Articles

The origin of monozygotic twinning

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Abstract

The incidence of monozygotic twinning appears to be increasing within the field of assisted human reproduction. Many theories have been put forward as to how and when this occurs. Whatever the cause, the normal events of embryo development, which necessarily involve axis formation, patterning and polarization, need to be adhered to in order to obtain a viable offspring. This paper describes the course of development in terms of axis formation and polarity and offers suggestions as to how either a disruption of this or duplication events in the course of the formation of these parameters could prevent or contribute to a twinning event. The likelihood of twinning occurring at any point is discussed in terms of the establishment of polarity and axes.

Keywords: *abnormal development, axis duplication, monozygotic twinning, polarity*

Introduction

Twinning is a widely used term that describes a number of different phenomena in mammalian development. In all situations, the result of twinning is the birth of two offspring from a single gestation or as the result of one pregnancy attempt. In species that are normally perceived as uniparous, twinning would refer to the birth of two offspring in a single pregnancy. The most common form of twinning is that of a pregnancy in which two fetuses are growing as the result of the ovulation and fertilization of more than one oocyte with subsequent implantation and fetal development, known as dizygotic twinning (polyovular, fraternal). Twinning can also occur from the splitting of one embryo after fertilization (monozygotic twinning; monovular, identical). In this new era of assisted reproduction, twinning could conceivably refer to the birth of two offspring at different time points that have originated from a single ovulation and fertilization event.

Some species have a tendency to twin during the majority of pregnancies. In sheep, this form of twinning has a genetic basis (Fahmy, 1996). In some strains of sheep the genes for twinning have been enhanced by purposeful or inadvertent (isolation) inbreeding of a high fecundity gene. The oldest known gene for high fecundity is that of the Finn sheep. This has been known for over 2000 years, and was described as the *F* gene in 1918 (Maijala, 1996). Through inbreeding, it has become very stable. More recent high fecundity genes include the *Fec B* or *Barroola* gene in Marino Barroolas, the *FecX* on the X chromosome in Inverdale Romney sheep, the *Thoka* gene in Icelandic sheep, and similar high fecundity or high prolificacy genes of Belle-Isle, Bluefaced Leicesters, Flemish Landrace, Galacian, Garole, Imertian, Olkusta, Teeswater and Virgin Isle White sheep (for a comprehensive review of high prolificacy sheep breeds, see Fahmy, 1996).

In the human, this form of twinning, dizygotic twinning, has also been suggested to have a genetic origin (Lewis *et al.*, 1996; McGillivary, 1986). Unlike sheep, a high fecundity gene in the human has not been isolated on chromosome 4 (Duffy *et al.*, 2001), although there is evidence that there is a region on chromosome 3 that may be involved with twinning (Busjahn *et al.*, 2000). There is 1.7 higher chance of having twins if one's sister did and 2.5 times higher chance if one's mother did (Lewis *et al.*, 1996). Dizygotic twinning is also regional or race related There is a rate of approximately 8/1000 births in the USA and Europe, 1.3/1000 in Japan and as many as 50/1000 in Nigeria (McGillivary, 1986).

In species that are multiparous, twinning takes on a totally different meaning. In the mouse, which routinely has more than one offspring, twinning could mean two or more embryos

implanted in a single crypt (implantation site) in the uterus with subsequent conjoined ectoplacental cones (Wan *et al.*, 1982) or the spontaneous/natural splitting of an embryo to form monozygotic twins (Hsu and Gonda, 1980).

Monozygotic twinning in humans is, however, constant across race, ethnic group and around the world. It is generally non-familial, and non-genetic, although there is some evidence of families with high incidences of monozygotic twinning (Harvey *et al.*, 1977).

In order to have monozygotic twins, an embryo needs to divide to produce two (or very rarely, more) identical copies from one fertilization. This twinning process can arise early, at the 2-cell stage or at later pre-compaction stages (4- to 16-cell). Here, the event of splitting would occur prior to the formation of the two cell lines that define the extra-embryonic and embryonic parts of the blastocyst. In this case, the resulting fetus would or could be dichorionic/diamnionic, since the embryos can develop as separate units with separate membranes. This form of twinning accounts for at least one-third of all monozygotic twins surviving to birth (Luebke *et al.*, 1990). If the original embryo divides at the blastocyst stage, after differentiation and formation of the inner-cell mass and trophectoderm, the resulting embryos should share the outer chorionic cavity but have separate anmions (diamnionic, monochorionic). This form of monozygotic twinning is the most common, constituting approximately two-thirds of all live births. If splitting occurs later, after the formation of the ectoplacental cone, the fetuses should share both amnion and chorion (monoamnionic/monochorionic), and this constitutes a small fraction of all surviving monozygotic twins. This last form of monozygotic twinning can also lead to incomplete separation of the two halves, leading to conjoined twins. All forms of monozygotic twins are found in human pregnancies. From the incidence of the types of twinning occurring that results in live births, it could be concluded that twinning in early cleavage stages is rare; it occurs more frequently at the blastocyst/early ectoplacental cone stage and rarely at later stages. If it does occur at the early or later stages, it is less likely to be compatible with viability (**Figure 1**).

Monozygotic twinning results in a very high rate of spontaneous abortions and fetal abnormalities (Livingstone and Poland, 1980; Jones and Benirschke, 1983). Approximately 15% of all twin pregnancies are aborted and this figure is higher for monozygotic twins (Greb *et al.*, 1987; the Wisconsin Stillbirth Service Project: Luebke *et al.*, 1990, WiSSP; www.wisc.edu/wissp). In these same surveys, 8.3% of stillbirths were from multiple births and more than half of these were from monozygtic twins (Greb *et al.*, 1987). Many abnormalities have been described in monozygotic twins at term and also in early abortions. The phenomenon of the vanishing twin (where one twin does not develop) is a well-described feature in many monozygotic twin pregnancies (Jones and Benirschke, 1983; Greb *et al.*, 1987; Luebke *et al.*, 1990). All the abnormalities point to aberrations in development.

Body stalk anomalies (Marinez-Frias *et al.*, 2000) are the absence of the umbilical cord, which is caused by maldevelopment of embryonic body folding. Body stalk anomaly is rare in dizygotic twinning but there are numerous reports of this defect in monozygotic twin gestations (term and early abortions) (Aksoy *et al.*, 2000), which might be the result of 'incomplete twinning' (Shih *et al.*, 1996). The body-stalk defect results from incorrect folding during gastrulation. There are other reports of gastrula defects in monozygotic twins that are linked/related to abnormal embryo folding and development that include amyoplasia, gastroschisis, bowel atresia and defects in the trunk wall muscles (Reid *et al.*, 1986). This is most likely to be the result of a late twinning event.

With the current IVF techniques, there has been a perceived (or real) increase in the overall incidence of monozygotic twins from the base line of 1–1.3% of births to reports of anywhere from 3 to 15% of recorded deliveries (Harvey *et al.*, 1977; Hall, 1996; Abusheika *et al.*, 2000; Saito *et al.*, 2000). It has been proposed that this is due to gonadotrophin stimulation, the micromanipulation of the oocyte (intracytoplasmic sperm injection; ICSI) or the embryo (assisted hatching) (Alikani *et al.*, 1994) or to culture conditions (blastocyst transfer) (Behr *et al.*, 2000). There are also reports showing that each of these agents is not the cause of the increased incidence (Schachter *et al.*, 2001; Sills *et al.*, 2000) What is evident, though, is that there is an increasing number of recognized multiple pregnancies that are monozygotic in origin and that this is clearly related to some aspect of the manipulation of reproduction *in vitro*.

The natural occurrence of monozygotic twinning is very rare, and in only one instance, the nine-banded armadillo, is it a regular event. In the nine-banded armadillo the embryo splits

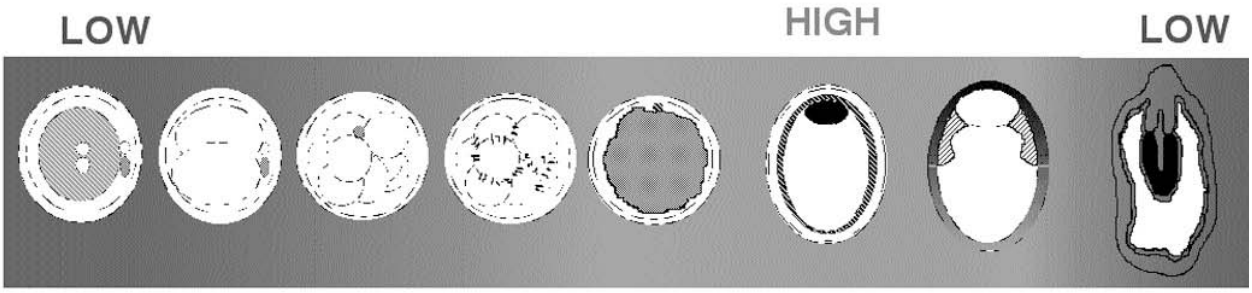

Figure 1. Embryo development and stages at which twinning is most likely to be successful.

into four, resulting in monozygotic quads at each pregnancy. The mechanisms behind this event could be an evolutionary adaptation based on the breeding patterns of these animals, which have a long diapause event (Enders, 1965) that is linked to food availability. Breeding occurs in April to May, the embryo enters the uterus 5–7 days after fertilization where it forms a blastocyst, hatches and then enters diapause with implantation occurring in November to December. During this phase, the inner cell mass remains totally undifferentiated (Enders, 1962). At the initiation of implantation, there is an increase in mitosis in the inner cell mass (ICM) and a syncytial trophoblast forms (Enders, 1962). Implantation occurs in the fundus region of the uterus, and there is a single implantation site and a single amnion and epiblast plate. The trophoblast has three layers, a layer of invasive trophoblast, an epithelial layer of absorptive trophoblast and a trophectoderm annulus, which is at the margin of the implantation site (Enders, 2002b). Implanting blastocysts have a single cavity formed by cavitation and the single epiblast plate. Cells from margin of the epiblast plate bud into the cavity forming an exocoelom, which displaces the amnion towards the epiblast plate. From this, two separated embryonic shields develop followed by the formation of two more that are close together. The growth or enlargement of the epiblast plate allowing enough cells to form four embryonic shields and the large exocoelom in which they can develop can be attributed to early and fast development of the extra-embryonic placental membranes, the large exocoelom and the slow development of the primitive streak (Enders, 2002b).

In this way, the animals maximize their fecundity in any one breeding season in the time span where suitable conditions for reproduction are restricted. These are not likely to be the mechanisms behind monozygotic twinning in the human, which is not a seasonal breeder and where dietary restrictions do not appear to lead to any increased incidence of monozygotic twinning. However, considerations of the mechanisms underlying the twinning in the nine-banded armadillo, i.e. splitting of the epiblast plate into multiple embryonic shields, should be considered in the human context. A case report in another primate, the Rhesus monkey, has shown a similar mechanism (Enders, 2002a).

Since monozygotic twinning is so rare in nature, and is associated with very high fetal demise *in utero*, it could easily be considered an anomaly of development. The recorded defects described above could all be linked to patterning defects or the breakdown of the normal events of patterning during the early stages of gastrulation. The establishment of axes, polarity and left–right asymmetries are recognized fundamental aspects of normal development of mammalian embryos and fetuses (Gardner, 2001; Yost, 2001) and through all animal species (for reviews, see Etkin and Jeon, 2001). In other words, monozygotic twinning could be associated with disruption of the fetal axes through events of abnormal axis formation, cytoplasm rotation or folding, leading to a duplication event. Since the axes are initiated very early in development (Edwards and Beard, 1997; Gardner, 2001), it could be caused by very early defects in embryonic polarity establishment and these could go back as far as oocyte development or fertilization. Any one of these stages could be accounted for in the diverse causes put forward for the increases of monozygotic twinning with assisted reproduction.

This review will concentrate on fertilization and the early events of embryo development with a view to understanding how the embryo could split and duplicate, what the consequences of this would be and how duplication could be incorporated into the events of early development. With this in mind, the reasons these events are probably so rare will be discussed and why it could lead to the vast number of abnormalities and fetal demise that are associated with monozygotic twinning.

Oocyte and embryo polarity and monozygotic twinning

Gonadotrophin-induced events

The coordination of oocyte and follicle development is crucial to ultimate success in reproduction (Eppig, 1991). The position of the oocyte in the follicle is also important to its later developmental potential (Van Blerkom *et al.*, 1997). The level of blood flow to the follicle can determine the metabolic ability of that follicle and oocyte, since the follicle cells deliver nutrients, and other products to the oocyte (Eppig, 1991, 2002; Van Blerkom *et al.*, 1997). Granulosa cell products move into the oocyte. These products could set up regions or polarized areas in the oocyte, which could be considered a follicle-directed polarization. In the mammalian oocyte, it has been proposed that a subset of follicle cells direct the distribution of STAT3 and leptin to the oocyte (Antczak and Van Blerkom, 1997). STAT3 is a protein involved with signal transduction and activation of transcription. Leptin is a cytokine product involved in the activation of STAT3. The area of hemisphere in the oocyte where the STAT3 and leptin are located corresponds with the region on the oocyte from which the first and second polar bodies are extruded.

The extrusion of the first polar body at the completion of the first meiotic division defines the polar region (not point) of the oocyte or the animal pole. The vegetal pole is located at the opposite side of the oocyte. Localization of molecules could conceivably establish gradients and boundaries, which could influence the developing embryo. If there is a disruption of this flow, through gonadotrophin stimulation, the normal course of polarized development may be disrupted. Any disruption in the fine-tuning of the co-ordinated development of follicle and oocyte could also lead to disruptions in message and axes formation. If the axes are disrupted and two sides are formed, this could lead to twinning. This event will be early and could be manifested during cleavage or at the time of blastocyst formation, the result being either two sets of blastomeres developing side by side or a duplication of the ICM at blastocyst formation. The former situation could be recognized *in vitro*, yet there have been no reports of this even though monozygotic twins have been reported from the transfer of embryos on day 2 or 3. Duplication of the inner cell mass could also be recognized *in vitro*.

Oocyte polarity considerations

Mammalian oocytes tend to be spheres and develop asymmetry through the placement of organelles, molecules or structures anywhere within them, such as the nucleus and the spindle. However, if they are not spheres, their shape automatically gives them asymmetry, which in turn will define

axes and polarity (Gardner, 1996b; Edwards and Beard, 1997; Scott, 2001). This polarity or asymmetry establishes or designates the polar axis. During the first meiotic division, the germinal vesicle breaks down, the spindle moves from a central position to a cortical region, the first polar body is extruded and the spindle attaches to the inner surface of the cell membrane (Edwards and Beard, 1997; Sousa and Tesarik, 1994; Silva *et al.*, 1999; Hardarson *et al.*, 2000). When the mature oocyte is ovulated (or retrieved in assisted reproduction) it has an animal–vegetal axis established which can be recognized through the first polar body. Disruptions in this polarity could lead to events that will persist in later development, resulting in duplications or axis disorientation, which could ultimately lead to twinning at the pre- or post-compaction period as described above.

Fertilization events

Fertilization of the mature oocyte sets up many new polarities. Sperm entry can be very directed as in Ascidians (Roegiers *et al.*, 1995), *Caenorhabiditis elegans* or *Drosophila*. Mouse oocytes have a region lacking surface microvilli at the polar end (where the first polar body is). Spermatozoa do not attach to the microvillus-free area so entry occurs away from this area and hence from the metaphase spindle. Human oocytes do not have a microvillus-free area (Pickering *et al.*, 1988; Santella *et al.*, 1992), and can theoretically bind spermatozoa anywhere. However, an area of broken or non-attached granulosa cells has been observed in oocytes being prepared for ICSI. This could be related to such an area in the mouse.

As the sperm and oocyte membranes fuse, there is a very rapid peri-peripheral calcium wave around the oocyte involving highly polarized mitochondria. This wave is very specific and only occurs at sites that have no contact with other cells as has been recorded in human oocytes (Van Blerkom *et al.*, 2002). This may also be the area observed in which there are few cumulus cells attached to the oocyte, a sight of directed sperm entry. The distribution after fertilization of aggregates of these excitable mitochondria is also not uniform and may have a profound effect on both the later ability of a blastomere to proceed with development (Van Blerkom *et al.*, 2000) and its ability to organize and polarize. When this pattern of distribution was disrupted, no or abnormal development ensued (Van Blerkom *et al.*, 2002). Thus, disruption of this event is unlikely to be the cause of duplication events leading to monozygotic twins.

An increase in intracellular calcium levels at fertilization triggers the transition from oocyte to embryo and is the key to development (Carroll *et al.*, 1996; Carroll, 2001). The process of fertilization and resultant signal transduction leading to calcium release is triggered by the fusion of the sperm and oocyte membranes. Subsequently, the spermatozoon releases 'factors' into the ooplasm, which augment the initial calcium release, the following calcium oscillations, release of corticle granules (CG) and a block to polyspermy (Abbott *et al.*, 1999). After the first very rapid wave of calcium release, many low frequency oscillations of calcium release persist for hours. The waves arise at the site of sperm entry. The point of sperm entry determines the polarity or co-ordinates of the eventual blastocyst and thus the fetus in the mouse (Pedersen, 2001; Plotrowska and Zernicka-Goetz, 2001), *Xenopus* and *Drosophila*. The progress of these waves or oscillations of calcium are either responsible for, or contribute to, the establishment of this axis development (Deguchi *et al.*, 1999). Wave propagation is both time and spatially regulated and involves InsP3/InsP3Rs, G proteins, and the endoplasmic reticulum (Lechleiter *et al.*, 1991; Lechleiter and Clapham, 1992; Atri *et al.*, 1993; Berridge, 1993; Clapham, 1995; Stehno-Bittel *et al.*, 1995).

The first wave is initiated at the sperm entry site and moves to the opposite side/pole of the oocyte. The second wave follows the same path. This wave is followed by movement or rotation of the cytoplasm. The movement follows the general direction of the wave. Calcium oscillations continue through the second meiotic division and stop at the stage of pronuclear formation. The waves alter through this cycle, becoming shorter as they progress. The speed of the waves also increases as meiosis proceeds. With each wave there is further cytoplasmic displacement. Thus, the point of wave initiation moves around the oocyte away from the point of sperm entry to the opposite, or vegetal, pole (Tang *et al.*, 2000). This wave can also be seen as a 'flare' within the cytoplasm (Payne *et al.*, 1997; Scott and Smith, 1998) and corresponds to the waves of calcium. This cytoplasmic streaming has also been seen in hamster and mouse oocytes where it is attributed to localization of mitochondria within the cytoplasm (Muggleton-Harris and Brown, 1988; Barnett *et al.*, 1996) and could also be connected to the calcium waves. If the waves are connected, it could also mean that the streaming is highly polarized, just as the calcium waves are. These waves start at the top or animal pole and move down to the vegetal pole, already established in the oocyte and by the first polar body.

With this in mind, if the spermatozoon enters the oocyte and initiates a calcium wave followed by cytoplasmic streaming at some point that sets up another set of poles other than the animal–vegetal one, it could result in either duplication of poles or cytoplasm that will eventually have the ability to set up a duplication event (see **Figure 2**). In ICSI, the point at which the needle enters the oocyte could be extremely important in determining polarity, axes and thus later development. If waves of calcium or cytoplasm streaming are not initiated at the correct point, aberrant development could ensue. This has been partly demonstrated in ICSI, where the placement of spermatozoa is seen to be crucial to outcome (Garello *et al.*, 1999; Van der Westerlaken *et al.*, 1999; Blake *et al.*, 2000). It will be interesting to determine if more monozygotic twins arise from ICSI versus IVF assisted cycles and if the injection site has any effect on the incidence of twinning.

A form of abnormal patterning can be induced artificially by disrupting cytoplasmic streaming in experimental situations. The normal or directional patterning of *Xenopus* development can be disrupted at the zygote stage (Scharf *et al.*, 1989). If fertilized *Xenopus* oocytes are briefly treated with D_2O during the first three cell cycles, a phenomenon results that could be likened to monozygotic twinning at the zygote phase. The resulting embryos show a shift in their patterning with excessive dorsal and anterior features with reduced ventral and posterior ones. Further, the embryos that result from these fertilized oocytes often have twinning of the dorsoanterior structures. It is speculated that the treatment increases the

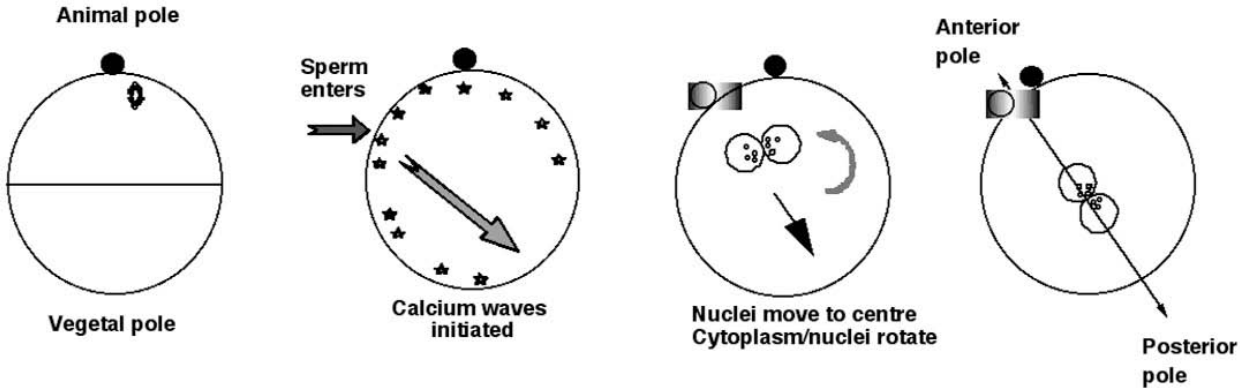

Figure 2. Early establishment of polarity.

effects of the cortical rotation resulting in the zygote setting up two or more directions of rotation. The result could be duplication or widened areas of key developmental structures involved in patterning and subsequent embryo formation, such as the Nieukoop centre, Spemann organizer region or the vegetal hemisphere. These experiments highlight the need for sequential pattern formation and adherence to the sequence for normal development. Similar effects have been recorded in mouse zygotes treated with lithium. If this is happening in human embryos *in vitro*, it could be speculated that two zones are forming in the embryo, which are manifested after compaction of cavitation as the cleaving embryos present with normal morphology.

Cleavage

The first cleavage division is always unequal and not random. The first mitotic cleavage plane is dictated by the position of the second polar body and the pronuclei and the resulting cells are elliptical. The second polar body is tethered at one end of this plane, which is also the long axis of the two elliptical cells coinciding with the polar axis of the embryo (Gardner, 1997). The embryo is unable to rotate freely inside the zona pellucida. With the first cleavage, there could also be unequal distribution of certain proteins (such as STAT3 and leptin; Antczak and Van Blerkom, 1997) to the two cells. This early difference has been linked to formation of distinct cell lines (Edwards and Beard, 1997, 1999), which will continue through the cleavage stages as distinct areas in the embryo. This could be similar to the early determinism that occurs in *Xenopus* (Gurdon, 1992), *C. elegans* (Kemphues and Strome, 1988), *Drosophilia* (St Johnston and Nusslein-Volhard, 1992). If some event has caused unequal or incorrect distribution of products to the cells or if the cleavage axis has been rotated or moved, there could be a duplication event, in a similar manner described above for *Xenopus*. This could lead to two sets of poles, two sets of blastomeres and ultimately a duplication of the embryo when the inner cell mass is formed.

Another stage at which an unequal distribution of cell products could occur, setting up duplicate embryos, would be during compaction. The formation of inside and outside cells at compaction has been seen as the first cell differentiation event in embryos (Johnson and Ziomek, 1981). During this time, refractile bodies or vesicles become differentially located in the embryos. These vesicles are present in the embryo from the zygote stage onwards (Calcaro and Brown, 1969). As the embryo compacts, they begin to fuse and locate in specific areas and are involved in formation of the blastocoel. The blastocoel forms initially as furrows between the inner and outer cells in the morula. If two focal areas for this clustering are initiated, perhaps caused by an earlier disruption of axes, two distinct blastocoels could be formed with two ICM leading to two embryos.

The sex ratio of monozygotic twins is skewed towards females (Machin, 1996), and especially so in the twins that are more closely joined, i.e. monochorionic twins (**Figure 3**). There are reports of a high incidence of skewed X inactivation in monozygotic twins. There has been some speculation that non-random X inactivation will lead to two populations of cells that repel each other leading to the possibility of two ICM (Goodship *et al.*, 1996). Studies on female monozygotic and dizygotic twin pairs discordant for X inactivation did not support this theory. However, that there is an excess of females in monozygotic twins, which is also linked to the form of twinning, would point to either a sex linked survival mechanism of a twinning event, a propensity for female embryos to twin (through perhaps slower development) or some involvement of X inactivation or differential expression of genes on the X chromosome.

Whatever mechanism is involved in early development, a duplication event would seem to be necessary in light of the lack of success in artificial twinning by separating blastomeres of embryos and allowing them to develop into blastocysts. Although blastomeres of pre-blastulation embryos are termed totipotent, there has been very limited success in getting these blastomeres to form viable blastocysts in isolated mouse, rabbit, sheep or cow embryos. The fewer cells the embryo has before the blastomeres are separated the more likely it is to form a viable blastocysts (Gardner and Nichols, 1991; Gardner, 1996b; Edwards and Beard, 1997; Gardner, 1999). Where twinning is concerned, this would tend to point away from a simple separation of blastomeres in the pre-compaction embryo to an event that causes two groups of blastomeres to be developing as separate polarized units. As an embryo divides and spreads its polarization through the cells, it is able to tolerate more and more cell removal (Edwards and Beard, 1999). This can also be true of a twinning event. As it grows, and has more cells the embryo could possibly duplicate or split, so long as axes of development are maintained. The

lower incidence of surviving offspring from early separation events may be due to this (**Figure 1**).

Blastocyst formation

At the blastocyst stage the polar body is still located at the apex of the blastocyst, near or over the ICM, at the site where the polar and mural trophectoderm meet (Gardner, 1997). This means that the blastocyst has a bilateral axis that is aligned, or the same as, the original animal–vegetal axis established in the zygote and that the embryonic–abembryonic axis is at right angles to this, again as established in the zygote (see Gardner, 1997; Gardner, 2001 for diagrams).

The cells in the trophectoderm proliferate in the early blastocyst but once expansion begins, mitosis in the cells in the mural area declines and they transform into giant cells (Gardner, 2000). At the time of implantation, trophectoderm cell proliferation is restricted to the area over the ICM (Gardner, 1996a). The polar trophectoderm retains mitotic activity only where the cells are connected to viable ICM cells (Gardner and Beddington, 1988; Nichols *et al.*, 1998), but there is no net increase in cell number due to a flow of cells from the polar region to the mural region. The cells migrate from the polar to the mural region in a very directed manner, not a radial manner, forming a coherent patch or cap in the mural region (Gardner and Nichols, 1991; Gardner, 2000). This movement could actually be specifying the bilateral symmetry of the embryo (Gardner, 2001). During the migration, the cells at the polar–mural junction extend transient processes to the ICM cells (Flemming *et al.*, 1984; Gardner, 2000). The processes are tongue like structures from the trophectoderm cells to a central area on the ICM and are withdrawn as the trophectoderm cells move to the mural region. The number of junction cells with processes varies inversely with rate of cell transfer from the polar to mural region.

The proliferation of polar trophectoderm in contact with viable ICM cells, directional movement of polar trophectoderm and the cytoplasmic processes could all be involved in a twinning process at the blastocyst stage. If an area of the ICM is atretic, or entering apoptosis, it could split the ICM into regions, causing the cells above it to transform into mural trophectoderm and potentially split the blastocyst into two. If polar trophectoderm moves to the mural region in two directions, it could conceivably set up two sides to the blastocyst, allowing the ICM to split, forming two embryos. Or, finally, if the processes that are anchored to the ICM do not withdraw, they could pull or displace ICM cells such that two groups are formed. From any of these events, two distinct embryonic poles could be formed within one blastocyst. The processes are easily seen in human blastocysts *in vitro* (Scott, 2001) but their persistence on expansion is linked to poor post-transfer development (author's unpublished data). Large and apparently duplicated ICM are also seen in human blastocyst grown *in vitro*. This may be as a result of unbalanced culture conditions but even if this is the case, the events of duplication still need to adhere to the establishment of polarity and axes for continued development of the fetus. It has been speculated that glucose-induced apoptosis in the ICM causes a furrow in the ICM and a duplication event (Menezo and Sakkas, 2002) Alternatively, duplication through excessive growth of the ICM, much like the duplication event that occurs in the nine-banded armadillo (Enders, 2002b) or as reported for a rhesus embryo (Enders, 2002a) could be the cause. This excessive growth again could be as a result of prolonged in-vitro culture.

Gastrulation

Once fully expanded, the blastocyst has definite polarity in that the ICM is located on one side of the blastocoel, at the embryonic pole, which corresponds with the polar region of the embryo, which has endured since the extrusion of the second polar body. This embryonic–abembryonic axis is the first defining axis of gastrulation in the fetus (Gardner, 1996b; Smith, 1980, 1985) and corresponds to the true dorso–ventral axis of the fetus. Since the blastocyst has true polarized symmetry and not radial symmetry, the establishment of the dorso–ventral axis necessarily sets up a true left and right to the embryo/fetus. This left–right asymmetry is essential for further development (Yost, 1995, 2001). If there are any duplication events early in development or at the blastocyst stage, there also needs to be duplication of all these axes, or further development will not occur naturally. The folding abnormalities associated with monozygotic twins could very

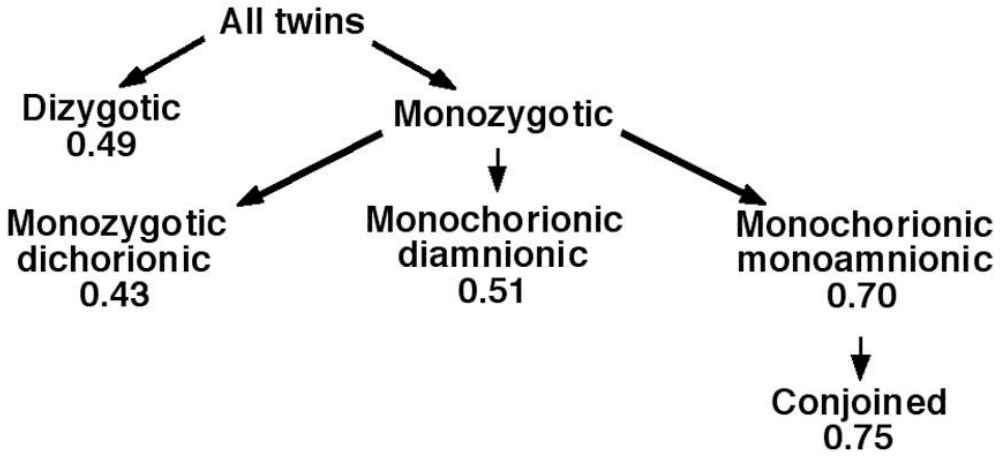

Figure 3. Female sex ratios of twin pregnancies.

easily be the result of anomalies in these axes establishment or alignment.

There is a growing body of literature (reviewed by Gardner, 2001) linking the events of gastrulation, formation of the fetus and establishment of the fetal axes to specific genes. Some of these genes are expressed asymmetrically and this is related to axis establishment. However, the expression of these genes is related to position within the early fetus, which is related to the formation of the blastocysts and this in turn goes back to the very original establishment of the animal–vegetal axis in the zygote by the extrusion of the second polar body. There is also detailed evidence of the abnormalities that result from lack of expression or abnormal expression of any of these genes. In some instances, this results in the disruption of folding. These facts all need to be taken into account when considering a later event of twinning, say at the stage of gastrulation. This may also be a reason that twinning events during this phase of development are rare and often not viable (**Figure 1**).

In conclusion, the timing of monozygotic twinning is probably not fixed and the mechanism varies from one set to another. What is not random is that the events of embryo development leading to a viable fetus need to be ordered and after a pattern. Disruption without duplication of the pattern will lead to abnormal, incomplete and supposedly non-viable development. How and why the duplication events occur is most likely as diverse as the times at which they can occur. What will be of extreme importance in elucidating the mechanisms of monozygotic twinning is to gather information on some important parameters and this needs to carried out on a multi-centre, world-wide basis. Questions to be raised include:

(i) What is the membrane status of the twins, as this will indicate when the embryo split?
(ii) What are the sexes of the babies? This should be compared with the sex ratio of dizygotic twin and assisted reproductive technology babies in general.
(iii) What form of assisted reproductive technology was performed (IVF versus ICSI)?
(iv) If ICSI was performed what was the timing and the orientation of the ICSI procedure?
(v) How long was the embryo maintained in culture and what medium and supplement was used?
(vi) Are there any discordant characteristics between the babies?
(vii) Are there any anomalies in the babies?
(viii) With careful ultrasound of all gestations to document the true incidence of twinning, how many monozygotic twin pairs result in a singleton or terminated pregnancy?
(ix) Analysis of all twins from assisted reproductive technology to establish true zygosity.

With this information, it will be easier to elucidate whether assisted reproductive technology procedures are contributing to the perceived increase in monozygotic twins and if and why they are. It may also help to develop an understanding of how this event does or can occur in nature.

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