On the Possible Cause of Monozygotic Twinning: Lessons From the 9-Banded Armadillo and From Assisted Reproduction

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Available hypotheses proposed to explain the mechanism of zygotic splitting fail to explain why monozygotic twins are more prevalent after all methods of assisted reproduction and which structure is likely to control this phenomenon. Arguably, a small proportion of oocytes might have an inborn propensity to undergo splitting upon fertilization leading to the constant prevalence of spontaneous monozygotic conceptions among different populations. Ovarian stimulation would then predictably increase the number of available splitting-prone oocytes and consequently would increase the chance for such oocytes to develop into monozygotic twins, leading to a 'dose'-dependent relationship between monozygosity rates and the combined effect of infertility treatment. Embryonic division into 2 distinct cell lines begins and accommodates within an intact zona pellucida that controls the process by preventing ill-timed hatching. Human fertilized oocytes are able to undergo 2 binary fissions, just as is the case for the 9-banded armadillo (the only other mammal that produces monozygotic quadruplets) and to give rise to a variety of combinations of monozygotic pregnancies. This hypothetical explanation does not negate the already existing and genetically sound hypotheses, but places them into a broader perspective that respects recent observations from modern infertility treatment.

The cause and mechanism of zygotic splitting are unknown. However, four axioms exist that relate to monozygotic twinning. First, monozygotic twins occur in a remarkably constant frequency among different racial groups. Second, the different types of monozygotic twin placentation appear to be related to the timing of zygotic splitting (Baldwin, 1994; Benirschke & Kim, 1973; Chitnis et al., 1999; Corner, 1955; Hall, 2003). Third, the frequency of monozygotic twins is increased by all methods of infertility treatment — the only factor known to consistently do so (Blickstein, 2005; Blickstein et al., 2003; Derom et al., 1987). Finally, general agreement exists that monozygotic twins are associated with the highest risk of morbidity and mortality for both fetus and neonate (Baldwin, 1994).

Despite major advances in reproduction biology during the past 3 decades, we remain unclear as to what might cause the splitting of a fertilized oocyte. Two major limitations are generally encountered in studies on monozygotic splitting. First, many studies fail to assess the true prevalence of monozygosity (Blickstein, 2005) because they either only look at monochorionic twins thus missing monozygotic–dichorionic pairs, or used extrapolations of the Hardy-Weinberg rule, which may not be appropriate in a case-mix of spontaneous and iatrogenic twins. The second, but not the least important, limitation is the acknowledged lack of animal models. Indeed, it appears that the human and the 9-banded armadillo (Dasypus novemcinctus) are the only mammals that regularly produce monozygotic gestations, although some species occasionally deliver monozygotic offspring.

Four theories are presently available to explain the mechanism of zygotic splitting: the so-called cell repulsion hypothesis (Hall, 1996), the existence of co-dominant axes (Baldwin, 1994), depressed calcium levels in the early embryo (Steinman & Valderrama, 2001), and the blastomere herniation hypothesis (Hall, 2003; Blickstein, 2005). Because neither offers a full nor a complete explanation, this review offers new insight to better explain this phenomenon.

Zygotic Splitting and Delayed Hatching

Whereas this discussion does not intend to compare in detail the reproductive mechanisms of the human and the armadillo, two striking and unique observations

should be considered. First and foremost is the fact that the armadillo consistently produce monozygotic quadruplets by two binary fissions (Enders, 2002; Prodohl et al., 1996). This is probably the only way that the armadillo can increase its litter size and enjoy a reproductive advantage. However, a study on the clonal nature of sibships in the 9-banded armadillos proposed that polyembryony may be associated evolutionarily with other reproductive peculiarities of this species, such as delayed implantation of a single oocyte (Prodohl et al., 1996). Indeed, implantation occurs after a prolonged (2–3 months) transport interval in the oviduct. Enders (2002) showed that after the armadillo's blastocyst implants in the fundic recess, a single amnion and cup-shaped epiblastic plate are formed, and an exocelom develops between the amnion and trophoblast of the implantation site.

The mere existence, albeit extremely rare, of monozygotic quadruplets in humans (Steinman, 1998; Timor-Tritsch et al., 1997) represents the potential of the human zygote to undergo two binary fissions. Furthermore, if the relationship between the timing of splitting and placentation is correct (Benirschke & Kim, 1973; Corner, 1955; Hall, 2003), early zygotic splits would necessarily occur during the first 3 to 4 days in the fallopian tube or when cultured in vitro, whereas all later splits (resulting in monochorionic twins) would have to occur in utero, as a peri-implantation event. However, the embryo usually hatches from its protective zona pellucida, before implantation occurs, albeit zygotic splitting was observed in zona-free embryo transfers (Frankfurter et al., 2004). Thus, zygotic splitting is likely to take place before hatching and therefore, delayed hatching would lead to delayed implantation and is conceivably a crucial step in human monozygotic splitting although monozygotic splitting is not always seen in other species with delayed implantation. This delay might also be the reason why monochorionic (as opposed to dichorionic) twins are not infrequently missed during a very early sonographic scan. Moreover, delayed hatching may explain why only dichorionic twins (irrespective of zygosity) are seen among tubal pregnancies.

Protection of the zygote that undergoes splitting and controlling of early embryonic events is accomplished by the zona pellucida which is there from the outset and also possesses appropriate biochemical and biophysical features to do so and, in pathological conditions, is known to interfere with normal hatching. It was suggested that familial monozygotic twinning may be associated with a single gene that causes inherited abnormalities of the zona pellucida that would allow cells to separate before implantation and placentation (Shapiro et al., 1978). As the zona pellucida behaves for all practical purposes as an elastic solid (Green, 1997), the inherent potential of the zona pellucida to restrain the division process is likely to determine the success or failure of embryonic splitting. There is compelling evidence that a controlled inter-

play exists between signals coming from the fertilized oocyte and the complex biochemical and biophysical properties of the zona pellucida (Green, 1997). Changes in the zona pellucida were suggested to facilitate early splitting, discordant X-inactivation (Burn et al., 1986), and abnormalities in the so-called developmental clocks (Boklage, 1987). Studies of in vitro fertilization (IVF) also suggested a link between the physical state of the zona pellucida, hatching, and generation of monozygotic twins (Alikani et al., 1994).

In practice, however, as long as the zona pellucida is present and determined to be intact, there is no visual clue that splitting occurred or will eventually occur. At the same time, it may well be that the embryo has already undergone the initial process of splitting. It follows that the timing of hatching — early or delayed — determines the likelihood that a fertilized oocyte will undergo early or late (i.e., at the balstocyst stage) splitting. Conceivably, most splits would occur in utero and result in monochorionic placentation, fewer will occur early, and very few (< 1%) would be delayed well beyond the time of implantation and would result in monoamniotic placentation. It seems that fertilization and maturation in vitro facilitates later splits and results in higher frequencies of monochorionic twins, increasing the monochorionic/dichorionic ratio from 2:1 to 3.5:1 (Derom & Derom, 2005).

The Trigger of Zygotic Splitting

The available theories endeavor to explain why one zygote would undergo unanticipated division to become monozygotic twins. The cell repulsion hypothesis maintains that cells in the developing zygote express subtle but specific genetic differences, which translate into a repulsive force that leads to splitting of the zygote. It then is presumed that the developing discordant cell lines recognize a need to separate in order to maintain their individual integrity (Hall, 1996).

This theory presents several difficulties. First, most monozygotic twins do not exhibit two distinct cell lines. Second, whereas this theory may explain very early splits, which should lead to development of monozygotic–dichorionic twins, it is less convincing in explaining later zygotic divisions when a blastocystic cavity has formed. Third, the repulsion theory cannot explain how ovulation induction and assisted reproductive technologies (ART) may cause genetic diversity in only part of the zygotes, as seen in dizygotic triplets in which one zygote splits and another does not in the same cohort of oocytes and under the same ovulatory conditions. Even more intriguing and equally unexplained by the repulsion theory is the occurrence of monozygotic triplets, in which a subsequent and distinct cellular differentiation event would have to occur in at least one of the resultant cell populations at the same embryological state. Moreover, if such an event were to occur in both cell populations, it would have to be followed by a spontaneous reduction of one of the resulting monozygotic quadru-

plets to finish with monozygotic triplets (Derom et al., 1987). In any event, this would mean that the resulting three monozygotic triplet embryos should be distinct in their genetic makeup following two successive repulsion events — an eventuality that has not been shown in vivo.

The second hypothesis maintains that an intrinsic propensity for embryonic splitting always exists as part of the so-called co-dominant axis theory of monozygotic twinning (Boklage, 2005). Under normal circumstances, one axis (out of more than one embryonic 'streak') dominates and suppresses the other(s). The continuous presence of a co-dominant axis is said to be the first step in monozygotic splitting. The main drawback of this theory is that it has not been shown to exist in mammals. In the early stages of avian development, many chick embryos consist of more than one primary axis, but at this crucial stage of embryogenesis, a streak-inhibitory factor is secreted to suppress the appearance of others (Levin, 1998).

The third theory suggests that the integrity of the zona pellucida is breached during embryonic development (Blickstein, 2005; Hall, 2003), thereby losing its sequestering and protective role (Norwitz et al., 2001) and permitting herniation of pluripotent cells through a gap in the zona pellucida as well as embryonic cleaving by an ill-defined pseudo-mechanical process. It has been speculated that changes in the physical properties of the zona pellucida following ovulation induction could lead to more traumatic hatching through a narrow opening to cause splitting of the blastomere as the conceptus emerges. This theory also presents several difficulties. First, advanced (late stage) mechanical zygotic splitting is likely to increase *separation* of the twins, whereas according to the accepted timeline of zygotic splitting, late divisions are supposed to exhibit increased *sharing* (i.e., ranging from the same chorion to the same organs). Second, ART procedures that involve zona pellucida micromanipulations were initially expected to have a higher frequency of monozygotic splits; however, although described in numerous case reports and short series (Schachter et al., 2001), the large study by Sills et al. (2000) casts doubt on the likelihood that zona manipulations play a substantial role in zygotic splitting. Finally, recent data collected by the East Flanders Prospective Twin Survey clearly show that the frequency of monozygotic twins is higher after ovulation induction than it is after ART (Derom & Derom, 2005; Derom et al., 1993). If indeed the common denominator of both ovulation induction and ART is ovarian stimulation, one would have to assume, according to the balstomere herniation theory, that some type of zonal damage had occurred prior to ovulation, that is, during folliculogenesis. Moreover, if the hatching event (normally on day 6 to 7 postfertilization) were to explain zygotic splitting, the normal timing of hatching cannot explain the occurrence of early splits.

The theory which suggests a 'splitting' effect of the in vitro (culture media) conditions (Steinman & Valderrama, 2001) also fails to explain the occurrence of monozygotic twins following ovulation induction. However, it is entirely possible that the trigger for splitting is regulated by calcium-mediated cellular adhesion (Steinman & Valderrama, 2001), which the zona pellucida is clearly a capable candidate to do both in vivo and in vitro.

Lessons From ART

How can one explain the constant prevalence of spontaneous monozygotic pregnancies in different races and populations, on one hand, and the increased prevalence of monozygosity in all forms of ART on the other? The most plausible explanation may envision a subpopulation of primary oocytes with an inborn and, as yet, unspecified propensity for zygotic splitting. The proportion of these oocytes is similar among different racial groups, and hence, if oocyte selection for folliculogenesis were a random event, the frequency of monozygotic twinning would be expected to be constant among different ethnic groups, as is actually the case. Consequently, the higher the number of ovulation events (i.e., following ovarian stimulation), the greater the chance of recruiting a splitting-prone oocyte for ovulation, as is indeed the case with all methods of assisted conceptions (Derom & Derom, 2005; Derom et al., 1987, 1993). In addition, the more fecund patients with a better chance to conceive are significantly more likely to have monozygotic twins, as seen in those receiving a less 'aggressive' regimen such as clomiphene citrate as the sole treatment, compared to other ovulation enhancing agents (Derom et al., 2006). It follows that the chance of a follicle that contains an oocyte with a propensity to undergo splitting is quasi-'dose'–dependant, whereby the term 'dose' refers to the combined effect of the patient's fecundity and the specific treatment administered. This finding is supported by the possibility that ovarian stimulation — the common denominator of all assisted procreation — may affect oocyte development that could predispose to splitting.

It is also possible that oocytes destined to undergo atresia, which will nevertheless ovulate following ovarian stimulation (Serna & Garcia-Velasco, 2005), might be involved in the splitting mechanism. It could be speculated that such rescue of atretic oocytes is lacking in spontaneous polyovulation (seen in advanced maternal age and in African women), and this, in turn, might explain why zygotic splitting is not age- and race-dependent.

Because the general view is that more twin pregnancies are formed than delivered, one may assume that the proportion of the primary oocytes with an inborn propensity for zygotic splitting is significantly higher.

Once a splitting-prone zygote is formed, splitting is initiated by whatever mechanism coordinates this

process. However, the embryonic cells, primed to undergo division, do not begin the physical separation as long as the zona pellucida is protecting the dividing but unhatched embryos. This is supported by the observation that monozygotic splitting occurs in similar frequency regardless of the embryonic condition (fresh vs. frozen–thawed) or whether embryo transfer was during a spontaneous or an induced cycle (Blickstein et al., 2003). The day 5 to day 6 hatching–splitting described by Behr and Milki (2003) lends support to our view that the prodromata for embryonic splitting may occur without a visual manifestation during the first 5 days of development and before the zona pellucida disintegrates during hatching and explains why embryologists do not observe any physical splitting in IVF programs.

The enhanced potential of the zygote to produce monochorionic twins — to about 5.6% of the cases — after blastocyst compared with cleavage stage transfers has been suspected to be the result of extended in vitro culture (Behr et al., 2000; Milki et al., 2003). However, some bias might be associated with this clinical impression because of improved implantation rates following blastocyst transfers. Nevertheless, if a zygote is predestined to undergo splitting, the extended culture in vitro might produce monochorionic twins more frequently by avoiding early splits to dichorionic twins.

The significant increase in anomalous embryonic development in the form of monozygotic splitting implies that all forms of assisted reproduction are teratogenic at a preembryonic level. It is unknown, however, whether the teratogenic effect involves a pathological transformation in otherwise normal oocytes or increases the probability of fertilization and implantation of an already anomalous oocyte.

Figure 1
Various combinations of two binary fissions of a cluster of cells forming the zygote. Dark color represents embryonic death. See text for discussion of the different panels. The similarity of the cluster of cells to a morula is only for artistic purposes.

Lessons From the 9-Banded Armadillo

None of the theories discussed above are able to explain the formation of monozygotic triplets and quadruplets unless the existing potential of a fertilized oocyte to undergo two binary fissions — as is the case in the armadillo — is acknowledged.

Figure 1 shows the various possibilities of two successive binary fissions. Panel A shows how monozygotic quadruplets are formed in the armadillo. The spontaneous reduction of one of the resulting embryos shown in panel A, is the best explanation available for human monozygotic triplets (panel B); although one may also envision a secondary fission of one embryo to reach the same result (Derom et al., 1987). Panel E shows the simplest construct and what is currently believed to occur in twins, when binary fission occurs once. However, if the potential of two binary fissions exists, two other possibilities for monozygotic twins also exist (panels C and D). The difference between the monozygotic twins in panels C, D and E is that those in panel E are the result of the first fission, whereas those in panels C and D are a
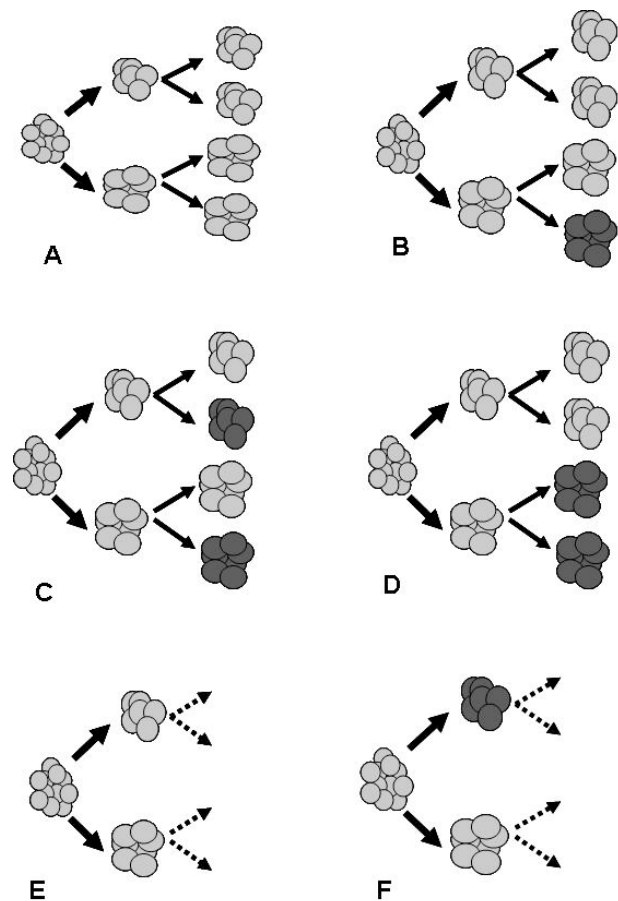
result of the second fission. The difference between the monozygotic twins in panels C and D is that those in panel D arise from one embryo whereas those in panel C arise from two different embryos. At this stage of the theory, it could be speculated that differences envisioned by comparing panels C, D, and E translate into diversity of the placental forms and might explain why only 25% of the monozygotic twins acquire the so-called 'mirror-image' characteristic (Teplica & Peekna, 2005) as well as some of the midline asymmetries (Boklage, 2006).

Of special interest is the situation depicted in panel F, suggesting the formation of monozygotic twins with a subsequent, but very early disappearance, of one embryo. This scenario would result in a singleton birth, but in theory, this singleton may retain characteristics of a monozygotic twin.

Finally, data from different sources show that the occurrence of dichorionic triplets is significantly more frequent among spontaneous than among induced triplets (Derom & Derom 2005; Geipel et al., 2005). In fact, when counting the splitting rate per available

zygotes (i.e., one for monozygotic twins, two for dizygotic twins or dizygotic triplets, and three for trizygotic triplets), not much difference in the splitting rate between spontaneous twins and triplets (28.9 vs. 30.9%, respectively) is seen. The difference becomes more meaningful (3.7 vs. 4.6% respectively) among multiples after ovulation induction and much more significant (1.1 vs. 2.5% respectively) after using ART. These figures are in accord with the theory of increased likelihood of selecting splitting-prone oocytes by induction of ovulation with and without in vitro fertilization.

The usual concept of the generation of dichorionic triplets — a combination triplets and monochorionic twins (Chasen et al., 2002; Geipel et al., 2005) — is a secondary split of one dizygotic twin. Indeed, various combinations of splits and subsequent losses of embryos suggest that more splits may occur but are unrecognized because one (or more) embryo is lost very early in odd monozygotic pregnancies.

Comment

As the first sentence of this review implies, *all* causes and mechanisms of zygotic splitting are at present, speculative (Steinman, 2000). The explanations compiled in this review combine the concepts of an inborn propensity of specific oocytes to split after fertilization and an important controlling role for the zona pellucida. One must admit that two binary fissions of the developing embryo might neatly explain what the existing hypotheses cannot, namely, the occurrence of monozygotic triplets and quadruplets. Following the same line of reasoning, monozygotic twins would not only result from a primary fission, but also from various combinations offered by subsequent secondary fissions. Such a construct may be the cause of inter-twin diversity among what is supposed to be 'identical' copies of a zygote.

Finally, the theory puts the existing speculative theories into a broader perspective without negating the existing and genetically sound hypothesis of cell repulsion, nor does it exclude the presence of a co-dominant axis, or calcium-mediated cell separation. Despite its speculative nature, this theory takes into consideration new observations from IVF and endeavors to fill specific gaps that had not been addressed in the prior hypotheses.

Acknowledgments

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