.1093/humrep/dep030

human reproduction

OPINION

Traces of embryogenesis are the same in monozygotic and dizygotic twins: not compatible with double ovulation

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Common knowledge of over a century has it that monozygotic and dizygotic twinning events occur by unrelated mechanisms: monozygotic twinning 'splits' embryos, producing anomalously re-arranged embryogenic asymmetries; dizygotic twinning begins with independent ovulations yielding undisturbed parallel embryogeneses with no expectation of departures from singleton outcomes. The anomalies statistically associated with twin births are due to the re-arranged embryos of the monozygotics. Common knowledge further requires that dizygotic pairs are dichorionic; monochorionicity is exclusive to monozygotic pairs. These are fundamental certainties in the literature of twin biology. Multiple observations contradict those common knowledge understandings. The double ovulation hypothesis of dizygotic twinning is untenable. Girl–boy twins differ subtly from all other humans of either sex, absolutely not representative of all dizygotics. Embryogenesis of dizygotic twins differs from singleton development at least as much as monozygotic embryogenesis does, and in the same ways, and the differences between singletons and twins of both zygosities represent a coherent system of re-arranged embryogenic asymmetries. Dizygotic twinning and monozygotic twinning have the same list of consequences of anomalous embryogenesis. Those include an unignorable fraction of dizygotic pairs that are in fact monochorionic, plus many more sharing co-twins' cells in tissues other than a common chorion. The idea that monozygotic and dizygotic twinning events arise from the same embryogenic mechanism is the only plausible hypothesis that might explain all of the observations.

Key words: twins / dizygotic / chimera / double ovulation / monozygotic

Background

Twins suffer a much greater frequency of birth anomalies than experienced by single-born infants. The anomalies that are more frequent among twins at birth include all of the most common major malformations, plus premature delivery and low birthweight, plus fetal, perinatal and infant mortality. Common knowledge has it that these anomalies and complications that are associated in excess with twin births are due more or less exclusively to monozygotic twins as result of the disrupted embryogenesis ('splitting') required for the initiation of monozygotic twin development.

The most common major malformations are the 'midline', or 'fusion', malformations. Those are anomalies of midline structures that are formed in embryogenesis by fusion of left- and right-half-structures followed by remodeling in the midline under the influence of neural crest mesenchyme. The best known of these are the neural tube defects, congenital heart defects and orofacial clefts (Boklage, 1984; 1987a,b,c; 2005a). These congenital anomalies have long been recognized as major correlates of infant mortality, and they associate strongly with prematurity, low birthweight and infant mortality among both twins and single-born infants. A sizable fraction of single-born infants are sole survivors of twin conceptions (Boklage, 1990, 1995); a fraction apparently large enough to include every live born case of every anomaly of asymmetry development.

All of those twin excesses of untoward developmental outcomes have consistently been attributed to the members of monozygotic twin pairs. We have even seen twin pairs declared monozygotic because they are of the same sex and one of them has one of those twin-excess malformations. The anomalies in question are uniformly found to be less frequent in boy–girl pairs than in same-sex twins. Because girl–boy pairs have been falsely assumed to be entirely developmentally representative of all dizygotic twins, the logic of the Weinberg difference method consistently translates the better outcomes in boy–girl pairs into the false conclusion that the excess anomalies belong more or less exclusively to the monozygotic twins (further explanation of Weinberg logic follows). Such a result, even including a high frequency of discordance for highly heritable anomalies, has been considered entirely to be expected as a result

of the strange embryogenesis assumed to be the origin of monozygotic twins. The core belief is that a cluster of cells which 'should' have formed one embryo somehow 'splits' to proceed with the building of two embryos. Every developmental anomaly found in excess among twins has been proposed to arise from the disruption of embryogenesis due to monozygotic twin 'splitting'. Always most prominent among the anomalies attributed in excess to monozygotic twins have been these anomalies of bilaterally a/symmetric development. It has been considered certain that the immediate consequence of splitting an embryo into halves is that any bilaterally asymmetric cellular organization under construction at the instant of splitting must be re-arranged to form two separate bilaterally a/symmetric cellular organizations, two systems of body-symmetry axes to serve as cellular and molecular armatures for two bodies. 'What should have become the left side of Lucie must now become the right side of Lisa.'

The splitting that has been assumed to initiate monozygotic twinning has generally been imagined to be a direct and immediate mechanical separation akin to the experimental production of twin newts from single embryos by Spemann and Mangold (1924). Their work seems to have been the origin of the belief in 'splitting', and it deserves a closer look.

The experimental procedure of Spemann and Mangold has been widely misunderstood as a splitting of an embryo into two fully separated masses of cells, which very importantly in fact it was not. In tying fine hairs around newt embryos for their experiments, Spemann and Mangold did divide some of them into two completely separated cell masses, in nearly all of which cases both pieces failed to develop further. Their most famous trick only worked—that is, they only got 'identical mirror-image' twin newts from single embryos—when the ligature lay along the anterior-posterior axis, near the midline, on or near the circumference of the midsagittal plane—and was incomplete, such that the two approximately half-embryo cell masses were left connected by a bridge of cells, and each 'half' included some of the cells that had begun forming the organizer tissue in the dorsal lip of the blastopore. They got few such results. For any mass of cells which included an insufficient representation of organizer cells, or which had no communication between certain counterpart cells on opposite sides of the midline, there would be no further development (Spemann, 1938).

The understandings that Spemann and Mangold gained from the results of these experiments, about the functions of those organizer cells moving through the embryo and inducing developmental changes in other cells, led to Spemann's 1935 Nobel Prize in Physiology for their discovery of the process we know as embryonic induction. Their insight is still a guiding principle in the ongoing progress of developmental biology.

Only with a double-extra dose of circumspection should we consider trying to map those results onto the circumstances of human twinning events—inside the mass of a single secondary oocyte inside its intact zona pellucida in the first 3, at most 4, days of embryogenesis. An extra difficulty of the situation is the requirement that such a mechanism must operate spontaneously for (conservatively estimated) as much as one-eighth of all human conceptions [conceptions, not births; live born twin pairs are closer to 1 in 80 live births; the difference is in the live births of sole survivors, products of twin conceptions born single, who are roughly 10 times as frequent as live born twin pairs (Boklage 1990, 1995)].

One difficulty might be considered obvious: there is no room inside the intact zona for 'splitting' an embryonic cluster of cells, if that requires the opening of any space between them such that they might undertake separate development while still inside the morula (cells of the morula have divided, without growth, from the original mass of the single zygote cell, inside the original intact zona pellucida). We must also recall that every time experimenters have artificially juxtaposed clusters of embryonic cells in any such situation, by adding any part of one inner cell mass even to the expanded blastocyst cavity of another embryo, even of different sex or species, the cell clusters have either fused to form single chimeric embryos or died; the procedure never produced twins. There is no reason to expect twinning to result from such an event even if it were physically possible.

Cellular origin of dichorionic twins

Twinning events initiating development of dichorionic twins must occur before differentiation of the mural trophoblast (from which the chorion will develop), such that each fraction of the original mass of cells may differentiate its own limiting membrane layer of trophoblast cells as precursor to chorion. This must occur within the first 3 or 4 days of embryogenesis, because differentiation of the trophoblast is visible, and hatching well under way, during about the fifth day. When the twinning event occurs before compaction of the morula and trophoblast differentiation, then two body-symmetry domains must be defined among the cells of the morula. Our best understanding is that the trophoblast arises from the first permanent differentiation of cells in embryogenesis. The trophectoderm layer of the blastocyst is the first embryonic cell type to exhibit highly differentiated functions (cf Red-Horse *et al*., 2004), before any axis of the embryogenic body symmetry becomes visible. At the compaction of the morula, inside cells connect and communicate with each other through gap junctions. Differentiating trophoblast cells on the exterior surface of the compacted morula define themselves as limiting membrane, forming a 'surface' layer of cells connected laterally by tight junctions. As blastulation proceeds, cells of the mural trophoblast pump fluid from outside in to form the blastocoel cavity, separating the mural trophoblast layer of cells from the inner cell mass attached to the polar trophoblast. At the same time, enzymes from trophoblastic 'zona breaker' cells weaken the zona, allowing it to relax to accommodate the swelling caused by the influx of fluid, and then to dissolve, allowing the blastocyst to escape the zona in hatching (Sathananthan *et al*., 2003).

Dichorionicity requires that two such shells of 'outside' cells must differentiate within the morula, to divide and enclose subsets of the inner cells into separate domains, each to form its own surrounding trophoblast layer, for further development as dichorionic twins.

Monochorionic twinning

When the twinning event occurs after a single trophoblast has differentiated to form a membrane around the inner cells of the compacted morula, then cells within the single contiguous cell mass must establish two systems of body-symmetry axes inside that single trophoblast. Those twins will be monochorionic, sharing that single trophoblast-chorion throughout the remainder of their gestation.

Monoamnionic twinning

Should the determination of two sets of body-symmetry axes wait for another several hours, until after the inner cell mass has flattened to form the bilaminar disk stage of embryogenesis and the cells of the future amnion have differentiated to line the inside of a second cavity on the other (dorsal) side of that bilaminar disk, then the twins will also share the amnion.

The monoamnionic option becomes available about the seventh day of embryogenesis (cf Boklage, 1981a). Not all monoamnionic twins are conjoined, but all conjoined twins are monoamnionic. Common knowledge has it, more basically, that all conjoined twins, being monoamnionic, are of course all monochorionic and are therefore all monozygotic. This is not the case. Kim *et al*. (2007) report a conjoined boy–girl pair. Girl–boy monochorionic diamnionic pairs are, of course, much less rare than that, just as with their same-sex counterparts.

The cellular logic of this situation and the absence of direct observations of normal human embryogenesis together cause me to believe that the difference which commits the twins to development as dichorionic versus monochorionic–diamnionic versus monoamnionic must arise from differences in the biochemistry of embryogenic induction as the origin of the relative timing (implicated here as if it were an independent variable). Relative timing of the twinning events is the way we have always talked about it; it makes a simple sort of sense and we have had no means to take the question deeper. There must be a molecular basis for the difference in timing; those cells cannot read clocks or graphs.

Among twins of African ancestry, about half of the number of twin pairs that are estimated to be monozygotic are monochorionic. Among white European monozygotic twins, two-thirds are monochorionic. Over 80% of monozygotic twins of East Asian ancestry are monochorionic (reviewed in Boklage, 2006). In the absence of credible data from competent zygosity diagnoses, the denominators of all of those fractions have always been derived from Weinberg difference method estimates of zygosity fractions. According to the logic of the Weinberg 'difference' method, the number of monozygotic pairs in any sample can be simply calculated as the 'difference' between the number of same-sex pairs and the number of boy–girl pairs. The logic of this algorithm absolutely requires the repeatedly discredited assumption that like-sex and unlike-sex dizygotic twin pairs are fully congruent in the range and quality of developmental experiences and outcomes, so that the presumed binomial distribution of sex-pairing at insemination of independent oocytes might endure through gestation to live birth (reviewed in Boklage, 2005a). The Weinberg algorithm has produced a reasonable approximation of genotyped zygosity fractions only in large samples of normal white European twins. With particular respect to zygosity distributions of the anomalies at issue here, the lower frequency of all congenital anomalies in girl–boy pairs (Boklage 1985, 1987d) makes the traditional outcome of the Weinberg algorithm inevitable when applied to samples with anomalies, always blaming the excess anomalies on the monozygotics. Where there is competent genotyping of each pair, if only by sound similarity questionnaire, there appears to be no evidence anywhere of excess anomalies among monozygotic versus dizygotic twins.

For twinning to occur, one mass of cells eventually must become two separate bodies, but there is no reason to suppose the separation required for human monozygotic twinning can occur by any mechanism related to the experiments of Spemann and Mangold. Physical separation is ultimately necessary, but it must come from within the embryo's contiguous cell mass, somehow defining two systems of cells each in progress toward defining its own system of embryogenic axes. The separation is not driven by external mechanical force, but is rather a developmental process of gene expression decisions and mechanochemical results thereof, involving among other component processes the equipping of cells with tight junctions instead of gap junctions to form two separate boundaries.

The strugglesome popping and pinching version of hatching reported in embryos from artificial fertilizations (in which the zona is hardened by incubation *in vitro*) is an artifact of that artificial process and cannot reasonably be considered representative of the natural process (Tarlatzis *et al*., 2002; Frankfurter *et al*., 2004; Aston *et al*., 2008). Twinning in the manner of Spemann's and Mangold's newts bears no relationship with the natural spontaneous process. Those colorful but baseless imaginings have led too many astray for too long already.

Dizygotic twins

Dizygotic twins, on the other hand, are presumed to have arisen from two separate and independent ovulations, and therefore to have come through parallel, independent and thus quite ordinary embryogeneses. Their development throughout gestation and the rest of life cannot be expected to differ from that of singletons, except perhaps for any consequences of the more-or-less doubled demands they will put on the uterus late in pregnancy.

This is a necessary prediction of the hypothesis of double ovulation as origin of dizygotic twinning. Given ovulations from two different follicles, there is no reason to imagine that the two oocytes and their respective fertilizations and subsequent development are anything but separate and independent. The entire literature reads as if it is based on that idea not as the hypothesis it is, but as if it were established fact. If their embryogeneses are parallel and independent, and they will have no interaction except perhaps when the excessive demands of their combined growth put them into competition for limited uterine resources, then we have no reason to suppose that they would share any part of the monozygotic excesses of developmental misfortunes rooted in the asymmetries of embryogenesis. But they do. All of it. In detail.

Double ovulation has never been observed or demonstrated to be the origin of even one pair of dizygotic twins, nor have any of the necessary direct predictions of that hypothesis been verified. The 'double ovulation' origin of naturally conceived dizygotic twins is entirely hypothetical. It remains unsupported by observation, but also remains in general unchallenged, accepted and handed down as if it were established fact.

Careful reading of the literature finds no credible evidence for multiple ovulation causing human multiple pregnancy. Every paper that has ever reported double ovulation as the origin of dizygotic twinning either states it as if it were fact without need of reference, or includes a reference to a previous writing as authority for the statement. That previous writing in its turn either states that dizygotic twinning arises from double ovulation as if it were fact without need of reference, or includes a reference to a previous writing as authority for the

statement. I have followed to its beginning at least nearly every such chain I have encountered. I have repeatedly discussed this problem in person and in print, and have never been offered any of the evidence I have reported missing. I have come to believe that there is no evidence anywhere directly associating human dizygotic twinning with double ovulation.

There is substantial credible evidence to the contrary. A body of evidence accumulated over the past 30 years shows that developmental anomalies arising in and from the cellular processes of dizygotic twin embryogenesis are for all practical purposes identical in nature, frequency and severity to those resulting from monozygotic twin embryogenesis. Every statement you may find to the contrary is based on the Weinberg difference method estimates of zygosity fractions instead of competent zygosity diagnosis. The facts of the matter are in irreconcilable conflict with the expectations and predictions of the hypothesis of double ovulation.

Results: a review of that evidence

Brain function asymmetry in twins and their families

The excess of non-righthandedness among twins (Boklage 1981b, 1987b; Derom *et al*., 1996) has been attributed primarily to the monozygotic twins, as a result of 'splitting' having disrupted embryogenic determinations of left–right asymmetries of brain function.

Non-righthandedness remains our simplest indicator of unusual brain function asymmetry. It has been supposed that unusual asymmetry of brain function might be the expected result of any departure from usual pathways through the processes of establishing left–right differences in embryogenesis of the brain.

In 773 three-generation twin families, the excess non-righthandedness among twins does not differ by twin zygosity. The first-degree relatives of twins are also more often non-righthanded than the singleton majority. There is no zygosity difference among twins or their first-degree relatives in the frequency of non-righthandedness (Boklage 1981b, 1987b).

Monochorionic twins have been assumed to be necessarily monozygotic. Monochorionic pairs are understood to have undergone twinning later in embryogenesis than dichorionic pairs, and are therefore presumed more likely to have suffered disturbances of embryogenic asymmetries, and especially discordant, 'mirror-image' handedness. In fact, monochorionic twins show no difference in frequency of, or matching for, non-righthandedness than their 'earlier-splitting' dichorionic counterparts. The timing of the twinning event in embryogenesis is of no interest to these questions—nothing about handedness in twins has anything to do with 'late' 'splitting' (Derom *et al*., 1996). Their work also confirms the absence of any relationship between non-righthandedness and twin zygosity.

The midline/fusion/symmetry malformations

Non-righthandedness is significantly associated, in singletons as well as twins and their first-degree relatives, with the same developmental malformations that are excessive in twins. Like non-righthandedness, the developmental anomalies that are excessively frequent among twins are all unusual variations on asymmetries in embryogenic development. The most common of them are known as the midline, or fusion, malformations, the best known being the neural tube defects, congenital heart defects and clefts of the lip and/or palate. The structures at issue are built during embryogenesis from bilateral half-structures fused in the midline and remodeled under the influence of mesenchyme cells from the neural crest.

The same developmental anomalies that are excessively frequent in twins are also excessively frequent in first-degree relatives of twins, and where it has been responsibly determined there is no zygosity difference in those observations. Well within sampling error, dizygotic twins and their relatives have the same numbers as the monozygotic twins and their relatives. There are a few statistically significant zygosity differences, in all of which situations the difference involves greater excess or stronger associations with dizygotic twins and/or their first-degree relatives (Boklage, 1987a, b; Kläning *et al*., 2002).

Embryogenesis of the first branchial arch craniofacial subsystems of the head

The design and construction of a set of teeth, as represented by covariance matrices of multiple measurements of dental diameters, serves well as a subsystem model of the building of the head. As might readily be supposed from their close proximity through all of development before and after birth, there are substantial overlaps in the molecular determination of the development of the mouth, face and head and that of the brain.

Defects of craniofacial development are strongly associated with defects of brain development. The holoprosencephaly sequence is a good example of the intersection of the embryogenic asymmetries of brain development with those of craniofacial development. Medial cleft lip is as much a neural tube defect as a facial clefting disorder. It is a failure of proper function of the frontonasal process, a manifestation of the holoprosencephaly sequence that is associated with mutations of the sonic hedgehog gene SHH along with other anomalies in the holoprosencephaly sequence, including cyclopia and diprosopus (Fernandes and Hébert, 2008; Geng *et al*., 2008). The sonic hedgehog gene product is also a major determinant of amelogenesis in the enamel organs of the embryogenic tooth buds (Dassule *et al*., 2000; Yamagishi *et al*., 2006; Takahashi *et al*., 2007). The system of structural relationships among tooth shapes and sizes is a sound subsystem model for the building of the head and brain. A 'set' of teeth is a highly integrated system of bilaterally approximately symmetrical parts, to be constructed in proper shapes and locations and relationships to all other components of the set of teeth and to the face and head in which the set of teeth is to fit and function.

Twins differ from singletons in these multidimensional developmental structures. Dizygotic twins differ from singletons as much as monozygotics do, in the same multidimensional directions. Dizygotic and monozygotic twins as groups both differ from singletons by much greater distances than they differ from each other. A few cases believed to be and entered in our samples as 'singletons' were classified as twins by these discriminant function analyses. This is to be expected from our knowledge that most products of twin conceptions who survive to delivery are delivered single. No twin in the samples was ever misclassified as a singleton in any of the analyses. Therefore it is not the case that some fraction of dizygotic twins experience embryogenesis

typical of monozygotic twins and some develop like singletons as predicted by the two-follicle hypothesis. There is no evidence that any naturally conceived twin pair ever experiences an embryogenesis typical of singleton development (Boklage, 1984, 1987c).

'Mirror-imaging' has a developmental reality (but nothing to do with zygosity)

Conversations with twins or parents of twins frequently include mentions of 'mirror-image twins' or 'mirror twins', assumed to be 'late-splitting identical' twins because they are discordant for handedness, representing presumed developmental consequences of their monozygotic 'split' having occurred after the beginnings of asymmetric cell differentiations.

Among singletons, left and right half-sets of teeth are built to different multivariate developmental patterns. Multivariate statistical analyses can robustly sort sets of singleton tooth-shape measurements between left versus right half-sets of tooth size and shape measurements. Among twins, those same multivariate statistical methods cannot find any difference on which to sort the left versus right half-jaw tooth-shape patterns, returning a *P*-value of 0.98 for the null hypothesis of no difference (*P*= probability that the two sets of measurements might just as well have come from repeated measurements from the same sample, differing only by way of errors in the measurements).

There is a complete absence, from twins of both zygosity groups quite equally, of the normal multivariate system of left–right asymmetries established early in embryogenesis in the craniofacial organization of the singletons (Boklage 1984, 1987c). Just like the handedness results, this is a reduction of/from the normal level of developmental asymmetry. Just as with handedness, the monozygotic and dizygotic twin groups show no significant difference in their respective departures from singleton results. Matching from the left side of one twin to the right side of the other (as usually understood by 'mirror imaging') appears to be a trivial manifestation of the side-to-side matching within individual members of each twin pair.

On the assumption that girl–boy twins are representative of all dizygotic twins

In these multivariate statistical analyses, tooth-shape patterns of singletons and same-sex twins can be robustly sorted by sex. Among single-born individuals and same-sex twins, discriminant function analyses can very accurately sort the sexes, based on these measurements of dental diameters. However, twins from male–female pairs cannot be sorted by sex with respect to those same patterns (Boklage 1984, 1987b).

Boy–girl twins are not developmentally equivalent to singletons of either sex or to same-sex twins of either sex. They are not suitable control subjects for considerations of differences between monozygotic twins and single-born individuals as groups. Girl–boy twins are not valid place-holders for Weinberg calculations. They are not developmentally representative of all dizygotic twins, which is a fundamental, necessary assumption of the Weinberg Difference Method logic, which is in its turn the only basis for all of the reports of developmental differences between monozygotic and dizygotic twins. Every assertion of developmental difference between monozygotic and dizygotic twins whose zygosity has not been demonstrated by competent genotyping is unsound and probably wrong.

Differences between all-same-sex twins as a group and girl–boy twins as another group are due at least as much to same-sex dizygotic twins as they are to the monozygotics among the same-sex pairs. Live born boy–girl twins are not reliably half of all live born dizygotic twins, and they are not developmentally equivalent to any other group of people, male or female, single or twin, monozygotic or dizygotic. Girl–boy twins have no equivalents in embryogenesis. Girls with twin brothers are developmentally distinct from other girls, and boys with twin sisters are (in fewer measures and often to lesser extents) developmentally distinct from other boys. Girls with twin brothers are 'masculinized' or 'defeminized' in: fertility (Lummaa *et al*., 2007), birthweight (Glinianaia *et al*., 1998; Goldman *et al*., 2003) and in several anatomical and behavioral measures [including but not limited to: brain function lateralization, aggression, risk-taking, eating disorders, spontaneous otoacoustic emissions, willingness to bend or break rules and finger-length ratios (Cohen-Bendahan *et al*., 2004a, 2005a; van Anders *et al*., 2006; Culbert *et al*., 2008)].

Most of the entries in a sizable literature about these differences depend upon the baseless assumption that the differences result from effects of testosterone from the boy fetus on his fetal twin sister (Cohen-Bendahan *et al*., 2004a, 2005a; Voracek and Dressler, 2007; Culbert *et al*., 2008). This notion apparently originates from a careless extrapolation from mixed-sex twinning in cattle, where a very different kind of placentation does in fact allow mixing of the blood of twin fetal calves, and also thereby all of their circulating developmental effector molecules including their hormones. The heifer calf of a male–female bovine pair usually develops to be called a freemartin. A freemartin heifer is infertile, with abnormal internal (and sometimes external) genitalia, and her most useful function having anything to do with her sex is likely to be as a spotter to indicate by mounting which of the normal cows is ready for the bull. Her twin brother bullock is usually not obviously built oddly, but is often not of much use for breeding and poorly fertile, if at all, if he does take a notion to breed. So, even in that situation, the male twin's developmental androgens cannot carry responsibility for the whole system of differences.

The closest human counterpart to the bovine placentation situation is opposite-sex monochorionic twins. It is entirely reasonable to assume that, like other monochorionic twins, they would almost always share circulation through placental anastomoses (Quintero *et al*., 2003; Souter *et al*., 2003; Williams *et al*., 2004; Ekelund *et al*., 2008), although this feature has apparently not been commented upon in every case (Nylander and Osunkoya, 1970; Iselius *et al*., 1979; Bieber *et al*., 1981; Mortimer, 1987; Miura and Niikawa, 2005). In large part, because common knowledge is firmly certain that they do not happen, few human monochorionic male–female pairs have been reported [I have seen some such pairs deleted from a large twin data set because they are 'obviously' errors, and a recent paper from Guilherme *et al*. (2008) excluded a set of triplets from their sample 'due to discordance in placental analysis and molecular zygosity'—? 'monochorionic, dizygotic, must be a mistake'?].

One fact worth remembering in these considerations is the impressive average sex difference in the speed of embryogenesis (Boklage 2005b). Relative timing of twinning events makes substantial differences in chorionicity. Female embryogenesis is slower, monochorionic twinning is later and monochorionic twins include a rather higher fraction of females than among the dichorionic. Monoamnionic,

and then conjoined, twinning events occur later and later still, and have higher and still higher fractions female.

Same-sex pairs are reported to be routinely in excess among genotyped live born dizygotics (James, 1971, 1979). This is in spite of prenatal losses concentrated in same-sex pairs (Rydhstroem and Heraib, 2001). The fraction of boy–girl pairs among all twins is inversely related with fraction male in all live births across major continental subpopulations. These effects bear consistent relationships with the speed of various reproductive and developmental processes and appear to depend upon some components or features of the paternal X-chromosome imprint (Boklage, 2005b).

Most girl–boy twins are dichorionic. Dichorionic placentae of either zygosity fuse about half the time, with the result that many mothers have been told their twins are 'identical' because they are of the same sex and had a 'single placenta.' However, competent pathological observation seldom gets chorionicity wrong, and dichorionic twins born with placental anastomoses indicating shared placental circulation are extremely rare.

A narrow majority of reported boy–girl monochorionic pairs have been from assisted reproduction technology (ART) pregnancies. Disorders of imprinting are reported at much higher frequency among ART births (Maher *et al*., 2003; Paoloni-Giacobino and Chaillet, 2004; Williams *et al*., 2004; Gardner and Lane, 2005; Maher, 2005; Miura and Niikawa 2005; Shiota and Yamada, 2005; Yoon *et al*., 2005; Aoki *et al*., 2006). Normal sex-dependent differences in the speed of human embryogenesis have been reported absent in IVF embryos (DuMoulin *et al*., 2005). Chang *et al*. (2008) later confirm drastic reduction from normal excess of males in cleavage-stage IVF transfers, but the normal excess is 'rescued', and amplified, by selecting for the faster males as blastocyst-stage embryos for transfer. Mixed-sex artificial mouse chimeras routinely appear at delivery as fertile males (Tarkowski, 1998). All this taken together suggests that boy–girl twin pairs are consistently and necessarily less than half of dizygotic pairs, and plausibly perhaps much less.

Testosterone concentration in amniotic fluid reflects the sex of the fetus it bathes. In boy–girl twin pairs, each fetus has a sex-appropriate concentration of amniotic fluid testosterone (Abeliovich *et al*., 1984), and the mother's serum shows no indication that her blood provides a path for the boy's testosterone to influence his fetal sister's development (Cohen-Bendahan *et al*., 2004b, 2005b). Several of the reported differences are bidirectional, and are therefore not due to testosterone from the boy fetus affecting his fetal sister *in utero*. To all appearances, when these assertions are closely examined, testosterone's reputed contributions to sex differentiation may have been over-rated.

Studies of structural relationships among the sizes and shapes of teeth in twins and singletons (detailed above) show perhaps the clearest evidence of these unusual features of boy–girl twins. How such differences are installed in the course of development will remain an excellent question for further study; there is more there to be learned.

Our understanding of the mechanisms that establish differences between left-side and right-side structures in embryogenesis has advanced dramatically in the past few years (Fukumoto *et al*., 2005; Levin, 2005). Our understanding remains incomplete, and might be considered negligible in view of all the embryo must do to build itself. To know that those mechanisms are not operating normally in the embryogenesis of twins of either zygosity must eventually tell us important things about embryogenesis for all of us.

The members of 'opposite-sex' twin pairs have other developmental distinctions as well. Both male and female members of boy–girl twin pairs have lower prenatal and infant mortality than their same-sex twin counterparts. Whatever makes boys generally more vulnerable to developmental failure throughout the fetal period is somehow substantially reduced among boys born alive with twin sisters, and it is not contagious. Girls born alive with twin brothers do not have their mortality statistics shifted in a masculine direction—in fact, they do better than same-sex female twins. Both members of live born girl–boy twin pairs are somehow better off, in several (developmental) ways, than their same-sex-twin counterparts (Boklage, 1985, 1987d).

Why it matters:

If dizygotic twins were indeed to arise from separate and independent processes of ovulation + syngamy + embryogenesis, there is no reason to expect their embryogeneses to differ from those of singletons. But they do, not slightly, not occasionally, but substantially and comprehensively. Dizygotic twins develop as differently from singletons as monozygotic twins do, from within the same system of deviations. Every anomaly of asymmetric embryogenesis that has been attributed to 'splitting' of monozygotic twin embryos happens identically or worse in dizygotic twin embryogenesis. Every naturally conceived, spontaneous twin pair examined to date in these ways shows the same developmental residuals of what has been visualized as 'splitting' to generate monozygotic twin embryos. Those erroneous attributions have been due primarily to falsely assumed premises and falsely drawn conclusions of the 'Weinberg difference method' tautology. This is the same source from which arises the conventional wisdom about the globally constant biology of [estimated] monozygotic twinning as a developmental error, in contrast to populational variations in frequency of dizygotic twinning which are presumed to arise from populational genetic variation in a tendency to double ovulation.

The monozygotic twinning process, with its need for an embryogenic cell mass to be divided into two parts, can be considered in and of itself to be an anomaly of embryogenic symmetry determination, and could be imagined to be likely to generate other related symmetry development anomalies. But dizygotic twinning generates all of the same symmetry development anomalies, at the same excess frequencies, and all assertions to the contrary arise from Weinberg method estimations.

How might dizygotic twinning happen, if not by double ovulation?

Both dizygotic and monozygotic twins, when naturally conceived, must arise by the same process of defining two embryogenic body symmetries within a contiguous mass of cells divided from the substance of a single secondary oocyte. That division must occur after some early determinants of structural asymmetries in embryogenesis have been established (at the 3-cell or 4-cell stage; Fukumoto *et al*., 2005), to cause disturbed asymmetries of embryogenesis to leave subtly anomalous traces in all twins.

The results defy essential predictions of the hypothesis of independent double ovulation. The hard part has been in knowing what to offer in its place. To that end, monochorionic dizygotic twins and other spontaneous embryonic chimeras provide gratifying insight (Boklage, 2006).

Monochorionicity has been adamantly considered to be absolutely diagnostic of monozygosity, with the result that all monochorionic pairs are believed to be monozygotic by default and are never genotyped unless grossly discordant for something more-or-less as compelling as sex.

Reports of monochorionic dizygotic twins have appeared occasionally for decades, the majority of which reports have involved some sort of pathology, and such twins have routinely been dismissed as freakishly rare, pathological anomalies.

It is absurd to imagine that the pathologies believed to be associated with those findings have caused monochorionicity for dizygotic twins who would otherwise have been dichorionic. It is absurd to suppose that the pathologies involved have caused mutations of alleles at multiple loci of at least one member of a monochorionic monozygotic twin pair to make them now have dizygotic genotypes. The pathologies involved have nothing to do with the zygosity or the chorionicity, but have served only to draw scrutiny that would not have occurred otherwise, in the course of which analyses dizygosity was discovered in twins that had previously been confidently classified monozygotic because of same sex and monochorionicity. The pathologies in question have drawn scrutiny because the twins are unignorably discordant in sexual development or for anomalies of known or supposed genetic or epigenetic origin.

Because they are discovered almost exclusively by accident, monochorionic dizygotic twins, like all other spontaneously chimeric individuals, must be supposed to be far more numerous than has so far been discovered.

Because independent parallel embryogeneses beginning from double ovulation have no known route to monochorionicity, normal, same-sexed, monochorionic twins are generally not genotyped to determine their zygosity. Frequent dizygosity among monochorionic twin pairs is a reasonable explanation for the observation that '. . . monochorionic MZ twins are more difficult than dichorionic MZ twins to diagnose by physical similarity . . .' (Forget-Dubois *et al*., 2003). In other words, monochorionic co-twins are often not as similar as dichorionic co-twins found to be monozygotic by genotyping. That would be exactly the result expected if a sizable fraction of monochorionic twins are in fact dizygotic. Mortimer (1987) reported finding three of 12 monochorionic pairs to be dizygotic. The results of a direct genotyping survey of consecutive Taiwanese twin pairs (Yang *et al*., 2006) place that value at just under one-third.

Many of the more recently reported monochorionic boy–girl pairs were artificially conceived. Something in ART disables or destabilizes parental genomic imprinting, which is normally responsible for the excess of males present through the fetal period from recognition to live birth, and also for the variation in frequency of unlike-sex pairs among race-group subpopulations (Boklage 2005b, 2006). Against that realization, a recently reported cluster of monochorionic girl–boy twin pairs have been seen as perturbations due to pathology inherent in technologically assisted reproduction. The effects of paternal X-chromosome imprinting, in reducing survival of female conceptuses by slowing female embryogenesis, variably across major continental subpopulations, provide a plausible explanation for the observation that most sex-chimeric mice are phenotypically fertile males (Tarkowski, 1998) and for the relative rarity of boy–girl twins of either chorionicity.

Monochorionic dizygotic twin pairs are necessarily chimeric, with one or both twins carrying cells of the co-twin's genotype (obviously at least the cells of the chorion, not necessarily or even typically blood cells, and not necessarily or even typically detectable in any one tissue sampled). Some of the reported pairs were at first diagnosed monozygotic by simple blood typing.

In order to explain the fact that some dizygotic twins are monochorionic, dizygotic twinning events must also occur in a single mass of cells, divided from the substance of a single secondary oocyte, inside a single zona pellucida. Dizygotic twinning events occur within a single contiguous mass of cells, sometimes before and sometimes after differentiation of a single trophoblast, at a cellular level exactly like the monozygotic events that have long been given exclusive claim to monochorionicity for that reason.

It is customary to declare that monochorionic or otherwise chimeric dizygotic twins are pathological and extremely rare. This assertion is without substance. The great majority of all chimeras, including monochorionic dizygotic twins, may be expected to have two normal cell lines of the same sex and will continue to be discovered only by accident (cf Boklage, 2006). The 'common knowledge' says it is a waste of time and reagents to genotype same-sex monochorionic twins. Any declaration of their extreme rarity, with so few properly examined, carries no logical weight.

Dizygotic twinning and spontaneous embryonic chimerism are one same thing, a single mechanism. Chimerism is not rare at all, but clearly common in direct assessments of its frequency, even according to methods that could never have identified more than a fraction of the chimerism present in the samples (van Dijk *et al*., 1996; Koopmans *et al*., 2005). Chimerism is, however, still generally considered freakishly rare and pathological, and is still discovered only rarely, still far more likely to be discovered when some anomaly draws investigation close enough to demonstrate dizygosity and chimerism.

It has long been supposed without evidence that chorions, placentas and placental blood vessels might fuse later in gestation, to produce 'twin' chimerism or 'blood' chimerism, in blood only (reported almost exclusively where only blood was examined). This notion departs from reality in several directions. (i) The making of many thousands of experimental chimeras has always and only occurred after either artificial removal of the zona or after hatching, as necessary to allow contact between cells of the embryos which might then fuse. (ii) Dichorionic placentae fuse spontaneously about half the time, regardless of zygosity; the chorions remain distinct, and placental circulations reliably remain separate. (iii) Even as undercounted as it has been, chimerism is several orders of magnitude more common than reported anastomoses between circulatory systems of dichorionic placentae. (iv) This is incompatible with the repeated observations of disturbed embryogenic asymmetries in dizygotic twinning.

If, however, spontaneous dizygotic twinning in general originates in a single mass of cells, this all makes sense.

So, how shall we routinely arrange for the nuclei we need to initiate two embryos from the substance of a single oocyte?

Dizygotic twinning inside a single trophoblast (the only credible route to monochorionic dizygotic twins) is also inside a single zona pellucida, and thus inside the substance of a single secondary oocyte, the product of a single ovulation. The secondary oocyte normally includes two haploid maternal half-genomes, usually aligned in second meiotic

metaphase at the time of ovulation, with one pole of the spindle against the membrane just inside where the first polar body was pinched out, awaiting a signal from sperm penetration to proceed to completion of the second meiotic nuclear division.

The secondary oocyte has a short useful life—it falls below 50% probability of being able to support normal embryogenesis within 12 h after ovulation, and that probability is close to zero after 24 h. In experimentally over-matured mammalian oocytes, the second meiotic spindle has been shown moving to the center of the cell, there to conduct a symmetrical division, both daughter cells of which have been shown being fertilized (still inside the single zona). Over-maturation of human oocytes increases the frequency of twinning and of aneuploidy, just as it does in experimental mammals (Harlap *et al*., 1985; cf Boklage, 1987a, b; Boklage *et al*., 1992).

It has been observed and photographed in experimental mammals that decay of the secondary oocyte may involve the movement of the second meiotic spindle back to the center of the cell, followed by a symmetrical division yielding two fertilizable daughter cells (cf Boklage, 1987a, b; Boklage *et al*., 1992). Some have erroneously supposed that these daughter cells (I have for a long time called the daughter cells of a divided secondary oocyte 'tertiary oocytes') should carry identical maternal half genomes. That notion ignores the effects of recombination, which will normally assure that those pairs of chromatids are no longer 'sister' duplicates of a single DNA strand, but are sufficiently mixed by recombination that they share, with small variance, the sib-pair average of 50% of their alleles.

One case of 'semi-identical' twins has finally been reported (Souter *et al*., 2007). Both of those twins are chimeric 46,XX/46,XY, and they were discovered because one of them was born with ambiguous genitalia, leading to a diagnosis of true hermaphroditism. They appear to have identical maternal contributions, for the generation of which two mechanisms can be imagined, neither of which has ever been proven to occur: it might happen by way of oogenic meiosis totally without recombination (not believed to be possible because successful meiotic chromosome synapsis is necessary for gametogenesis and is initiated by crossing over), or by 'endoreduplication' of the genomic contents of the maternal pronucleus (not believed to be possible without centrosomal machinery left behind in meiosis I, normally to be reacquired from the sperm). Against those alternatives, a very improbable segregation that generated sibling matches for all the tested alleles does not really seem so extreme.

Once upon a time, and for a long time, I believed and argued that the chromosomes of tertiary-oocyte twins should retain traces of their origin in the form of excessive matching for DNA sequences in and near the centromeres, where we had believed for decades that there was little or no recombination (Elston and Boklage, 1978). It escapes me now how we reconciled that belief in the absence of recombination near all centromeres with the understanding that the hierarchical repeats in the alpha-satellite centromeric DNA can only be explained by unequal recombination, although perhaps non-meiotic (Waye and Willard, 1986).

Genome-scan linkage studies here and elsewhere (Derom *et al*., 2006) have found no evidence for any excess of dizygotic co-twins matching for DNA sequences near their centromeres.

Our belief that recombination simply does not occur within centromeric regions was based on long-ago microscopic observations of chiasmata during spermatogenesis (it always will be much easier to obtain samples of human meiotic cells in spermatogenesis than in oogenesis). That was the state of the art; it was what everybody believed with whom I ever discussed it. It has since become clear, through several reports published over the time since then, that recombination actually occurs freely in pericentromeric regions of most chromosomes in human oogenesis (Mahtani and Willard, 1988; Wu *et al*., 1990; Weeks *et al*., 1995), which is of course where it matters for these considerations. There was never good reason to believe that tertiary-oocyte twinning should leave a trace in the form of excess matching for maternal alleles, even for loci near centromeres. Allele matching between dizygotic co-twins should have exactly the same probability distribution as that in non-twin sib pairs, even for loci quite close to centromeres, for which reason it never could have provided useful evidence for or against double ovulation.

A series of publications explaining the signaling system required for non-randomly establishing proper asymmetry of heart-looping in vertebrate embryos represents a masterful body of work (Levin, 2005). However, that work begs a question and hits the wall: how does ongoing embryogenesis 'know' which side of the primitive streak is the 'right' side on which to inhibit sonic hedgehog? Something reliably inhibits the appearance of the sonic hedgehog protein on the right side of the primitive streak, such that the asymmetric left-side expression of sonic hedgehog then sets off a cascade of signals on the left side that results in properly asymmetric heart looping. Without that specification, the development of the visceral asymmetries becomes coin-toss random. Whether that inhibitor of sonic hedgehog on the right side of the primitive streak is *activin*, or *bcl*2, or *bmp*4 or some combination or interaction thereof—whatever gene product it is, and of course it need not be the same one in every organism that uses such a mechanism—the reliably asymmetric presence of that gene product cannot be the 'symmetry-breaking' event, simply because the embryonic territory in question is already reliably asymmetric.

Serotonin does not make the answer entirely clear, but moves the question back to the first two or three cell divisions. Serotonin is a signaling molecule, a close relative of the amino acid tryptophan, best known as a monoamine neurotransmitter. Fukumoto *et al*. (2005) have shown that serotonin has work to do very early in development, long before there are nerve cells among which to carry signals, and that it is required for the development of proper asymmetries in many embryonic structures. Proper function of two different enzymes involved in the biochemistry of serotonin is required for normal embryogenic left–right patterning, working well before the functions of the asymmetric inhibitor of sonic hedgehog beside the primitive streak. In *Xenopus*, 'the descendants of the right ventral blastomere' are the most dependent upon serotonin-transporter signaling in left–right patterning.

We do not yet know how serotonin does what it seems to do in triggering these molecular functional asymmetries. It is clear, however, that serotonin's functions in patterning operate from the basis of an asymmetry that is already established in the first two or three cell divisions of embryogenesis. Results from embryos of the domestic chicken are consistent with those from *Xenopus*. It is not clear how those same serotonin-dependent functions are performed in mammalian embryos, but they have been shown to be essential to normal mammalian embryogenesis (Côté *et al*., 2007; Dubé and Amireault, 2007).

The 'breaking of symmetry' in embryogenesis, to assign structural and functional differences between left-side and right-side structures, occurs for different components at different times and places, by mechanisms that seem to operate stepwise. Two sources of developmental asymmetry are known to exist before the asymmetries of serotonin biochemistry become apparent in the first four blastomeres: (i) there is a multi-axially asymmetric distribution of sequestered maternal messages and signaling molecules in the ooplasm (Braude *et al*., 1988; Schultz, 2002; Dobson *et al*., 2004; Hamatani *et al*., 2006), and (ii) the DNA—every organism appropriately tested to date has means to know and use the differences between leading and lagging strands and between mother and daughter strands (Pierucci and Zuchowski, 1973; Klar, 2004, 2007).

For these reasons, any division of the substance of a single ooplasm between two embryos will disturb to some degree the early steps in establishing the axes of asymmetry for embryogenesis. It has been standard belief for decades that this accounts for the anomalies of embryogenic asymmetry observed among twins and long attributed entirely to the monozygotic twins. Since dizygotic twins show the same anomalies of embryogenic asymmetry as seen in monozygotics, it is reasonable to hypothesize that the two systems of anomalies are in fact a single entity and have the same origin.

The twinning mechanism proposed here could explain all of these observations that have previously been ignored because of their contradiction of the assumptions and predictions of the deeply rooted double-ovulation hypothesis for dizygotic twinning. The results summarized here make it clear that the realities of dizygotic twin developmental asymmetry are virtually identical to those of monozygotic twin development and incompatible with independent double ovulation. In the absence of similarly simple alternative mechanism, that message has fallen on deaf ears (cf Hoekstra *et al*., 2008). Simplicity has been soundly called a hallmark of truth—but it is no guarantee. Not all true things are simple and not all simple things are true.

The frequency of spontaneous embryonic chimerism and especially, repeated observations of definitely monochorionic dizygotic twins, brings the answer almost into focus. The embryogenesis of dizygotic twins leaves the same traces in lifelong development as that of the monozygotics. All naturally conceived twin embryos become twin embryos by establishing a doubled system of axial patternings within a single contiguous mass of cells divided from the substance of a single secondary oocyte. This process has the same system of developmental consequences for embryogenic asymmetries in all naturally conceived twins. The difference between monozygotic and dizygotic twinning is, exactly as it should be by definition, all and only about the difference between the presence of only one, or of two, diploid nuclei in the zygote, and thus embryonic cells of one genotype or two.

The establishment of double axial patterning occurs after some embryogenic cell function asymmetries have been set at least as biases. That situation is demonstrable at least as early as the first two or three cell divisions of embryogenesis. Asymmetries with fundamental consequences for the remainder of embryonic and fetal development are in place by that time. As another consequence, all twin embryos grow from fractions of a single ooplasm, and therefore cannot both have the full normal set of asymmetry-determining substances brought to fertilization sequestered in the oocyte and then distributed in specific patterns among the early blastomeres. From this beginning in which embryogenic symmetry operations are compromised, dizygotic twins have the same array of anomalies of embryogenic asymmetries as the monozygotics have, with the exception of a few situations in which the results are worse for the same-sex dizygotics.

If these disturbances of embryogenesis can serve as reasons because of which monozygotic twinning has been considered inherently a symmetry anomaly and has for so long been considered to be mechanistically associated with numerous other symmetry anomalies, then those same reasons are the only plausible candidates as causes of the very same system of anomalies of embryogenic symmetry operations that is observed in dizygotic twins.

The double-ovulation hypothesis for the origin of dizygotic twinning owes its continued presence in the folklore entirely to uncritical repetition. Not a single naturally conceived pair of dizygotic twins has ever been seen or shown to have arisen from oocyte cells that became available via separate and independent ovulations.

In clear contradiction, the development of dizygotic twins demonstrably differs from that of singletons at least as much as the embryogenesis of monozygotic twins does, and in the same ways, involving anomalous variations in the same system of embryogenic asymmetries. Whatever is odd about the embryogenesis of monozygotic twins is just as odd in the embryogenesis of dizygotic twins, in the very same ways. It is compatible with all of these accumulated observations to suppose on the contrary that dizygotic and monozygotic twinning must arise from the same cellular event, by the same mechanism, with the same resulting collection of anomalies. The hard part remaining is about arranging for a single zygote to have two diploid nuclei of different genotypes.

Where binuclear zygotes come from:

Triploidy has on several occasions been reported to be the most common aneuploidy among early failures of recognized pregnancies (11, 12 and 16% according to Brajenović-Milić *et al*., 1998, Wołczyński *et al*., 1993 and Be *et al*., 1997, respectively). Given the much greater frequency of triploidy observed among spontaneous miscarriages than among live births, we must suppose that triploidy should be much more common among the at least two-thirds of all conceptions that fail before the recognition of pregnancy (Boklage, 1990, 1995). Of those triploids surviving to be found among miscarriages, some papers have reported the majority to be digynic, primarily from 'retention of the second polar body'. Other papers report that most triploids are diandric, with two paternal half-genomes. Perhaps a few represent diploid sperm, but most by far are products of dispermy. The near absence (0–3% of diandric triploids versus the expected one-fourth) of 69,XYY karyotypes among triploid abortions testifies to dramatic losses before recognition (cf Huang *et al*., 2008; Mcfadden and Langlois, 2000; Zaragosa *et al*., 2000).

It is clear from the abundance of triploids that embryogenesis begins from tripronuclear zygotes with a substantial frequency, yielding products that survive to the recognition of pregnancy rather more often than they survive even a very few weeks beyond that. Those triploid 'embryos' represent only that fraction in which the three pronuclei have formed a single mitotically functional zygote nucleus. Mitotic functionality in turn requires that the zygote nucleus use a single pair of centrioles to form a single functional bipolar spindle. Dispermic triploid nuclei might be expected to contain a centrosome from each

sperm, and probably two pairs of centrioles, and might therefore be more prone to forming multipolar spindles when there is only one maternal pronucleus.

Observed frequencies of digynic triploids show that the presence of both maternal half-genomes in a zygote, forming two maternal pronuclei and a tripronuclear zygote, cannot be assumed to be limiting the possibility of tetragametic zygosis. The frequency of diandric triploids shows that penetration of the oocyte by two sperm cells to yield two paternal pronuclei in tripronuclear zygotes cannot be assumed to be limiting the possibility of tetragametic zygosis.

The joint occurrence, with the presence of both maternal half-genomes and two paternal pronuclei in the same 'tetragametic' zygote, can reasonably be hypothesized to occur with sufficient frequency to explain the frequency of naturally conceived dizygotic twins (Golubovsky, 2003). Dispermy, to put in place two paternal pronuclei, also implies the presence of two centrosomes, one to make each zygote nucleus mitotically functional.

If we may thus suppose the reasonable availability of tetragametic zygotes, the rest follows readily. All that remains is the establishment of two axial patternings among the cells of a single morula before trophoblast differentiation (for dichorionic twins of either zygosity) or within a single cell mass in the morula (for monochorionic twins of either zygosity).

Predictions and tests

The events in question in the stages of spontaneous human embryogenesis at issue here are ethically and technically out of reach of direct observation or testing. The underlying hypotheses do make a number of testable predictions, which might productively be examined as follows:

(i) Cancel all further appearances of the uncritical assumption that all monochorionic twins are monozygotic. Genotype monochorionic twins for zygosity, from at least two tissue samples each, at least one of which is not blood. Many monochorionic twin pairs, when properly genotyped, will prove to be dizygotic (Yang *et al*., 2006). Given the sharing of the chorion, they are by definition chimeric. Sampling of blood alone for traditional blood typing has been found to give wrong first answers in cases of chimerism not involving large admixture in the blood. Genotyping multiple samples can improve the odds, but realize that some will still be missed, due to sampling by chance only non-chimeric tissues and/or only one of the two cell lines. The results of Yang *et al*. (2006) are in fact almost certain to underestimate the frequency of monochorionic dizygotic twins, because of the probability that some of their pairs with matching blood genotypes are chimeric in tissues that were not examined.

(ii) Test dizygotic co-twins directly, blood-to-serum, for immunological cross-tolerance. In many (chimeric) dizygotic pairs, at least one twin will show tolerance of the other twin's tissue antigens (Vietor *et al*., 2000).

(iii) Lower the threshold for recognition of extra alleles in DNA genotype profiles. Even a very small admixture of extra alleles at multiple loci is deeply meaningful, unless either your technology or your technical staff is untrustworthy. (In other words, run the laboratory so that you have no reason to suppose that all 'extra' signals arise from background noise or contamination.)

(iv) Genotype a statistically useful number of failed, cleavage stage, IVF 'embryos', preferably from separated single cells. If cells cannot be separated, realize that the polar bodies will supply extra maternal alleles, in inverse proportion to the total number of cells in the aggregate. There is no excuse but chimerism for more than one set of paternal alleles.

(v) Genotype all available clinical 'mosaic' cell-line samples. With rare exception, those have been diagnosed 'mosaic' because of mixtures of cells with and without a single cytogenetic anomaly, and have not been tested for any other departures from normal diploidy—specifically not for extra alleles at multiple loci.

Funding

This work was supported in part by NIH grants R01-HD-22507 and N01-HG-65403, grants from the Children's Miracle Network, and by the Brody School of Medicine at East Carolina University.

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Submitted on November 21, 2008; resubmitted on January 16, 2009; accepted on January 22, 2009